Biochemical Pharmacology, Vol. 30, No. 8, pp. 833–837, 1981.
Printed in Great Britain.

0006-2952/81/080833-05 $02.00/0

POLYPHOSPHOINOSITIDE METABOLISM IN ERYTHROCYTES OF SPONTANEOUSLY HYPERTENSIVE RATS

GURIJ KISELEV‡, AUGUSTINA MINENKO*, VERA MORITZ† and PETER OEHME*

*Institute of Drug Research, †Central Institute of Cardiovascular Regulation Research, Academy of Sciences of the GDR, Berlin,—GDR, and ‡Pavlov Institute of Physiology Academy of Sciences of the USSR, Leningrad, U.S.S.R.

(*Received* 27 *March* 1980: *accepted* 16 *October* 1980)

Abstract—Content of mono-, di- and triphosphoinositides as well as ^{32}P incorporation into these phospholipids was investigated on erythrocytes from spontaneously hypertensive rats at 1, 2 and 4 months of age. Already at 1 month the diphosphoinositide content was higher and the ^{32}P incorporation into di- and triphosphoinositides lower, compared with the controls. During development of hypertension ^{32}P incorporation rates for hypertensive rats decreased more slowly then for controls, so that the initial differences decreased and were then even reversed.

Polyphosphoinositides (PPI) as well as their synthesizing [1] and hydrolysing [2] enzymes are known to be located on the inner surface of erythrocytes. Their metabolism causes changes in calcium binding [3, 4] and, consequently, in membrane permeability to monovalent cations [5, 6].

On the other hand SHR show disorders of potassium transport [7] and passive sodium transport in arterial muscle tissue [8, 9] and erythrocyte membranes [10, 11]. The disorders in transport of monovalent cations are assumed to be associated with changes in calcium binding on membranes [12–14]. Increased calcium efflux was observed for both smooth aortic muscle and adipose tissue of SHR [13, 15].

The membrane alterations in hypertension might be connected with disorders of PPI metabolism. Changes in PPI context of erythrocyte membranes of three-month-old SHR were reported in [16].

Our aim was to study PPI content and ^{32}P incorporation into PPI of erythrocytes throughout development of hypertension in SHR.

MATERIALS AND METHODS

In vivo *experiments*

Kinetics of ^{32}P *incorporation into erythrocyte phospholipids.* We used two-month-old male Wistar rats weighing 230 to 250 g. Carrier-free ^{32}P disodium hydrogen phosphate was intraperitoneally injected (2 mCi/kg body wt) and the animals were decapitated after 20, 45, 90 and 180 min respectively. The blood of each of the rats was collected in a solution that contained 0.9% of NaCl, 8 mM of glucose and 20 U/ml of heparin. Haematocrit was determined in the blood solution thus obtained, and samples were taken for both extraction of phospholipids and determination of specific activity of inorganic phosphate.

^{32}P *incorporation into erythrocyte phospholipids from rats of various age.* One-, two- and four-month-old male SHR and normotensive Wistar rats (NWR) of the same age and sex were used. 90 min after ^{32}P injection the animals were decapitated and the blood was collected as described above. Erythrocytes were prepared, lipids extracted and relative specific activities determined as follows.

Lipid extraction and phospholipid fractionation. The blood samples were centrifuged at 1500 *g* for 0.5 min. The supernatant as well as most of the leukocytes were removed by suction. The lipids were extracted with a mixture of chloroform–methanol–conc. hydrochloric acid (100:100:0.6). The lipid extracts (after washing with 1-N HCl and then with a chloroform–methanol–0.2-N HCl mixture (3:47:50)) were evaporated under nitrogen and the phospholipids chromatographically separated on formaldehyde-treated paper [17].

Determination of relative specific activity of phospholipids. The content and radioactivity of the phosphate groups were determined for monophosphoinositides (MPI), diphosphoinositides (DPI) and triphosphoinositides (TPI) as well as for total phospholipids (total PL). Radioactivity was measured by means of a LKB Wallac 8100 liquid scintillation counter, the phospholipid paper strips being directly applied to the dioxan scintillation fluid. The same phospholipid-containing paper strips were then used for phosphate determination [18]. Phospholipid contents are given in μg P/ml of erythrocytes and specific activities (SA_{PL}) in cpm/μg P. To determine the specific activity (SA_{Pi}) of inorganic phosphate, protein was precipitated using 10% trichloroacetic acid. Afterwards the amount of phosphate [19] and its activity was determined in the supernatant. ^{32}P incorporation rates of phospholipid fractions are given as relative specific activities (RSA):

$$RSA = \frac{SA_{PL}}{SA_{Pi}} \times 100.$$

Please address reprint requests to A. Minenko, DDR-1136 Berlin, Alfred-Kowalke Str. 4, Institut für Wirkstofforschung.

In vitro *experiments*

Experiments with fresh erythrocytes. Erythrocytes were tested from one- and four-month-old animals. We used an incubation solution of the following composition: 154.0 mM NaCl, 5.6 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.2 mM Na_2HPO_4, 8.0 mM glucose, 1.0 mM adenosine, 16.5 mM Tris buffer (pH 7.4). The packed erythrocytes were washed three times with heparinized (20 U/ml) incubation solution, pre-incubated for 30 min (in incubation medium) at 37° and exposed for another 30 min to ^{32}P after addition of 10 mCi/ml $Na_2H^{32}PO_4$. The final volumes were 15 ml for four-month-old rats and 10 ml for one-month-old rats, haematocrit being about 10 per cent. After incubation samples of the erythrocyte suspension were used for lipid extraction and for determination of specific activity of inorganic phosphate (carried out just as those *in vivo*).

Experiments with erythrocytes stored for 48 hours. These experiments were performed on four-month-old rats. The blood was collected and the erythrocytes were washed as already described for the *in vivo* experiments. The erythrocytes were kept in incubation solution at 5° for 48 hr. Then they were washed once in incubation solution, pre-incubated for 1.5 hr at 37° and exposed for 30 min to ^{32}P (incubation vol., 10 ml). The other procedures that followed were identical with those already described.

RESULTS

In vivo *experiments*

The time course of ^{32}P incorporation into erythrocyte phospholipids is given in Fig. 1. Obviously incorporation into phospholipids did not reach its maximum within the time interval chosen. The increase of specific activity of the phospholipids is nearly linear.

The dependence of the phospholipid content in erythrocytes of NWR and SHR on age is given in Table 1.

In one-month-old SHR the content of phospholipids analyzed is higher than in the corresponding NWR (significantly for total PL and DPI). This difference is smaller after two months. For phosphoinositides it is even reversed in four-month-old SHR (significantly for DPI).

The content of phosphoinositides has a differing age dependence between NWR and SHR. When comparing one- and four-month-old NWR, TPI and DPI increases strongly (to 138% and 203%, respectively). Yet, in SHR there was only a small increase of TPI (115%), whereas DPI and MPI decreased (to 67 per cent and 85 per cent).

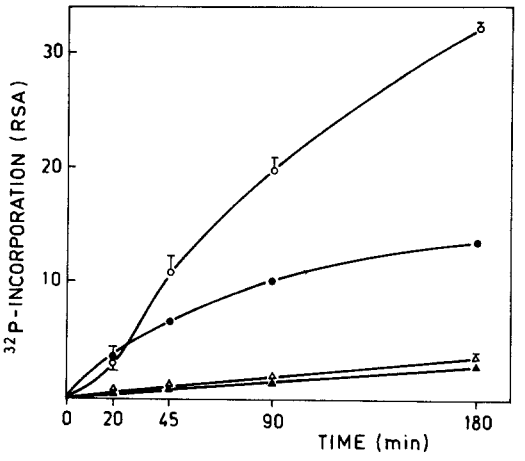

Fig. 1. Time course of ^{32}P incorporation into phospholipids of erythrocytes from NWR (*in vivo*). Key: TPI (○): DPI (●); MPI (△); total PL (▲). Relative specific activity = RSA = $(SA_{PL}/SA_{Pi} \cdot 100$. SA_{PL} = specific activity of phospholipid (cpm·μg P_{PL}^{-1}). SA_{Pi} = specific activity of inorganic phosphate (cpm·μg P_{Pi}^{-1}). $n = 3$. Mean values ± S.E.M.

Figure 2 shows ^{32}P incorporation into phospholipids of erythrocytes from NWR and SHR in dependence on age.

^{32}P incorporation rates of phospholipids always decreased with age, most strongly for TPI and DPI and more expressed in NWR. The relative specific activities of TPI and DPI were lower in one-month-old SHR compared with NWR, while in two-month-old animals there were no differences between SHR and NWR.

In vitro *experiments*

Total PL content in erythrocytes of SHR of either age group was higher than in that of NWR (Table 2,A) similar to the *in vivo* experiments (Table 1).

Phospholipid contents were higher in one-month-old SHR (as in the *in vivo* experiments). The PPI contents in four-month-old SHR were also higher (contrary to the *in vivo* experiments). ^{32}P

Table 1. Phospholipid content of erythrocytes from SHR and NWR in dependence on age (*in vivo*)

Age (months)	Animals	Content (μg P/ml erythrocytes)			
		TPI	DPI	MPI	total PL
1	SHR	7.3 ± 0.24	3.6 ± 0.20***	10.4 ± 0.44	174 ± 9**
	NWR	6.5 ± 0.18	1.6 ± 0.10	9.5 ± 0.42	142 ± 3
2	SHR	6.4 ± 0.47	1.6 ± 0.14	6.9 ± 0.16*	144 ± 2**
	NWR	5.4 ± 0.43	2.0 ± 0.32	7.5 ± 0.19	132 ± 2
4	SHR	8.4 ± 0.45	2.4 ± 0.20*	8.9 ± 0.40	158 ± 9.7
	NWR	9.0 ± 0.19	3.3 ± 0.21	9.7 ± 0.47	149 ± 5.3

$n = 5$. Mean values ± S.E.M. Significant differences between SHR and NWR: *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

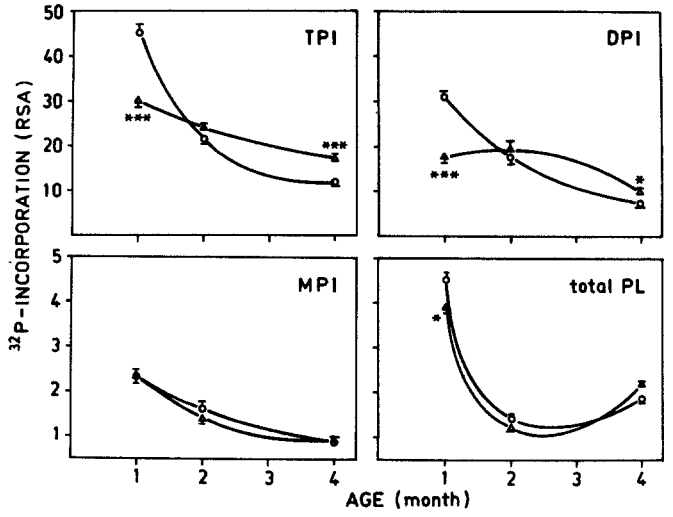

Fig. 2. ^{32}P incorporation into phospholipids of erythrocytes from SHR and NWR in dependence on age (*in vivo*). Key: SHR (△) and NWR (○). RSA = relative specific activity. $n = 5$. Mean values ± S.E.M. Significant differences between SHR and NWR: *P < 0.05; ***P < 0.001.

incorporation rates of PPI as well as of total PL (Table 3,A) showed an age dependence similar to the *in vivo* experiments.

Stored erythrocytes of four-month-old rats showed no differences between SHR and NWR as to the content of total PL and TPI (Table 2,B). The DPI content, however, differed, just as is the case with fresh erythrocytes (Table 2,A).

The total PL content of stored erythrocytes from SHR as well as TPI contents from SHR and NWR (Table 2,B) were lower than the corresponding values of fresh erythrocytes (Table 2,A). ^{32}P incorporation into PPI of stored erythrocytes from four-month-old SHR was lower than that from NWR (Table 3,B) and reached values similar to the *in vivo* experiments with one-month-old SHR (Table 3,A).

Table 4 shows the dependence of blood pressure on age.

While the blood pressure of one-month-old SHR scarcely differed from that of NWR, two-month-old SHR had a slightly and four-month-old SHR a strongly elevated blood pressure.

DISCUSSION

Under our experimental conditions the most rapid ^{32}P incorporation was found for DPI and TPI (Fig. 1) containing about 70 per cent of the total phospholipid radioactivity. Autoradiography of the paper chromatograms showed very low ^{32}P incorporation into phosphatidic acid.

From Figure 1, it follows, with regard to the 1:2:3 ratio of the phosphate groups in MPI, DPI and TPI, that ^{32}P incorporation into DPI is about 10 times and into TPI about 25 to 30 times faster than into MPI.

Because of the low incorporation rate of MPI and since MPI is a precursor for the PPI, the diester phosphate groups must be stable also in the PPI. Hence ^{32}P incorporation is confined to the monoester phosphate groups and consequently results from phosphorylation of MPI (DPI) by kinases and from dephosphorylation of TPI (DPI) by phosphomonoesterases.

A low rate of ^{32}P incorporation into PPI of erythrocytes from one-month-old SHR was found *in vivo*

Table 2. Phospholipid content of erythrocytes from SHR and NWR in dependence on age *in vitro*¶

Age (months)	Animals	Content (μg P/ml erythrocytes)			
		TPI	DPI	MPI	total PL
1	SHR	7.7 ± 0.39**	2.7 ± 0.24*	9.0 ± 0.47**	197 ± 2.5***
(n = 5)	NWR	5.9 ± 0.34	1.9 ± 0.10	6.6 ± 0.50	148 ± 6.5
(A)					
4	SHR	8.9 ± 0.38**	1.8 ± 0.11***	8.5 ± 0.48	174 ± 9.0*
(n = 8)	NWR	7.5 ± 0.23	1.3 ± 0.04	8.4 ± 0.21	146 ± 6.7
(B)					
4	SHR	5.1 ± 0.40	2.0 ± 0.05***		139 ± 4.4
(n = 5)	NWR	5.3 ± 0.62	1.4 ± 0.09		142 ± 5.6

¶ Without (A) and with (B) cold storage.

Mean values ± S.E.M. Significant differences between SHR and NWR: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 3. ^{32}P incorporation rate (per cent of control) of phospholipids of erythrocytes from SHR in dependence on age (*in vitro*¶ and *in vivo*‖)

Age (month)	Incubation (*n*)	RSA (per cent of control)			
		TPI	DPI	MPI	total PL
1 (A)	*in vivo* (5)	65.6 ± 1.5×**	57.5 ± 2.5***	100.4 ± 5.3	86.4 ± 2.1*
	in vitro (5)	50.5 ± 2.8×**	58.0 ± 5.1×*	70.0 ± 15.2	89.3 ± 17.5
4	*in vivo* (5)	144.0 ± 7.7***	136.7 ± 10.9*	104.21 ± 3.9	114.3 ± 5.4
	in vitro (8)	131.7 ± 7.2*	129.5 ± 3.7**	160.1 ± 15.1**	119.5 ± 7.0
(B) 4	*in vitro* (5)	65.8 ± 6†	84.9 ± 10.6	—	103.9 ± 17.5

¶ Without (A) and with (B) cold storage.
‖ Fig. 2 in the present study.
RSA = relative specific activity. (RSA of NWR = 100 per cent).
Mean values ± S.E.M. Significant differences between SHR and NWR: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 4. Blood pressure of NWR and SHR in dependence on age

Age (months)	NWR		SHR	
	systol.	diastol.	systol.	diastol.
1	109 ± 3.1	70 ± 2.14	119× ± 4.09	73 ± 2.80
2	128 ± 1.9	73 ± 2.14	162×* ± 2.49	94** ± 2.49
4	121 ± 2.7	62 ± 1.79	204** ± 3.96	132×* ± 3.39

n = 70; mm Hg, mean value ± S.E.M.; Significant differences between SHR and NWR: * $P < 0.05$, ** $P < 0.001$.

(Fig. 2) as well as *in vitro* experiments (Table 3). This low ^{32}P incorporation together with higher PPI contents (Tables 1 and 2, A) can be due to a decrease of dephosphorylation or a decrease of both, phosphorylation and dephosphorylation with that of the latter predominating.

Figure 2 shows that ^{32}P incorporation decreases age-dependently in both, NWR and SHR with a stronger decrease in NWR. Because of this stronger decrease in NWR the incorporation rates at four month are higher in SHR than in NWR, which is in contrast to the values at one month.

After cold storage of erythrocytes and subsequent incubation under normal conditions, however, ^{32}P incorporation in SHR is lower than in NWR, both four month of age (Table 3). On cold storage breakdown of PPI gets activated and on subsequent reestablishment of physiological conditions resynthesis is activated as well [20, 21].

We thus suppose the lower ^{32}P incorporation found in SHR to be linked with a decreased capability of activating phosphorylation/dephosphorylation reactions. We consequently believe that regulation of phosphoinositide phosphorylation in erythrocytes of SHR is altered in the prehypertensive as well as in the hypertensive stage (at one and at four months, resp.).

These alterations may even be connected with the development of hypertension. Since phosphoinositide phosphorylation is known to cause changes in the Ca^{2+}-binding capacity of membranes [3, 4] and consequently in transport of monovalent cations across the membrane [5, 6], these alterations in phosphoinositide phosphorylation might also cause the changes in Ca^{2+}-binding [12–14] and membrane transport of monovalent cations [7–11] observed in essential hypertension.

This had to be tested also on other objects (e.g. in the humoral system or on neuronal tissue).

Acknowledgement—The authors thank Mrs. Gabriela Vogelreiter and Mrs. Andrea Geelhaar for skilful technical assistance.

REFERENCES

1. R. J. Garrett and C. M. Redman, *Biochim. biophys. Acta* **382**, 58 (1975).
2. N. E. Garrett, R. J. Garrett, R. T. Talwalkar, and R. L. Lester, *J. cell. Physiol.* **87**, 63 (1976).
3. J. T. Buckley and J. N. Hawthorne, *J. biol. Chem.* **247**, 7218 (1972).
4. T. Kawaguchi and K. Konishi, *Biochim. biophys. Acta* **597**, 577 (1980).
5. H. S. Hendrickson and J. L. Reinertsen, *Biochem. biophys. Res. Commun.* **44**, 1258 (1971).
6. R. A. Akhtar and A. A. Abdel-Latif, *J. Pharmac. exp. Ther.* **204**, 655 (1978).
7. A. W. Jones, *Circulat. Res.* **33**, 563 (1973).
8. J. Altman, R. Garay, A. Papadimitrion and M. Worcel, *Br. J. Pharmac.* **59**, 496 P (1977).

9. J. Altman, F. Da Ponte and M. Worcel, *Br. J. Pharmac.* **59**, 621 (1977).
10. J. V. Postnov, S. N. Orlov, P. V. Gulak and A. S. Shevchenko, *Pflügers Arch.* **365**, 257 (1976).
11. S. M. Friedman, M. Nakashima, R. A. McIndol and C. L. Friedman, *Experientia* **32**, 476 (1976).
12. J. V. Postnov, *Kardiologia (Moscow)* **18**, 15 (1978).
13. T. T. Zsotér, C. Volchinsky, N. F. Henein and L. C. Ho, *Cardiovasc. Res.* **11**, 353 (1977).
14. J. V. Postnov, S. N. Orlov and N. I. Pokudin, *Pflügers Arch.* **379**, 191 (1979).
15. J. V. Postnov and S. N. Orlov, *Experientia* **35**, 1480 (1979).
16. P. V. Gulak, G. M. Boriskina and J. V. Postnov, *Bull. exp. Biol. Med. (USSR)* **8**, 155 (1977).
17. G. V. Kiselev and L. I. Pavlinova, *Ukrain. biochim. Zurnal* **47**, 389 (1975).
18. G. R. Bartlett, *J. biol. Chem.* **234**, 466 (1959).
19. G. E. Delory, *Biochem. J.* **32**, 1161 (1938).
20. S. C. Peterson and L. B. Kirschner, *Biochim. biophys. Acta* **202**, 295 (1970).
21. R. P. Schneider and L. B. Kirschner, *Biochim. Biophys. Acta* **202**, 283 (1970).