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SODIUM ION AND THE NEUTROTRANSMITTER-STIMULATED ^{32}P LABELLING OF PHOSPHOINOSITIDES AND OTHER PHOSPHOLIPIDS IN THE IRIS MUSCLE

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Summary

The effects of Na^+, other cations and the neurotransmitters, acetylcholine and norepinephrine on $^{32}P_i$ incorporation into phospholipids of the rabbit iris smooth muscle were investigated [1]. The basal ^{32}P-labelling of phospholipids, including phosphatidic acid, phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine and the polyphosphoinositides increased with Na^+ concentration [2]. The neurotransmitter-stimulated ^{32}P labelling of phosphatidic acid, phosphatidylinositol and phosphatidylcholine is dependent on the presence of extracellular Na^+ [3]. The monovalent cation requirement for Na^+ is specific. Of the monovalent cations Li^+, NH_4^+, K^+, choline$^+$ and Tris, only Li^+ partially substituted for Na^+ [4]. A significant decrease in ^{32}P labelling of phospholipids in response to acetylcholine was observed when Ca^{2+} and/or K^+ were added to an isoosmotic medium deficient of Na^+ [5]. Ouabain, which blocks the Na^+-pump, inhibited the basal $^{32}P_i$ incorporation into phosphatidylcholine and the acetylcholine-stimulated ^{32}P labelling of phosphatidic acid, phosphatidylinositol and phosphatidylcholine [6]. It was suggested that phosphoinositide breakdown is associated with Ca^{2+} influx as we have previously reported (Akhtar, R.A. and Abdel-Latif, A.A. (1978) J. Pharmacol. Exp. Ther. 204, 655—668) and that the enhanced ^{32}P-labelling of phosphoinositides could be associated with Na^+ outflux, via the Na^+-pump mechanism.

Introduction

The response of a variety of tissues to neurotransmitters, neurohumors, neuropharmacological agents and electrical pulses involves increases in the

phosphate turnover of certain phospholipids, such as phosphatidic acid and the phosphoinositides (for reviews see Refs. 1 and 2). Neither the molecular mechanism nor the physiological significance of this 'phosphoinositide effect' in cell membranes is well understood. However, it is well established that this phenomenon is mediated through muscarinic cholinergic and α-adrenergic receptors [1]. While activation of these receptors is known to lead either to changes in the intracellular ionic environment, or to changes in intracellular concentration of a second messenger such as cyclic AMP or cyclic GMP, or to changes in relevant enzymes, there has been only few reports which showed that some of these changes could mediate the increase observed in phospholipid phosphate turnover in response to receptor activation [2]. Michell and his colleagues [3] proposed that the agonist-stimulated breakdown of phosphatidylinositol in target tissue leads to the release of Ca^{2+} which in turn triggers the observed cellular responses. In certain tissues there is good experimental evidence to support this hypothesis (for summary see Refs. 1 and 4).

In previous communications from this laboratory we reported on the characteristics of the phosphoinositide effect in the rabbit iris smooth muscle (for summary see Refs. 5 and 6). Among key findings in these studies were the following: (a) That in iris muscle which was prelabelled with $^{32}P_i$, acetylcholine stimulates the breakdown of triphosphoinositide and $^{32}P_i$ labelling of phosphatidic acid and phosphatidylinositol [7]. (b) That in iris muscle which was prelabelled with $^{32}P_i$ and its Ca^{2+} content depleted with EGTA, the acetylcholine-stimulated breakdown of polyphosphoinositides and ^{32}P-labelling of phosphatidic acid and to a much lesser extent phosphatidylinositol is dependent on the presence of extracellular Ca^{2+} [8]. (c) That in iris muscle which was prelabelled with [^{3}H]myoinositol, acetylcholine stimulates the release of inositol monophosphate and inositol triphosphate [9]. In light of these findings we suggested that the interaction of acetylcholine (or norepinephrine) with its receptor results in an activated complex, which in turn could lead to the enhanced phospholipid metabolism via a Ca^{2+}-mediated step (Scheme I) and consequently to muscle response [6,8]. More recently the dependency of the phosphoinositide effect on the presence of extracellular Ca^{2+} has been demonstrated in synaptosomes [10—12].

Neurotransmitter + Receptor → Neurotransmitter — Receptor → Ca^{2+}_{int} ↑ →

Phosphoinositide breakdown → Diacylglycerol + inositol phosphates

Scheme I. A possible role for Ca^{2+} in the neurotransmitter-stimulated breakdown of phosphoinositides in the iris muscle.

Acetylcholine and norepinephrine act to increase the intracellular concentration of Ca^{2+} [13] and both phosphatidylinositol-phosphodiesterase [14] and triphosphoinositide-phosphodiesterase [15] are stimulated by Ca^{2+}.

Activation of muscarinic cholinergic and α-adrenergic receptors in smooth muscle also leads to increase in cell-surface permeability to Na^+ and K^+ in addition to Ca^{2+} [16]. The question arises as to whether these monovalent cations are involved in the chain of events leading from receptor activation to phospholipid metabolism and subsequently to muscle response. The present study

was initiated to provide evidence pertinent to this issue. In contrast to our previous studies which were run under breakdown conditions [6—8], the present studies were conducted under synthetic conditions. We present data in the present study which suggest that the rephosphorylation of diacylglycerol (Scheme I) to phosphatidic acid and subsequently to the phosphoinositides could be associated with the Na^+-pump mechanism.

Materials and Methods

Materials

Acetylcholine, eserine sulfate, norepinephrine and ouabain were purchased from Sigma Chemical Co., St. Louis, MO; and ^{32}P carrier-free was obtained from New England Nuclear Corp., Boston, MA. Phospholipids were from sources described previously [7,8]. All other chemicals were of reagent grade.

Methods

Preparation and incubation of iris muscle. In the present work albino rabbits of either sex, weighing approx. 2 kg were used. The unanesthetized rabbits were stunned by a blow to the head and the eyes were enucleated immediately and placed in ice and transported from the slaughter house to the laboratory in less than 30 min. The irises were removed and placed, in pairs from the same animals, in tubes containing 3 ml of the isoosmotic medium.

In general irises were incubated singly (of the pair one was used as control) in isoosmotic medium that contained 10 μCi $^{32}P_i$ at 37°C for 1 h in the presence and absence of the pharmacological agent as indicated. The neurotransmitters, acetylcholine and norepinephrine were added 30 min after preincubation of the tissue with $^{32}P_i$. Where the Na^+ concentration was varied isoosmolar substitution of NaCl was made by sucrose. The reactions were ended by addition of ice-cold trichloroacetic acid to a final concentration of 10%.

Extraction and isolation of phospholipids. After ending the reaction with trichloroacetic acid, the acid was aspirated and the muscle was washed twice with distilled water. The phospholipids were extracted and isolated by means of two-dimensional thin-layer chromatography (TLC) as described previously [7]. In brief, the iris muscle was homogenized in 2 ml of chloroform/methanol/HCl (300 : 300 : 1.5 by volume). The residue was first extracted with the same solvent, then with chloroform/methanol/HCl (400 : 200 : 1.5 by volume). The three extracts were pooled and evaporated under N_2. The residue was dissolved in 2 ml of chloroform and washed twice with 1 ml of 0.1 N HCl. The chloroform phase was dried under a stream of N_2 and the residue was dissolved again in 2 ml of chloroform. Hereafter, this will be referred to as 'total phospholipid extract'. Aliquots (50 μl in duplicate) of the total phospholipid extract were removed and counted. The remaining lipid extract was dried under N_2 and the residue dissolved in 70 μl of chloroform. The lipids were detected by means of I_2 vapor and spots corresponding to each phospholipid were scraped off the plate and counted in 10 ml of scintillation fluid in a Beckman liquid-scintillation spectrometer. All the data reported in this paper on the ^{32}P radioactivity in phospholipids are expressed as cpm/iris.

All of the experiments reported in this paper were carried out either in duplicate or triplicate.

Results

Effects of extracellular Na^+ concentration on basal ^{32}P labelling of total phospholipids

The basal ^{32}P incorporation into total phospholipids of the iris muscle is dependent on the extracellular Na^+ concentration (Fig. 1). The ^{32}P labelling of total phospholipids increased with Na^+ concentration and levelled off around 75 mM concentration of the cation. In the absence of Na^+ the ^{32}P radioactivity recovered in total phospholipids is only 7% of that obtained in the presence of 100 mM Na^+. Under these experimental conditions an increase in intracellular Na^+ concentration is presumably due to passive flux of the cation down its electrochemical gradient.

Effects of extracellular Na^+ concentration on neurotransmitter-itimulated ^{32}P labelling of phospholipids

To show whether Na^+ is required for the enhanced ^{32}P labelling of phosphatidic acid the phosphoinositides of the iris muscle in response to acetylcholine and norepinephrine we have investigated the effects of the neurotransmitters in the presence of various concentrations of this monovalent cation. In the absence of Na^+ acetylcholine had little influence on the ^{32}P labelling of all of the phospholipids investigated, however, at 5 mM concentration of the cation

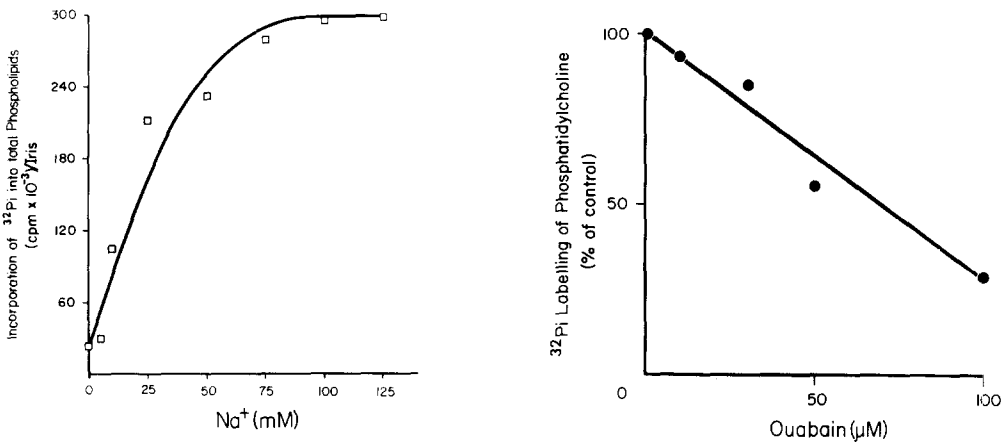


Fig. 1. Effects of extracellular Na^+ concentrations on basal ^{32}P labelling of total phospholipids in the rabbit iris smooth muscle. Irises were incubated in an isoosmotic medium (2.5 mM $CaCl_2$/1.3 mM $MgCl_2$/ 5 mM KCl/26 mM Tris-HCl buffer, pH 7.4, 10 mM D-glucose and sucrose to maintain the osmolarity) that contained 10 μCi of $^{32}P_i$ in a total volume of 1 ml at 37°C for 1 h with Na^+ at 2.5—125 mM. ^{32}P labelling of phospholipids was determined. The results are expressed as the mean of five separate experiments.

Fig. 2. Effects of ouabain on ^{32}P labelling of phosphatidylcholine. Irises were incubated singly (of the pair one was used as control) in an isoosmotic medium (its composition was the same as that given under Fig. 1 except that 125 mM NaCl was substituted for the sucrose) that contained 10 μCi $^{32}P_i$ at 37°C for 1 h in the absence and presence of ouabain at 10—100 μM. Phosphatidylcholine was isolated from the lipid extract as described in the text. The results are expressed as the mean of three separate experiments.

TABLE I

EFFECTS OF EXTRACELLULAR Na^+ CONCENTRATION ON ^{32}P LABELLING OF PHOSPHOLIPIDS IN PRESENCE AND ABSENCE OF ACETYLCHOLINE

Irises were incubated singly (of the pair one was used as control) in isoomotic medium (its composition was the same as that given under Fig. 1) that contained 10 μCi of $^{32}P_i$ at 37°C for 30 min with Na^+ at 2.5—125 mM; then acetylcholine plus eserine (0.05 mM each) was added and the incubation was continued for an additional 30 min. Individual phospholipids were isolated and their ^{32}P-radioactivities determined. The results are expressed as the mean of four separate experiments. %, percent of control.

Na^+ (mM)	^{32}P labelling of phospholipids (cpm · 10^{-2})/iris									
	Phosphatidic acid	%	Phosphatidyl-inositol	%	Phosphatidyl-choline	%	Phosphatidyl-ethanolamine	%	Polyphospho-inositides *	%
0	5 (4.5) **	90	4 (4)	100	3.5 (3.6)	103	1.6 (1.6)	100	24.6 (22.8)	93
2.5	17.9 (22.5)	126	20 (21)	105	9.5 (9.9)	104	3 (3.2)	107	38 (37)	97
5	18 (35.8)	199	18.7 (45)	241	27.6 (30)	109	14.8 (13.2)	89	54.7 (50)	91
10	15 (33)	220	19.5 (43)	221	28 (30.8)	110	14.8 (16.9)	114	71 (73)	103
25	98.7 (165)	167	237 (356)	150	418 (373)	89	44.7 (42.5)	95	563 (550)	98
50	86.6 (180)	208	230 (359)	156	623 (693)	111	49.3 (50)	101	684 (640)	94
125	126 (250)	198	222 (380)	171	715 (944)	132	53.9 (54.9)	102	731 (720)	98

* Phosphatidylinositol 4-diphosphate and phosphatidylinositol 4,5-triphosphate.

** In the presence of acetylcholine plus eserine.

TABLE II

EFFECT OF NOREPINEPHRINE ON ^{32}P LABELLING OF PHOSPHOLIPIDS IN PRESENCE AND ABSENCE OF Na^+

Conditions of incubation were the same as described under Table I except that acetylcholine was replaced by norepinephrine (0.05 mM). The results are expressed as the mean of two separate experiments. Values in brackets represent the fold increase over control.

Additions (mM)	Effect of norepinephrine on ^{32}P labelling of phospholipids (cpm · 10^{-2})/iris				
	Phosphatidic acid	Phosphotidyl-inositol	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Polyphospho-inositides
None *	15	25	12	2	111
Norepinephrine (0.05)	20 (1.3)	32 (1.3)	13 (1.1)	2 (1.0)	87 (0.78)
Na^+ (125)	118 (7.9)	358 (14.3)	506 (42.2)	66 (33.0)	980 (8.8)
Norepinephrine (0.05) + Na^+ (125)	243 (16.2)	579 (23.2)	521 (43.4)	68 (34.0)	895 (8.1)

* Control, in absence of norepinephrine and Na^+.

the neurotransmitter provoked a 99%, 141% and 9% increase in the labelling of phosphatidicacid, phosphatidylinositol and phosphatidylcholine respectively (Table I). The neurotransmitter had little effect on ^{32}P labelling of phosphatidylethanolamine and the polyphosphoinositides. Treatment of the muscle with chloroform/methanol/HCl extracts all of the tissue lipids including triphosphoinositide, but under these experimental conditions the plasmalogens are hydrolyzed [6]. Under the same experimental conditions the basal ^{32}P labelling of the various phospholipids increased appreciably with Na^+ concentration (Table I). Thus at 125 mM Na^+ the basal ^{32}P labelling of phosphatidic acid, phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine and the polyphosphoinositides increased by 25-, 56-, 204-, 34- and 30-fold respectively (Table I). Acetylcholine, in the presence of 125 mM Na^+, enhanced the

TABLE III

EFFECTS OF MONOVALENT CATIONS ON ^{32}P LABELLING OF PHOSPHOLIPIDS IN PRESENCE AND ABSENCE OF ACETYLCHOLINE

Conditions of incubation were the same as described under Table I except that Na^+ (125 mM) was replaced by various cations (125 mM) as indicated. The results are expressed as the mean of two separate experiments. %, percent of control.

Cation (125 mM)	^{32}P labelling of phospholipids (cpm × 10^{-2})/iris					
	Phosphatidic acid	%	Phosphatidyl-inositol	%	Phosphatidyl-choline	%
None	11 (12.9) *	117	15.6 (16.2)	104	7 (8)	114
NaCl	126 (390)	310	397 (698)	176	486 (594)	122
LiCl	64.7 (100)	155	55.9 (50)	89	63.9 (69)	108
NH_4Cl	13.9 (13.6)	98	6 (7)	117	1.6 (1.8)	113
KCl	12.9 (14)	109	9.5 (11.6)	122	3.9 (2.9)	74
Choline Cl	1.5 (1.2)	80	1.8 (2)	111	1.3 (1.1)	85
Tris-HCl	8.2 (8.1)	99	3.7 (4.5)	122	1.3 (1.5)	115

* In the presence of acetylcholine plus eresine (0.05 mM each).

^{32}P labelling of phosphatidic acid, phosphatidylinositol and phosphatidylcholine by 98%, 71% and 32% respectively.

Similarly when Na^+ was ommitted from the incubation medium norepinephrine had little effect on ^{32}P labelling of phospholipids, however, in the presence of 125 mM Na^+ the neurotransmitter provoked a 106%, 62%, and 3% increase in the ^{32}P labelling of phosphatidic acid, phosphatidylinositol and phosphatidylcholine respectively (Table II).

Effects of monovalent cations on the acetylcholine-stimulated ^{32}P labelling of phospholipids

The specificity of the phospholipid effect for monovalent cations was investigated. Na^+ is required for both basal and acetylcholine-stimulated ^{32}P labelling of phospholipids (Table III). Li^+, NH_4^+, K^+, choline$^+$ or Tris did not substitute for Na^+. However Li^+ is the only monovalent cation which substituted partially for Na^+. In smooth muscle Li^+ can substitute for Na^+ in the Na^+-Ca^+ exchange carrier [16].

Effects of Na^+, K^+ and Ca^{2+} on the acetylcholine-stimulated ^{32}P labelling of phospholipids

Results from studies on the effects of Na^+, K^+ and Ca^{2+} on the phospholipid effect in response to acetylcholine are given in Table IV. In general the phospholipid effect was enhanced by addition of Na^+ and depressed by addition of K^+ and/or Ca^{2+} to the basic incubation medium. Thus addition of Na^+ increased the acetylcholine-stimulated ^{32}P labelling of phosphatidic acid, phosphatidylinositol and phosphatidylcholine by 131%, 112% and 27% respectively, while addition of K^+ and/or Ca^{2+} depressed the ^{32}P labelling by 15 to 22% of that of the control. In contrast addition of Na^+ plus K^+ increased the acetylcholine-stimulated ^{32}P labelling of phosphatidic acid, phosphatidylinositol and phosphatidylcholine by 306%, 290% and 158% respectively (Table IV).

TABLE IV

EFFECTS OF Na^+, K^+ AND Ca^{2+} ON THE ACETYLCHOLINE-STIMULATED ^{32}P-LABELLING OF PHOSPHOLIPIDS

Conditions of incubation were the same as described under Table I except that the basic incubation medium consisted of: 1.3 mM $MgCl_2$/26 mM Tris-HCl buffer, pH 7.4, 10 mM-D-glucose and sucrose to maintain the osmolarity. The concentrations of Na^+, K^+ and Ca^{2+} were (mM): 125, 5 and 2.5 respectively. The results are expressed as the mean of 2 separate experiments.

Cation added	Effect of acetylcholine on ^{32}P labelling of phospholipids (% of control)		
	Phosphatidic acid	Phosphatidylinositol	Phosphatidylcholine
None	117	119	105
Na^+	231	212	127
K^+	94	85	92
Ca^{2+}	89	91	83
$Na^+ + K^+$	406	390	259
$Na^+ + Ca^{2+}$	168	126	128
$K^+ + Ca^{2+}$	85	81	78
$Na^+ + K^+ + Ca^{2+}$	218	161	111

TABLE V

EFFECT OF OUABAIN ON THE ACETYLCHOLINE-STIMULATED ^{32}P-LABELLING OF PHOSPHOLIPIDS

Conditions of incubation were the same as described under Table I except that the irises were incubated in a complete medium that contained 125 mM Na^+/5 mM K^+/2.5 mM Ca^{2+}/1.3 mM Mg^{2+}/26 mM Tris buffer and 10 mM D-glucose.

Additions (mM)	Effect of acetylcholine on ^{32}P labelling of phospholipids (% of control)		
	Phosphatidic acid	Phosphatidyl-inositol	Phosphatidyl-choline
Ouabain (0.1)	123	110	51
Acetylcholine (0.05)	309	176	138
Ouabain (0.1) + acetylcholine (0.05)	99	92	105

Effect of ouabain on ^{32}P labelling of phospholipids in absence and presence of acetylcholine

The finding that the phospholipid effect was maximal in the presence of Na^+ plus K^+ (Table IV) suggested to us that in the iris smooth muscle this phenomenon could be linked to the Na^+-pump mechanism. To test this hypothesis we investigated the effect of ouabain, which blocks the Na^+-pump, on the phospholipid effect. Ouabain, at 0.1 mM concentration, abolished the stimulatory effect of acetylcholine on ^{32}P labelling of phosphatidic acid, phosphatidylinositol and phosphatidylcholine and furthermore it inhibited the basal ^{32}P labelling of phosphatidylcholine by 49% and increased that of phosphatidic acid by 23% (Table V). The inhibitory effect of the glycoside on the basal ^{32}P labelling of phosphatidylcholine is concentration-dependent (Fig. 2). Thus the inhibitory effect of the drug increased from 7% at 10 μM to 68% at 100 μM.

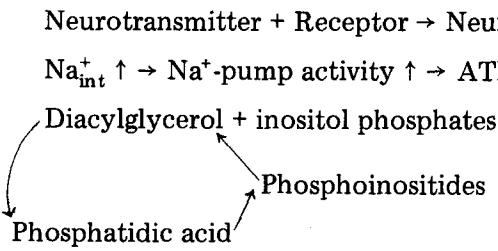
Discussion

It is clear from the data presented in this paper that in the rabbit iris smooth muscle the turnover of the polarhead groups of phospholipids are profoundly affected by the cationic composition, specifically Na^+, K^+ and Ca^{2+}, of the medium and that the presence of Na^+ is required for the phospholipid effect. The role the neurotransmitters acetylcholine and norepinephrine play in this phenomenon is presumably to raise the membrane permeability to the cations. This conclusion is in accord with our previous studies with this tissue [5—8] and those of others working with a variety of tissues [10—12; 17—19]. Thus several years ago Brossard and Quastel [17], working with rat brain slices, reported that acetylcholine stimulation of $^{32}P_i$ incorporation into phospholipids is dependent on the presence of Na^+. More recently Keryer et al. [19] reported that in rat parotid glands cholinergic stimulation of *myo*-[2-^{3}H]inositol into phosphatidylinositol is dependent on extracellular Na^+.

The findings in the present studies, namely: (a) the significant increase in the basal ^{32}P labelling of phospholipids, including phosphatidic acid, phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine phosphate turn-

over with Na^+ concentration (Fig. 1 and Table I); (b) the dependency of the neurotransmitter-stimulated ^{32}P labelling of phosphatidic acid, phosphatidylinositol and phosphatidylcholine on Na^+ (Tables I and II), presumably through an increase in Na^+ permeability by the neurotransmitters; (c) the specificity of the basal and acetylcholine-stimulated ^{32}P labelling of phospholipids to Na^+ (Table III); (d) the maximal increase in the acetylcholine-stimulated ^{32}P labelling of phosphatidic acid, phosphatidylinositol and phosphatidylcholine obtained only when both Na^+ and K^+ are present in the medium (Table IV); and (e) the ouabain inhibition of both the basal ^{32}P labelling of phosphatidylcholine (Fig. 2) and the acetylcholine-stimulated ^{32}P-labelling of phosphatidic acid, phosphatidylinositol and phosphatidylcholine (Table V), all point to a close link between the phospholipid phosphate turnover and the Na^+-pump mechanism in this tissue. This relationship could be interpreted as follows: Na^+ plays an important role in delivery of Ca^{2+} to the cytoplasm [4,20] and muscarinic receptor activation is thought to activate the Na^+ channel which depolarizes the membrane and activates the Ca^{2+} channel [21,22]. First, the Ca^{2+} entering the smooth muscle cell in response to receptor activation [13] could activate the enzymes involved in phosphoinositide breakdown to diacylglycerol and inositolphosphates [9]. The increased turnover of phosphatidic acid and phosphatidylinositol phosphate appears to be a consequence of a stimulation of phosphatidylinositol breakdown to diacylglycerol and inositol phosphate [23,24]. The enzymes involved in the hydrolysis of phosphoinosites to diacylglycerol and inositolphosphates have been reported in a variety of tissues [25,26] including the iris muscle [14,15] and are activated by Ca^{2+} in vitro, which supports the view that increased phospholipid phosphate turnover is a consequence of receptor-mediated Ca^{2+} entry [8,10,12]. Acetylcholine markedly increased the production of [^{3}H]inositol monophosphate and [^{3}H]inositol triphosphate from [^{3}H]inositol prelabelled iris muscle [9]. In the present studies when Ca^{2+} and/or K^+ were added to a Na^+-deficient isoosmotic medium there was a significant decrease in ^{32}P labelling of phospholipids in response to acetylcholine (Table IV). No requirement for K^+ in the phosphoinositide effect was observed in the present work. This could be due to the eflux of K^+ from the muscle cell, where it is high, into the incubation medium. Removal of the polarhead groups from these phospholipids could serve to facilitate the cationic fluxes through the Na^+-Ca^+ channels. In this connection Kai and Hawthorne [27] suggested that polyphosphoinositides could be involved in control of permeability of nerve cell membrane. Secondly, the Na^+ and Ca^{2+} entering the cell, through passive flux and in response to the neurotransmitters, lead to an increase in intracellular Na^+ and Ca^{2+} concentrations which in turn could stimulate the cation transport processes. Restoration of the polarhead groups to the diacylglycerol backbone at the Na channel which is reflected in the enhanced ^{32}P labelling, could be associated with the extrusion of Na^+ and Ca^{2+}, presumably via the Na^+- and Ca^{2+}-pumps. There is evidence that in smooth muscle aerobic glycolysis is specifically coupled to Na^+ and K^+ transport processes [28]. It has been suggested by several investigators that metabolic and active transport are mutually regulated [29]. Thus it is not unreasonable to assume that an increase in the activities of the transport processes could be accompanied by an increase in the turnover of ATP which in turn could lead to the observed enhanced ^{32}P

labelling of phospholipids during the recovery process. In this connection we have recently investigated the level of ATP in the iris muscle and the effect of acetylcholine thereon (Akhtar, R.A. and Abdel-Latif, A.A., unpublished observations). Muscles were first incubated in the presence and absence of the neurotransmitter then the nucleotides were separated by means of paper PEI-cellulose chromatography and the concentration of ATP determined by the firefly bioluminescence assay. We found the concentration of ATP to be 4.615 nmol/5 irises and its specific radioactivity to be 153 cpm/pmol and 179 cpm/pmol in the absence and presence of the neurotransmitter respectively. Acetylcholine does not appear to alter the level of ATP. The inhibitory effect of ouabain on the basal ^{32}P labelling of phosphatidylcholine and increase in that of phosphatidic acid and phosphatidylinositol could be due to the increase in diacylglycerol, caused by the glycoside inhibition of phosphatidylcholine synthesis, which is then phosphorylated to phosphatidic acid, via diacylglycerol kinase and subsequently to phosphatidylinositol. Several years ago Nichols et al. [30] reported both inhibitory and stimulatory effects of ouabain upon the incorporation of ^{32}P into the phosphatidic acid fraction of rabbit brain slices. Inhibition of the acetylcholine-stimulated ^{32}P labelling of phosphatidic acid, phosphatidylinositol and phosphatidylcholine by ouabain could be due to blockade of the Na^+-pump by the drug. This latter observation is further evidence in support of our suggestion that the enhanced ^{32}P labelling of phospholipids in response to a Na^+ gradient or to a neurotransmitter-induced Na^+ permeability change is closely linked to the Na^+-pump mechanism in the rabbit iris smooth muscle. A possible link between the enhanced phosphoinositide phosphate turnover in response to Na^+ and the neurotransmitters and the Na^+ pump mechanism is given in Scheme II. The precise link between the enhanced phospholipid phosphate turnover and the active transport of cations remains to be elucidated.



Scheme II. A possible role for Na^+ in the neurotransmitter-stimulated ^{32}P labelling of phosphoinositides in the iris muscle.

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