Henry Ford Hospital Medical Journal

Volume 17 | Number 4 Article 3

12-1969

Cation Transport I. Metabolic Activity of the Polyphosphoinositide Complex in Isolated Renal Cortex Tubules

Paul Bartlett

James F. Bossart

Henry Ford Hosp. Med. Journal
Vol. 17, No. 4, 1969

Cation Transport

I. Metabolic Activity of the Polyphosphoinositide Complex in Isolated Renal Cortex Tubules

Paul Bartlett, Ph.D. and James F. Bossart, B.S.*

Although several mechanisms have been proposed to explain the transport of hydrophylic cations across lipoprotein barriers, the nature and identity of the carrier mechanism have not yet been elucidated. The present communication is concerned with a mechanism postulating that monoesterified phosphate on the inositol moiety of phosphatidyl inositol might provide anionic sites that function as cation carriers via a phosphorylation-dephosphorylation cycle, catalyzed in its simplest sequence by phosphatidyl inositol kinase and diphosphoinositide phosphomonoesterase activities. In this context, one might reasonably expect that turnover of monoesterified phosphate in the polyphosphoinositides of isolated renal cortex tubules would be reduced in tubules in which movement of sodium out of, and of potassium into, the tubule cells had been inhibited by ouabain. Contrary to this expectation, turnover of monoesterified phosphate, as evidenced by ^{32}P-orthophosphate incorporation into the monoesterified phosphate of the polyphosphoinositides, was strikingly increased when such tubules from rabbits were incubated in the presence of ouabain. This increase appeared to correlate only with ouabain-inhibited extrusion of sodium from the intracellular compartment of tubule cells, and not at all with the inhibitory effects of ouabain on K^+-reaccumulation.

Although a variety of mechanisms have been proposed to explain the transport of hydrophylic cations across lipoprotein barriers, the nature and identity of the carrier mechanism have not yet been elucidated. Work in this respect has focused largely on mechanisms involving: (a) a Na^+- and K^+-activated ATP phosphohydrolase present in all membranous structures capable of transporting cations against an electrochemical gradient[1-10]; or (b) the net ATP phosphohydrolase activity resulting from a combination of enzymes, such as phosphokinase and phosphohydrolase, acting in sequence. In this respect, combination mechanisms proposed by the Hokins,[11-13] involving a diglyceride kinase and phosphatidate phosphohydrolase, i.e. the phosphatidic acid cycle; and by Heald[14] and Judah et al,[15] involving phosphorylation and dephosphorylation of a protein, have been both provocative and stimulating to investigators interested in carrier mechanisms.

A third combination mechanism, which involves phosphorylation of mono-phosphoinositides to di- or triphosphoinositides with subsequent de-

* Edsel B. Ford Institute for Medical Research, Department of Biochemistry and Molecular Biology.

This work was supported in part by a grant from the National Institutes of Health, AM-06983.

phosphorylation of the resulting polyphosphoinositides, has also been suggested. While studies focused on the phosphorylating step of this last combination mechanism have failed, for the greater part, to provide experimental evidence to support the proposed phosphorylation-dephosphorylation cycle, certain other experimental findings caution against exclusion of the polyphosphoinositides from a role in membrane transport. Thus, under incubation conditions which one might expect to lead to an accumulation of phosphorylated carrier intermediate, no labeling of polyphosphoinositides from γ-^{32}P-ATP was observed in the Na^+-K^+-ATPase system of the electric organ of the Torpedo.[16] Furthermore, time-course studies of the labeling of polyphosphoinositides of human erythrocyte membranes failed to provide evidence for a polyphosphoinositide intermediate which equilibrates rapidly with labeled ATP.[17] On the other hand, results of studies of guinea pig brain slices, presented by Hayashi et al,[18] are compatible with the view that turnover of polyphosphoinositide monoesterified phosphate may be involved in cation transport. Thus, incorporation of ^{32}P-orthophosphate into the inositides was stimulated by incubation of the slices in the presence of sodium-Ringers, and this effect was depressed by ouabain. Pertinent also is the preliminary report of Standefer and Samson[19] suggesting involvement of the phosphoinositides in the Na^+-K^+-activated ATPase system of rat brain. During neonatal maturation, a marked increase in Na^+-K^+-ATPase is paralleled by incorporation of^{32}P-orthophosphate into the phosphoinositides. Furthermore, these parameters respond in parallel to temperature change, to Na^+ and K^+ concentrations, and to the transport inhibitor, ouabain.

If one considers the foregoing in conjunction with the widespread distribution of the polyphosphoinositide synthesizing system[20] and the relatively greater activity of this system observed in brain, kidney and salt gland (tissues having a relatively higher rate of cation transport than liver, heart, or pancreas), it is readily apparent that a more extensive study is needed of systems which may involve the polyphosphoinositides. In an effort to obtain new information bearing on the validity of the proposed combination mechanism, we have studied ^{32}P-orthophosphate incorporation into the polyphosphoinositide complex of a kidney cortex tubule system in which demonstrable changes in cation and water content can readily be brought about either by incubating the system in the presence of the transport inhibitor, ouabain, or by changing the ionic environment of the system.

Procedures

Freshly prepared rabbit kidney cortex tubules, isolated by the procedure of Burg and Orloff,[21] were used in all studies. In the first of three pairs of experiments, the effects of ouabain on water and cation content (first example of pair), and on the associated ^{32}P-orthophosphate incorporation into the polyphosphoinositide complex (second example of pair) in the tubules were studied by the following procedure. Tubules were pre-incubated for 30 minutes at 0° C in standard medium of the composition shown in the footnote to Table I, and subsequently incubated for 30 minutes at 25° C in

TABLE I

EFFECTS OF OUABAIN ON WATER AND CATION TRANSPORT IN RABBIT KIDNEY CORTEX TUBULES

Treatment of Tubules *	Total Water	Inulin Space	Intracellular Water		Total Cations Na^+	Total Cations K^+	Intracellular Na^+	Intracellular K^+	Apparent Intracellular Na^+	Apparent Intracellular K^+
	% Wet Wt.		% Wet Wt.	kg/kg D. S.	m.e./kg D. S.				m.e./kg $water_i$	
a. Pre-incubated †	79.7	23.8	55.3	2.64	453	142	318	131	$120^{(107)}$	$50^{(86)}$
b. Pre-incubated, then incubated ‡	78.7	32.4	46.4	2.18	346	179	175	167	$80^{(112)}$	$76^{(79)}$
c. As in a, then incubated in incubation medium containing 0.3 mM ouabain	80.5	30.3	50.0	2.58	514	86	345	69	$133^{(109)}$	$27^{(108)}$

*Pre-incubation carried out for 30 minutes at 0°C; incubation for 30 minutes at 25°C.

†*Composition of pre-incubation medium*: 125 mM Na^+, 6.7 mM K^+, 1.3 mM Mg^{+2}, 125 mM Cl^-, 1.3 mM SO_4^{-2}, 4 mM HCO_3^-, 2.3 mM $H_2PO_4^-$, 0.95 mM Tris (pH 7.4) + 5% calf serum, v/v. Osmolality = 281 mOsM. Aerated with 100% oxygen.

‡*Composition of incubation medium*: 121 mM Na^+, 6.0 mM K^+, 1.2 mM Mg^{+2}, 0.2 mM Ca^{+2}, 113 mM Cl^-, 1.2 mM SO_4^{-2}, 3.6 mM HCO_3^-, 2.1 mM $H_2PO_4^-$, 4 mM α-KG, 19.1 mM Tris (pH 7.4), 11.6 mM Glucose + 5% calf serum, v/v. Osmolality = 290 mOsM. Aerated with 100% oxygen.

D.S. = Dry Solids.

$water_i$ = intracellular water. Superscripts are apparent extracellular cation concentrations (i.e. m.e./kg extracellular water).

a medium of the composition given in the companion footnote.

The second and third pairs of experiments studied effects of altering respectively the Na^+ and the K^+ concentration of the ionic environment, along with the effects of ouabain, on the same parameters (water, cation content, and ^{32}P-orthophosphate incorporation). Tubules were pre-incubated in Tris-Ringers solution for 30 minutes at 25° C and subsequently incubated for 30 minutes at the same temperature in either Na-Ringers solution (high Na^+) or K-Ringers (high K^+) in the presence and absence of ouabain.

Thus, in the first of each pair of experiments, the effects of the experimental conditions on tubular cellular water and cation content were determined; while in the second experiment, activity of the polyphosphoinositide complex, measured in terms of ^{32}P-orthophosphate incorporation into the various fractions of the complex, was determined. Experimental conditions were identical except that in the latter experiments, the cold NaH_2PO_4 in the incubation medium was replaced with an equivalent amount of ^{32}P-labeled compound containing 200 μC ^{32}P.

In the second experiment of each pair, the tubules were separated by centrifugation, and the tubular pellets resuspended in 5% trichloroacetic acid (TCA), homogenized, and separated again by centrifugation. This operation was repeated an additional time, the 5% supernatants combined, and incorporation of ^{32}P into the acid-soluble, heat-labile phosphate deter-

mined by a modification[20] of the method of Crane and Lipmann.[22] TCA pellets were then processed for isolation of the polyphosphoinositide complex, as described by Huggins and Cohn,[23] and Andrade and Huggins.[24] The phosphoinositide complex was then subjected to either of two treatments: (a) alkaline hydrolysis,[24] resulting in the formation of inositol phosphates, which were then chromatographed on Dowex-1-X8[25]; or (b) hydroxylamine deacylation ,[26] followed by column chromatography of the glycerol inositol phosphates on Dowex-1-Cl^- eluting with a linear gradient of lithium chloride.[26, 27] Peak fractions were pooled and concentrated in a flash evaporator at room temperature. ^{32}P-activity was measured in suitable aliquots of the concentrate. Specific activities of the various fractions were calculated from the mean of ten 10-minute counts and the mean of duplicate phosphorus determinations on each fraction.

Inulin space measurements were made essentially as described by Rosenberg et al,[28] using inulin-carboxyl-^{14}C. Na^+ and K^+ concentrations were determined by flame photometry of suitable aliquots of nitric acid extracts of the tubular pellets, separated from incubation media in specially designed centrifuge tubes.[21] A Model 143 Instrumentation Laboratories Flame Photometer with digital read-out was used for such measurements. Carbon-14 measurements were made in the Packard Model 3003 Tri-Carb Scintillation Spectrometer, equipped with Automatic Standardization Model 3950. Counting efficiency was 86%.

Results

Effects of Ouabain. Table I shows that, on both a m.e./kg D.S. basis and on a m.e./kg intracellular water basis, the potassium content of pre-incubated (treatment *a*) rabbit kidney tubules was strikingly increased by subsequent incubation in Na-Ringers solution (treatment *b*). As in experiments with kidney cortex slices,[29] such alterations were completely inhibited in tubules incubated in the presence of ouabain (treatment *c*). In the presence of the transport inhibitor, the apparent intracellular K^+ concentration was actually reduced to levels below those seen in the pre-incubated tubules.

In contrast to effects on potassium content, the Na-Ringers incubation (treatment *b*) resulted in extrusion of Na^+ from the tubules. Both on a m.e./kg D.S. basis and on a m.e./kg intracellular water basis, the Na^+ concentration in the tubular pellet was reduced. The presence of ouabain, as shown in *c*, completely inhibited such alterations and, in fact, resulted in intracellular Na^+ concentrations slightly higher than those in the preincubated tubules. Incubation in Na-Ringers (treatment *b*) resulted in an inulin space of 32.4% wet weight compared with a value of 23.8% for pre-incubated tubules, and in a reduction in intracellular water, expressed either on a wet or a dry weight basis. The addition of ouabain, as in treatment *c*, resulted in a partial reversal of these alterations.

Table II summarizes the results of the companion experiment in which metabolic activity of the polyphosphoinositide complex was examined in terms of ^{32}P-orthophosphate incor-

TABLE II

EFFECTS OF OUABAIN ON ^{32}P-ORTHOPHOSPHATE INCORPORATION INTO RABBIT KIDNEY CORTEX TUBULE PHOSPHOINOSITIDES

Fraction	Tubules pre-incubated (see a, Table I), then incubated: Without ouabain (See b, Table I)		With ouabain (0.3 mM) (See c, Table I)	
	S.A. †	R.S.A. ‡	S.A. †	R.S.A. ‡
TCA-Soluble	143,088		158,795	
7'-Acid Heat-Labile P	130,450		144,260	
TCA Protein	2,518		2,029	
Phosphoinositide Complex	8,963		7,589	
Fraction I	7,396	56.7	5,733	39.8
Fraction II	218	1.7	466	3.2
Fraction III	82	0.3	1,661	11.5
Fraction IV	1,009	7.7	2,603	18.0

†S.A. = Mean Specific Activity = Counts/min/μgm Phosphorus (P).

‡R.S.A. = Relative Mean Specific Activity (Actual values X 1,000) $= \frac{\text{S.A. of Fraction}}{\text{S.A. 7'-Acid Heat-Labile P}}$

Fraction I: Inositol Phosphate + inorganic orthophosphate.

Fraction II: Inositol Diphosphate.

Fraction III: Inositol Triphosphate.

Fraction IV: More highly phosphorylated analogs of inositol phosphate.

poration into the various components of the complex. In the presence of the transport inhibitor, ouabain, which completely blocked the ability of the pre-incubated tubules to reaccumulate K^+, striking alterations were induced in both the specific and relative specific activity of the various fractions of the tubular phosphoinositide complex. Fraction I, reflecting the metabolic activity of the diester phosphate atom of phosphatidyl inositol, clearly indicates that such activity was reduced in tubules incubated in the presence of ouabain. Results for fractions II, III, and IV, which reflect metabolic activity of the monoesterified phosphate atom, were characterized by strikingly higher specific and relative specific activities in tubules incubated in the presence of ouabain than in tubules incubated in ouabain-free medium.

Effects of Ionic Environment and Ouabain. As can be seen from Table III, pre-incubation of rabbit kidney cortex tubules in Tris-Ringers having a relatively low Na^+ concentration, followed by incubation in Na-Ringers in which the Na^+ concentration was increased 30-fold (treatment *b*), resulted in increases in intracellular water and in both intracellular Na^+ and K^+ concentrations, expressed on a m.e./kg D.S. basis. Addition of ouabain to the Na-Ringers incubation medium (treatment *c*), resulted in virtually a three-fold increase in intracellular Na^+ concentration, and not only in complete inhibition of the ability of the tubules to reaccumulate K^+ but also in a

TABLE III

EFFECTS OF HIGH Na^+ IONIC ENVIRONMENT AND OUABAIN ON WATER AND CATION TRANSPORT IN RABBIT KIDNEY CORTEX TUBULES

Treatment of Tubules *	Total Water	Inulin Space	Intracellular Water		Total Cations		Intracellular		Apparent Intracellular	
					Na^+	K^+	Na^+	K^+	Na^+	K^+
	% Wet Wt.		% Wet Wt.	kg/kg D. S.	m.e./kg D. S.				m.e./kg $water_i$	
a. Pre-incubated in Tris-Ringers †	77.0	28.0	48.9	2.13	141	152	97	137	$46^{(37)}$	$64^{(12)}$
b. As in a, then incubated in Na-Ringers ‡	80.3	26.4	54.0	2.72	251	233	113	228	$42^{(102)}$	$83^{(4)}$
c. As in a, then incubated in Na-Ringers ‡ containing 0.3 mM ouabain	81.3	28.1	54.2	3.07	484	85	329	70	$107^{(97)}$	$23^{(9)}$

*All incubations carried out for 30 minutes at 25° C.

†*Composition of low Na^+ Tris-Ringers Solution*: 159 mM Tris (pH 7.4), 7.4 mM K^+, 3.9 mM Na^+, 1.3 mM Mg^{+2}, 5.2 mM Cl^-, 1.3 mM sulfate, 3.9 mM bicarbonate, 2.2 mM acid phosphate ($-H_2PO_4$). Total osmolality = 279 mOsM. Aerated with 100% oxygen.

‡*Composition of Na-Ringers Solution*: 19 mM Tris (pH 7.4), 113 mM Na^+, 6 mM K^+, 1.2 mM Mg^{+2}, 0.2 mM Ca^{+2}, 4 mM αKG, 12 mM glucose, 113 mM Cl^-, 1.2 mM sulfate, 3.6 mM bicarbonate, 2.2 mM acid phosphate ($-H_2PO_4$). Total osmolality = 275 mOsM. Aerated with 100% oxygen.

D.S. = Dry Solids.

$water_i$ = intracellular water. Superscripts are apparent extracellular cation concentrations (i.e. m.e./kg extracellular water).

further reduction in intracellular K^+ to levels below those found in the pre-incubated tubules.

Table IV summarizes the results of the companion experiment conducted for the purpose of ascertaining the effects of alterations in ionic environment and ouabain on the metabolic activity of the polyphosphoinositide complex. As seen in the first pair of experiments (Table II), the specific activity of the 7′-acid heat-labile P was higher in tubules incubated in the presence of ouabain than in tubules incubated in the absence of the transport inhibitor. In contrast to results summarized in Table II, however, metabolic activity of the diester phosphate atom (Fraction I) was not altered in kidney cortex tubules incubated in the presence of the transport inhibitor, ouabain, and that of monoesterified phosphate, as reflected in Fractions II and IV, was sharply reduced.

Effects of altering the K^+ concentration in the ionic environment are tabulated in Tables V and VI. Preincubation of kidney cortex tubules in K^+-free Tris-Ringers and subsequently in Na^+-free, high K^+-Ringers solution (treatment *b*) resulted in a reduction in inulin space, a marked rise in intracellular water, a reduction in intracellular Na^+, and a particularly striking increase in intracellular K^+ concentration. With the exception of the intracellular Na^+ concentration, incubating the tubules in the presence of ouabain (treatment *c*) did not alter

TABLE IV

EFFECTS OF HIGH Na^+ IONIC ENVIRONMENT AND OUABAIN ON ^{32}P-ORTHOPHOSPHATE INCORPORATION INTO RABBIT KIDNEY CORTEX TUBULE PHOSPHOINOSITIDES

Fraction	Tubules pre-incubated (see a, Table III), then incubated: Without ouabain (See b, Table III)		With ouabain (0.3 mM) (See c, Table III)	
	S.A. †	R.S.A. ‡	S.A. †	R.S.A. ‡
TCA-Soluble	382,172		389,481	
7' -Acid Heat-Labile P	219,381		302,743	
TCA Protein	5,523		5,969	
Phosphoinositide Complex	27,974		28,027	
Fraction I	9,201	42	9,362	39
Fraction II	21,484	100	9,191	30
Fraction IV	62,021	280	47,760	157

†S.A. = Mean Specific Activity = Counts/min/μgm Phosphorus (P).

‡R.S.A. = Relative Mean Specific Activity = $\frac{\text{S.A. of Fraction}}{\text{S.A. 7'-Acid Heat-Labile P}}$ (Actual Values X 1,000)

Fraction I: Glycerophosphate, Inositol Phosphate, and inorganic orthophosphate.

Fraction II: Glycerophosphoinositolphosphate.

Fraction IV: Glycerophosphoinostoldiphosphate.

TABLE V

EFFECTS OF A HIGH K^+ IONIC ENVIRONMENT AND OUABAIN ON WATER AND CATION TRANSPORT IN RABBIT KIDNEY CORTEX TUBULES

Treatment of Tubules *	Total Water	Inulin Space	Intracellular Water		Total Cations Na^+	Total Cations K^+	Intracellular Na^+	Intracellular K^+	Apparent Intracellular Na^+	Apparent Intracellular K^+
	% Wet Wt.		% Wet Wt.	kg/kg D. S.	m.e./kg D. S.				m.e./kg $water_i$	
a. Pre-incubated in K^+-free Tris-Ringers †	82.2	41.2	40.8	2.28	139	28	76	26	$33^{(27)}$	$11^{(8)}$
b. As in a, then incubated in K-Ringers ‡ (Na^+-free)	88.2	35.1	53.1	4.48	69	859	43	537	$10^{(9)}$	$120^{(109)}$
c. As in a, then incubated in K-Ringers ‡ containing 0.3 mM ouabain	87.2	36.0	51.3	4.03	94	798	69	489	$17^{(9)}$	$122^{(109)}$

*All incubations carried out for 30 minutes at 25° C.

†*Composition of K+-free Tris-Ringers Solution*: 145 mM Tris (pH 7.4), 11.3 mM Na^+, 1.1 mM Mg^{+2}, 4.4 mM Cl^-, 1.1 mM sulfate, 2 mM acid phosphate ($-H_2PO_4$), 3.3 mM bicarbonate ($-HCO_3$). Total osmolality = 295 mOsM. Aerated with 100% oxygen.

‡*Composition of K-Ringers Solution*: 30 mM Tris (pH 7.4), 115.7 mM K^+, 1.2 mM Mg^{+2}, 111 mM Cl^-, 1.2 mM sulfate, 2.1 mM acid phosphate ($-H_2PO_4$), 4 mM α-KG, 11.4 mM glucose. Total osmolality = 298 mOsM. Aerated with 100% oxygen.

D.S. = Dry Solids.

$water_i$ = intracellular water. Superscripts are apparent extracellular cation concentrations (i.e. m.e./kg extracellular water).

TABLE VI

EFFECTS OF HIGH K^+ IONIC ENVIRONMENT AND OUABAIN ON ^{32}P-ORTHOPHOSPHATE INCORPORATION INTO RABBIT KIDNEY CORTEX TUBULE PHOSPHOINOSITIDES

Fraction	Tubules pre-incubated (see a, Table V), then incubated: Without ouabain (See b, Table V)		With ouabain (0.3 mM) (See c, Table V)	
	S.A. †	R.S.A. ‡	S.A. †	R.S.A. ‡
TCA-Soluble	299, 109		308, 102	
7' - Acid Heat-Labile P	272, 483		184, 833	
TCA Protein	1, 977		1, 966	
Phosphoinositide Complex	7, 274		7, 762	
Fraction I	7, 318	27	5, 539	30
Fraction II	5, 140	19	6, 159	33
Fraction III	2, 095	8	8, 849	48

†S.A. = Mean Specific Activity = Counts/min/μgm Phosphorus (P).

‡R.S.A. = Relative Mean Specific Activity (Actual Values X 1,000) $= \frac{\text{S.A. of Fraction}}{\text{S.A. 7'-Acid Heat-Labile P}}$

Fraction I: Glycerophosphate, Inositol Phosphate, and inorganic orthophosphate.
Fraction II: Glycerophosphoinositolphosphate.
Fraction III: Inositol Diphosphate.

any of these results. Ability of the tubules to extrude Na^+, as reflected in intracellular Na^+ concentration, however, did appear to be significantly reduced.

From the ^{32}P-orthophosphate incorporation data (Table VI), it can be seen that in the experiments using a high K^+-Ringers solution, the specific activity of the 7'-acid heat-labile P fraction was sharply reduced in tubules incubated in the presence of the transport inhibitor, ouabain. This is in sharp contrast to the effects of ouabain on tubules incubated in the high Na^+-Ringers (Table IV). The effects of ouabain on incorporation of the isotope into the monoesterified phosphate of phosphatidyl inositol phosphate (Fraction II) were also strikingly different in these two experiments. A two-fold increase in the relative specific activity of this fraction (Table VI) clearly indicates an increase in its metabolic activity, in contrast to the reduced activity observed in the experiments with a high Na^+ incubation medium (Table IV). An increase in the metabolic activity of monoesterified phosphate in the polyphosphoinositide complex of tubules incubated in the presence of ouabain is also indicated by the data for Fraction III. Both the specific and the relative specific activity of this fraction were strikingly higher in tubules incubated in the presence of the transport inhibitor, ouabain, than in its absence.

Discussion

In terms of a theory of cation transport which postulates that monoesterified phosphate on the inositol moiety of phosphatidyl inositol might provide anionic sites that act as cation carriers via a phosphorylation-dephos-

phorylation cycle, catalyzed in its simplest sequence by phosphatidyl inositol kinase and diphosphoinositide phosphomonoesterase activities; one might reasonably expect that turnover of monoesterified phosphate would be reduced in tubules in which movement of sodium out of, and of potassium into, tubule cells had been inhibited by ouabain. Results of studies summarized in Tables I and II clearly demonstrate that this is not the situation. Metabolic activity of monoesterified phosphate in the polyphosphoinositides was strikingly higher in tubules incubated in the presence of ouabain than in tubules incubated in its absence.

Now although it is clearly recognized that the concomitant occurrence of high metabolic activity of the monoesterified phosphate of the inositol moiety of the polyphosphoinositides together with complete inhibition of extrusion of Na^+ out of, and of K^+ into, the intracellular compartment does not provide *per se* unequivocal proof of a metabolic relationship between these parameters; nevertheless, it hardly seems fortuitous that the metabolic activity of the monesterified phosphate was greatly reduced in the presence of ouabain (see Table III) and a high extracellular Na^+ — the same experimental conditions in which movement of Na^+ into the intracellular compartment was greatly stimulated. Furthermore in the third model system, in which tubules were preincubated in Tris-Ringers solution and then incubated in a high K^+ environment in the presence and absence of ouabain, it will be noted that again in the presence of ouabain extrusion of Na^+ from the intracellular compartment was largely inhibited while the metabolic activity of the monoesterified phosphate on the inositol moiety of the tubular polyphosphoinositides was strikingly increased (see Tables V and VI). Inhibitory effects of ouabain on reaccumulation of intracellular K^+ were largely negated by the high extracellular K^+ environment.

From the foregoing observations, it seems quite clear that increased turnover of monoesterified phosphate on the inositol moiety of tubular polyphosphoinositides is correlatable only with ouabain-inhibited extrusion of Na^+ from the intracellular compartment. On the other hand, inhibitory effects of ouabain on tubular cell reaccumulation of K^+, as seen in the experiments summarized in Tables I and III, appears to be completely dissociable from such activity. In both the first (Tables I and II) and second pair of experiments (Tables III and IV), ouabain completely inhibited reaccumulation of K^+ by the K^+-depleted cells; yet in the first case turnover of the monoesterified phosphate on the inositol moiety of the polyphosphoinositides was strikingly higher in the presence of ouabain, while in the second pair of experiments turnover was strikingly lower in the presence of ouabain. In terms of a peritubular cation pump providing for a coupled movement of Na^+ out of, and of K^+ into, the intracellular compartment, our experimental findings obviously are not in accord with the proposed polyphosphoinositide phosphorylation-dephosphorylation cation transport mechanism.

An alternative to the above discussed mechanism of membrane transport, however, is the theory that changes in monoesterified phosphate

on the inositol moiety of the polyphosphoinositides, arising as a result of the balance of phosphophosphoinositide kinase and polyphosphoinositide phosphomonoesterase activities, might affect control of the flux of univalent cations by causing variations in the anionic surface charge of membranes. In this respect, the strongly electronegative phosphate groups of polyphosphoinositides — closely oriented in a bimolecular lipid membrane largely composed of neutral molecules such as lecithin and cholesterol — might be expected to mutually repel each other. Alterations in the spacing of the lipid molecules in the membrane could thus arise from head group repulsion and bring about changes in the membrane's permeability to cations.[30]

In this context, factors affecting the charge on the head group of the polyphosphoinositides become of paramount importance in the regulation of membrane permeability. Two of these: (a) the relative rates of the phosphorylative and dephosphorylative activities, and (b) the neutralizing effect of counter-ion binding on the electronegative monoesterified phosphate groups on the inositol moiety of the polyphosphoinositides seem fairly obvious. Attempts by our group to procure information concerning the first of these have thus far failed. The assay of both phosphoinositide kinase and polyphosphoinositide phosphomonoesterase activities, as well as the estimation of the level of ATP available for phosphorylation, is complicated by the presence of considerable ATPase activity in tubular systems subjected to the treatment described in Table I.

In regard to the second factor, i.e. counter-ion binding, in view of the equivalent charge-neutralizing capacity of Na^+ and K^+, one might expect an environment high in either of these ions to give rise to comparable effects on membrane permeability and turnover of monoesterified phosphate on the inositol moiety of the polyphosphoinositides. From the results summarized in Tables III-IV and Tables V-VI, it can readily be seen that diverse rather than comparable effects were obtained.

While the experimental data thus support neither the proposed phosphoinositide phosphorylation-dephosphorylation cation transport mechanism nor the head group repulsion concept of regulation of membrane permeability, the striking alteration in metabolic activity of monoesterified phosphate on the inositol moiety of tubular polyphosphoinositides during ouabain inhibited transport still remains an exciting finding that challenges explanation. The increasing tempo of investigative effort focusing on the purification and characterization of the enzyme systems involved in the metabolic turnover of kidney phosphoinositides can be expected to contribute significantly to the understanding of membrane physiology and pathology. Progress in the development of sophisticated physiochemical methods for demonstrating conformational changes, which may be associated with membrane phosphoinositide phosphorylation and polyphosphoinositide dephosphorylation, may also add to our knowledge of membrane transport.

REFERENCES

1. Skou, J.C.: The influence of some cations on an adenosine triphosphatase from peripheral nerves. *Biochim Biophys Acta* 23:394-401, 1957.

2. Skou, J.C.: Further investigations on a $Mg^{++} + Na^{+}$-activated adenosintriphosphatase, possibly related to the active, linked transport of Na^{+} and K^{+} across the nerve membrane. *Biochim Biophys Acta* 42:6-23, 1960.

3. Bonting, S.L.; Simon, K.A., and Hawkins, N.M.: Studies on sodium-potassium-activated adenosine triphosphatase. I. Quantitative distribution in several tissues of the cat. *Arch Biochem Biophys* 95:416-23, Dec 1961.

4. Whittam, R., and Wheeler, K.P.: The sensitivity of kidney ATPase to ouabain and to sodium and potassium. *Biochim Biophys Acta* 51:622-4, 1961.

5. Skou, J.C.: Preparation from mammallian brain and kidney of the enzyme system involved in active transport of Na^{+} and K^{+}. *Biochim Biophys Acta* 58:314-25, 1962.

6. Wheeler, K.P., and Whittam, R.: Some properties of a kidney adenosine triphosphatase relevant to active cation transport. *Biochem J* 85:495-507, Oct-Dec 1962.

7. Kinsolving, C.R.; Post, R.L., and Beaver, D.L.: Sodium plus potassium transport adenosine triphosphatase activity in kidney. *J Cell Comp Physiol* 62:85-93, Aug 1963.

8. Landon, E.J., and Norris, J.L.: Sodium- and potassium-dependent adenosine triphosphatase activity in a rat-kidney endoplasmic reticulum fraction. *Biochim Biophys Acta* 71:266-76, 1963.

9. Sen, A.K., and Post, R.L.: Stoichiometry and localization of adenosine triphosphate-dependent sodium and potassium transport in the erythrocyte. *J Biol Chem* 239: 345-52, Jan 1964.

10. Skou, J.C.: Enzymatic basis for active transport of Na^{+} and K^{+} across cell membrane. *Physiol Rev* 45:596-617, Jul 1965.

11. Hokin, L.E., and Hokin, M.R.: The mechanism of phosphate exchange in phosphatidic acid in response to acetylcholine. *J Biol Chem* 234:1387-90, Jun 1959.

12. Hokin, L.E., and Hokin, M.R.: Phosphatidic acid metabolism and active transport of sodium. *Fed Proc* 22:8-18, Jan-Feb 1963.

13. Hokin, M.R., and Hokin, L.E.: The synthesis of phosphatidic acid and protein-bound phosphorylserine in salt gland homogenates. *J Biol Chem* 239:2116-22, Jul 1964.

14. Heald, P.J.: Phosphoprotein metabolism and ion transport in nervous tissue: A suggested connection. *Nature* 193:451-4, Feb 3, 1962.

15. Judah, J.D.; Ahmed, K., and McLean, A.E.M.: Ion transport and phosphoproteins of human red cells. *Biochim Biophys Acta* 65:472-80, 1962.

16. Glynn, I.M., et al: The adenosine-triphosphatase system responsible for cation transport in electric organ: Exclusion of phospholipids as intermediates. *Biochem J* 94:692-9, Jan-Mar 1965.

17. Hokin, L.E., and Hokin, M.R.: The incorporation of ^{32}P from triphosphate into polyphosphoinositides [γ-^{32}P]adenosine and phosphatidic acid in erythrocyte membranes. *Biochim Biophys Acta* 84:563-75, 1964.

18. Hayashi, K.; Nakamura, I., and Yamazoe, S.: Effect of sodium ions on the incorporation of P^{32}-labeled orthophosphate into phosphatidic acid and inositides in guinea pig brain slices. *J Biochem* 56:381-91, Nov 1964.

19. Standefer, J.C., and Samson, F.E.: Phosphoinositides related to the (Na+ + K+) activated ATPase in rat brain. *Fed Proc* 26:765, Abstract 2837, Mar-Apr 1967.

20. Santiago-Calvo, E., et al: The chromatographic separation of polyphosphoinositides and studies on their turnover in various tissues. *Biochim Biophys Acta* 84:550-62, 1964.

21. Burg, M.B., and Orloff, J.: Oxygen consumption and active transport in separated renal tubules. *Amer J Physiol* 203:327-30, Aug 1962.

22. Crane, R.K., and Lipmann, F.: The effect of arsenate on aerobic phosphorylation. *J Biol Chem* 201:235-43, Mar 1953.

23. Huggins, C.G., and Cohn, D.V.: Studies concerning the composition, distribution, and turnover of phosphorus in a phosphatido-peptide fraction from mammalian tissue. *J Biol Chem* 234:257-61, Feb 1959.

24. Andrade, F., and Huggins, C.G.: Myo-inositol phosphates in a phosphoinositide complex from kidney. *Biochim Biophys Acta* 84:681-93, 1964.

25. Seiffert, U.B., and Agranoff, B.W.: Isolation and separation of inositol phosphates from hydrolysates of rat tissues. *Biochim Biophys Acta* 98:574-81, 1965.

26. Brockerhoff, H., and Ballou, C.E.: The structure of the polyphosphoinositide complex of beef brain. *J Biol Chem* 236:1907-11, Jul 1961.

27. Palmer, F.B., and Rossiter, R.J.: A simple procedure for the study of inositol phosphatides in cat brain slices. *Canad J Biochem* 43:671-83, Jun 1965.

28. Rosenberg, L.E.; Downing, S.J., and Segal, S.: Extracellular space estimation in rat kidney slices using C^{14} saccharides and phlorizin. *Amer J Physiol* 202:800-4, Apr 1962.

29. Kleinzeller, A., and Knotkova, A.: The effect of ouabain on the electrolyte and water transport in kidney cortex and liver slices. *J Physiol* (Lond.) 175:172-92, Dec 1964.

30. Dawson, R.M.C.: "The metabolism and function of polyphosphoinositides in the nervous system," in *Cyclitols and Phosphoinositides*, edited by H. Kindl. Pergamon Press, 1966, pp 57-67.