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Sodium ion and the effect of acetylcholine on phospholipid and phosphoprotein phosphate turnover in the rabbit iris smooth muscle*

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The responses of a variety of tissues to neurotransmitters, neurohumors, neuropharmacological agents and electrical pulses involve increases in the phosphate turnover of certain phospholipids, such as the phosphoinositides [1], and certain phosphoproteins [2]. Neither the molecular mechanism nor the physiological significance of the "phosphoinositide effect", which may be defined as a change in the rate of metabolism or turnover of the inositol-containing phospholipids when the tissues in which they occur are stimulated, is well understood. It is well established that the phosphoinositide effect is mediated through muscarinic cholinergic and α-adrenergic receptors [1]. Although activation of these receptors is known to lead to changes in the intracellular ionic environment, changes in intracellular concentration of a second messenger such as cyclic AMP or cyclic GMP, or changes in relevant enzymes, there has been little work done to show that these changes could mediate the increase in phosphoinositide turnover in response to receptor activation [1]. This has lead Michell and his colleagues [1, 3] to postulate that the agonist-stimulated breakdown of phosphatidylinositol (PI) in target tissue leads to the opening of Ca^{2+} gates and thus, to increased intracellular Ca^{2+} which, in turn, triggers the observed cellular responses. In previous communications from this laboratory, we reported on the characteristics of the phosphoinositide effect in the rabbit iris (for summary see Refs. 4 and 5). A key finding in these studies was the observation that in iris muscle, which was prelabeled with $^{32}P_i$ and in which the Ca^{2+} content was depleted with EGTA,† the acetylcholine (ACh)-stimulated breakdown of triphosphoinositide (TPI) and the ^{32}P-labeling of phosphatidic acid (PA) and to a much lesser extent PI are dependent on the presence of extracellular Ca^{2+} [6]. More recently the dependence of the phosphoinositide effect on the presence of extracellular Ca^{2+} has been demonstrated in synaptosomes [7, 8].

Activation of muscarinic cholinergic and α-adrenergic receptors in smooth muscle also leads to an increase in cell-surface permeability to Na^+ and K^+ in addition to Ca^{2+} [9]. In the present study, the effects of Na^+ and other cations on ^{32}P-labeling of phospholipids and phosphoproteins in the absence and presence of ACh were investigated.

Materials and Methods. Irises for the following experiments were obtained from albino rabbits at a local slaughterhouse. In general, whole irises were incubated singly (of the pair, one was used as control and the other as experimental) in isosmotic medium that contained 10 μCi $^{32}P_i$ (carrier free, New England Nuclear Corp., Boston, MA), at 37° for 1 hr in the presence and absence of the pharmacological agent as indicated. ACh was added 30 min after preincubation of the tissue with $^{32}P_i$. When the Na^+ concentration was varied, isosmolar substitution of NaCl was made by sucrose. The reaction was ended by addition of ice-cold trichloroacetic acid (TCA) to a final concentration of 10%. Phospholipids were extracted from the tissue with chloroform–methanol–HCl (400:200:1.5, by vol.), then separated into individual phospholipids by means of two-dimensional t.l.c. with silica gel H, and their radioactive contents determined as described previously [10]. After extraction of the lipids, phosphoproteins were determined in the residual-insoluble precipitate as alkali-labile phosphate by a modification of the procedures of Ahmad and Judah [11]. Briefly, the residual-insoluble precipitate was washed three times with 3 ml of 5% TCA containing 0.1 M KH_2PO_4. The tubes were centrifuged after each

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† EGTA, ethyleneglycolbis (amino-ethylether)tetra-acetate.

Table 1. Effects of extracellular Na^+ concentration on ^{33}P-labeling of phospholipids and phosphoproteins in the presence and absence of acetylcholine*

Na^+ (mM)	^{32}P-labeling of phospholipids and phosphoproteins (cpm × 10^{-2})/iris									
	PA	%†	PI	%	PC	%	TPI	%	Phosphoproteins	%
0	5 (4.5)‡	90	4 (4)	100	3.5 (3.6)	103	24.6 (22.8)	93	27.6 (35)	127
2.5	17.9 (22.5)	126	20 (21)	105	9.5 (9.9)	104	38 (37)	97	89.5 (96)	107
5	18 (35.8)	199	18.7 (45)	241	27.6 (30)	109	54.7 (50)	91	122 (131)	107
10	15 (33)	220	19.5 (43)	221	28 (30.8)	110	71 (73)	103	113 (129)	114
25	98.7 (165)	167	237 (356)	150	418 (373)	89	563 (550)	98	370 (365)	99
50	86.6 (180)	208	230 (359)	156	623 (693)	111	684 (640)	94	886 (828)	93
125	126 (250)	198	222 (380)	171	715 (944)	132	731 (720)	98	932 (1119)	120

* Irises were incubated singly (of the pair, one was used as control and the other as experimental) in isosmotic medium (the composition was the same as that given under Fig. 1) that contained 10 μCi $^{32}P_i$ at 37° for 30 min with 2.5 to 125 mM Na^+; then acetylcholine plus eserine (0.05 mM each) was added and the incubation was continued for an additional 30 min. Individual phospholipids and total phosphoproteins were isolated and their ^{32}P-radioactivities were determined as described in the text. Each result is the mean of four separate experiments. Abbreviations: PA, phosphatidic acid; PI, phosphatidylinositol; PC, phosphatidylcholine; and TPI, triphosphoinositide.

† Per cent of control (in the absence of ACh).

‡ In the presence of ACh plus eserene.

washing and the supernatant fraction was discarded. All centrifugations were carried out in a refrigerated centrifuge at 3000 *g* for 20 min. After washing the precipitate twice with 5 ml of deionized water, the residue was incubated with 3 ml of 3 M KOH at 37° for 18 hr. Perchloric acid was added to make the final concentration 0.6 M, the tubes were centrifuged, and the precipitate was discarded. The supernatant fraction was then neutralized with 1 M KOH, the tubes were centrifuged, and the precipitate was discarded. The resulting supernatant fraction was passed over activated charcoal, the tubes were centrifuged, and the ^{32}P-radioactivity was determined in the supernatant. All the data reported in this paper on the ^{32}P-radioactivity in phospholipids and phosphoproteins are expressed as cpm/iris.

Results and Discussion. The data presented show that addition of Na^+ was required for the incorporation of $^{32}P_i$ into both phospholipids and phosphoproteins of the rabbit iris smooth muscle. Thus, in the absence of Na^+ the ^{32}P-radioactivity that was recovered in total phospholipids and phosphoproteins was, respectively, only 7 and 14 per cent of that obtained in the presence of 100 mM Na^+ (Fig. 1). Furthermore, the ^{32}P-labeling of both phospholipids and phosphoproteins increased with Na^+ concentration and levelled off around 75 mM. In the absence of Na^+, therefore, both the ^{32}P-labeling of the phospholipids and that of phosphoproteins were comparable, and at 100 mM Na^+ the ^{32}P-radioactivity in the former was more than twice that of the latter (Fig. 1). To determine whether Na^+ was required for the enhanced ^{32}P-labeling of phosphoinositides in response to ACh, we investigated the effect of the neurotransmitter on labeling of the various phospholipids and total phosphoproteins in the presence of various concentrations of the monovalent cation. In the absence of Na^+, ACh had little influence on the ^{32}P-labeling of PA, PI or phosphatidylcholine (PC); at a 5 mM concentration of the cation, however, the neurotransmitter caused, respectively, a 99, 141 and 9 per cent increase in the labeling of these phospholipids (Table 1). Using the same experimental conditions, ^{32}P-labeling of the individual phospholipids and total phosphoproteins increased significantly with Na^+ concentration. Thus, at 125 mM Na^+ the labeling of PA, PI, PC and phosphoproteins increased respectively, by 25-, 56-, 204- and 34-fold over that of the control, and when ACh was added it increased, respectively, by 98, 71, 32 and 20 per cent (Table 1). In contrast to our previous studies which were done using breakdown conditions [5], with the present experimental conditions (synthetic) ACh had little effect on ^{32}P-labeling of TPI. Omission of Ca^{2+} depressed ^{32}P-incorporation into phospholipids of the iris muscle (data not shown). In this connection, Keryer *et al.* [12] reported recently that in rat parotid glands cholinergic stimulation of *myo*-[2-^{3}H]inositol into PI is dependent on extracellular Na^+. The dependence of the ACh-stimulated ^{32}P-incorporation into phospholipids on Na^+ could be due to the fact that activation of muscarinic cholinergic receptors in smooth muscle brings about an increase in cell-surface permeability to the cation. The requirement for Na^+ in ^{32}P-labeling of phospholipids, both in the absence and presence of ACh, is specific. Thus, Li^+, NH_4^+, K^+, choline, or Tris did not substitute for Na^+ [13]. Li^+ was the only monovalent cation to partially substitute for Na^+. In smooth muscle Li^+ can substitute for Na^+ in the

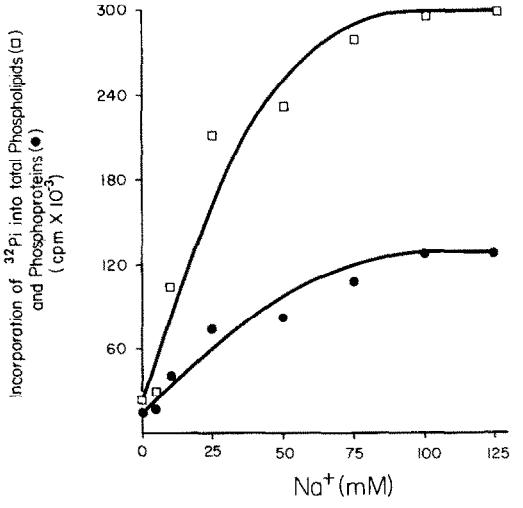

Fig. 1. Effects of extracellular Na^+ concentration on ^{32}P-labeling of total phospholipids and phosphoproteins in the rabbit iris smooth muscle. Irises were incubated in an isosmotic medium [2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 5 mM KCl, 26 mM Tris/HCl buffer (pH 7.4), 10 mM D-glucose, and sucrose to maintain the osmolarity] that contained 10 μCi $^{32}P_i$ in a total volume of 1 ml at 37° for 1 hr, with 2.5 to 125 mM Na^+. ^{32}P-labeling of phospholipids and phosphoproteins was determined as described in the text. Each result is the mean of five separate experiments.

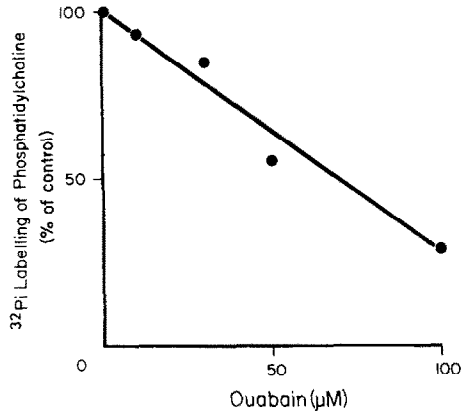

Fig. 2. Effects of ouabain on ^{32}P-labeling of phosphatidylcholine. Irises were incubated singly (of the pair, one was used as control and the other as experimental) in an isosmotic medium (its composition was the same as that given under Fig. 1. except that 125 mM NaCl was substituted for the sucrose) that contained 10 μCi $^{32}P_i$ at 37° for 1 hr in the absence and presence of 10–100 μM ouabain. Phosphatidylcholine was isolated from the lipid extract as described in the text. Each result is the mean of three separate experiments.

Na^+–Ca^{2+}-exchange carrier [14]. In view of the fact that omission of Na^+ from the incubation medium leads to inhibition of the Na^+-pump, the above findings on the requirement for this cation in the ^{32}P-labeling of phospholipids and phosphoproteins suggest a possible relation between phospholipid- and phosphoprotein phosphate turnover and the Na^+-pump mechanism in this tissue, possibly through ATP turnover. Whole iris (iris-ciliary body), which has a high rate of Na^+ transport [15] and a high Na^+–K^+ ATPase activity (W. Taft and A. A. Abdel-Latif, unpublished results), exhibits a high rate of aerobic glycolysis in addition to its respiratory activity [16]. Oxygen uptake and glycolysis have been shown to be inhibited by ouabain [17] and, furthermore, a link between oxidative phosphorylation and glycolysis and ion transport has been demonstrated in this tissue [17]. It has been suggested by several investigators that metabolism and active transport are mutually regulated [18, 19]. Thus, it is not unreasonable to assume that an increase in the activities of the transport processes can be accompanied by an increase in the turnover of ATP which, in turn, can lead to the observed enhanced ^{32}P-labeling of phospholipids and phosphoproteins during the recovery process. To test this assumption we measured (1) the effect of ouabain, which blocks the Na^+-pump, on phospholipid phosphate turnover, and (2) the effects of Na^+, ACh and ouabain on the specific radioactivity of ATP in the iris. Ouabain was found to inhibit ^{32}P-labeling of PC, and this increased with the glycoside concentration (Fig. 2). Thus, at 0.1 mM this drug inhibited the labeling of PC by 70 per cent. Under the same experimental conditions, there was an increase in the labeling of PA and PI (data not shown). The latter observation could be due to the increase in diacylglycerol (the precursor of PA) caused by ouabain inhibition of PC synthesis, which is then phosphorylated to PA via diacylglycerol kinase, and, subsequently, to PI. In more recent studies we have investigated the effects of Na^+, ACh and ouabain on the specific radioactivity of ATP in the rabbit iris (R. A. Akhtar and A. A. Abdel-Latif, unpublished observations). Muscles were first incubated in the presence and absence of Na^+ (50 mM) and ACh (50 μM) or ouabain (50 μM), then the ^{32}P-labeled nucleotides were separated by means of paper PEI cellulose chromatography, and the concentration of ATP was determined by the firefly bioluminescence assay. We found the specific radioactivities of ATP (cpm/pmole ATP) in the absence of Na^+ and ACh, in the presence of Na^+, and in the presence of Na^+ plus ACh to be, respectively, 5.4, 148.0 and 199.0. ACh did not appear to alter the level of ATP. Ouabain lowered the specific radioactivity of ATP by about 35 per cent of that of the control.

The above findings, namely (a) the significant increase in ^{32}P-labeling of phospholipids, including PA, PI and PC, and phosphoprotein phosphate turnover with increased Na^+ concentration (Fig. 1, Table 1); (b) the dependence of the ACh-stimulated ^{32}P-labeling of PA, PI and PC on Na^+, presumably through an increase in Na^+ permeability caused by the neurotransmitter (Table 1); (c) the specificity of Na^+; (d) the inhibition of PC labeling by ouabain (Fig. 2); and (e) the changes in the specific radioactivity of ATP[^{32}P] suggest a close link between phospholipid and phosphoprotein turnover and the Na^+-pump mechanism, probably through ATP metabolism (Scheme 1). This conclusion can be interpreted as follows: Na^+ plays an important role in delivery of Ca^{2+} to the cytoplasm [14, 20]; muscarinic receptor activation apparently activates the Na^+ channel, thus depolarizing the membrane and activating the Ca^{2+} channel [21, 22]. First, the Ca^{2+} entering the smooth muscle cell stimulates the enzymes that are involved in phosphoinositide breakdown, to form diacylglycerol [5, 23, 24]. Removal of the polar head groups from these phospholipids facilitates the cationic fluxes through the Na–Ca^{2+} channels. Second, the Na^+ entering the cell, through passive flux and in response to ACh, leads to an increase in intracellular Na^+ concentration which, in turn, stimulates the Na^+-pump activity and ATP turnover. Restoration of the polar head groups to the diacyl glycerol backbone at the Na^+–Ca^{2+} channels, which is reflected in the enhanced ^{32}P-labeling from ATP[^{32}P], could be associated with the extrusion of Na^+, presumably via the Na^+-pump mechanism. Although in some tissues PI breakdown has been reported to be involved in the control of Ca^{2+} gating [1, 3], the above findings support the hypothesis that in the iris muscle the increase in phosphoinositide phosphate turnover (breakdown and resynthesis) follows ACh-induced cationic permeability changes [5, 6, 13, 25].

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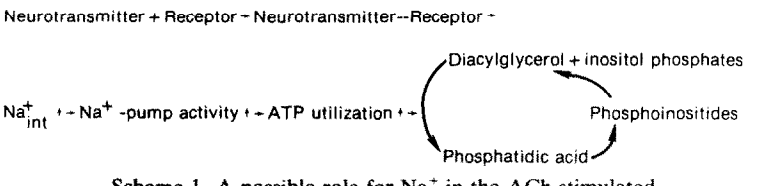

Scheme 1. A possible role for Na^+ in the ACh-stimulated ^{32}P-labeling of phosphoinositides in the iris muscle.

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Acute and chronic effects of barbiturates and ethanol on phospholipid and sulfatide content of rat brain regions

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The cellular mechanisms underlying the sedative effects of barbiturates and ethanol remain obscure. It is generally accepted, however, that the major sites of anesthetic action reside within the synapse [1].

Recent studies have shown that both acute and/or chronic administration of barbiturates and ethanol produce changes in a number of membrane lipid parameters. Acute pentobarbital administration, for example, significantly increases the ratio of triphosphatidylinositol (TPI) to diphosphatidylinositol (DPI) in rat brain microsomes [2]. Barbiturates, added *in vitro*, decrease the incorporation of ^{32}P into synaptosomal phosphatidylinositol and phosphatidic acid [3]. Both short-term (2 hr) and long-term (10 days) ethanol administration to mice have been shown to reduce the relative proportion of polyunsaturated fats in synaptosomal phospholipids [4]. *In vitro* ethanol increases the fluidity of spin-labeled synaptosomes, whereas the result of chronic ethanol administration is an adaptational resistance to the *in vitro* fluidizing action of ethanol [5]. Chronic administration of ethanol also causes an increase in the cholesterol content of erythrocyte and brain membranes [6]. This adaption by membranes may expalin the development of resistance [5] to the fludizing actions of *in vitro* ethanol exposure, although more recent work suggests that the presence of cholesterol in membranes may be required for the expression of tolerance but not for the attenuation of ethanol-induced membrane fluidization [7].

Although it is clear that barbiturates and ethanol alter a variety of important membrane variables, few studies have directly compared the acute or chronic effects of these drugs on brain membrane lipids. Johnson *et al.* [8] have shown recently that artificial membranes, formed from lipid extracts of synaptosomal membranes from ethanol-tolerant mice, exhibit tolerance to the fluidizing effect of ethanol and cross-tolerance to pentobarbital, but not to morphine. In the present paper, we report the results of a comparative study on the effects of acute and chronic administration of ethanol and barbiturates on the content of brain regional phospholipids and sulfatides.

Male Spargue–Dawley rats (250–350 g) were kept on a 12/12 hr light–dark cycle with food and water *ad lib.* for at least 5 days. The rats were then randomly divided into three experimental groups: control, acute, and tolerant. For the barbiturate studies, acute animals received a single dose of pentobarbital (50 mg/kg, i.p.) and were killed 30 min later. Animals in the tolerant group received a food cup containing a milled diet (Purina Lab Chow) thoroughly mixed with 2.5 to 3.5 mg phenobarbital/g diet as their sole food source. Animals in the tolerant group were maintained on the phenobarbital diet for 13 days and were killed on the morning of day 14.

A treadmill apparatus ("moving belt") was used to determine the degree of intoxication and tolerance development for animals in the tolerant group. Briefly, this