Gen. Physiol. Biophys. (1982), **1**, 413—421

Phosphoinositides in the Flight Muscle of *Periplaneta americana*: Incorporation of Labelled Phosphate and Inositol

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Abstract. The content of phosphatidylinositol phosphate (DPI) in the flight musculature of the cockroach *Periplaneta americana* was found to be 30 nmol g^{-1} wet weight, that of phosphatidylinositol-bis-phosphate (TPI) 6.5 nmol g^{-1} wet weight. After administration of ^{32}P-orthophosphate in vivo, the specific radioactivity (S.A.) of arginine phosphate reaches a plateau after 2 hours and remains constant for the subsequent 22 hours. The S.A. of all phospholipid phosphates except the phosphate in position 5 of TPI rises during this time. The diesteric phosphate of DPI and TPI has lower S.A. than the diesteric phosphate of phosphatidylinositol (PI) after 2, 6 and 24 hours of incorporation in vivo. The phosphates in position 4 of DPI and TPI incorporate with identical rate, the phosphate in position 5 of TPI incorporates very rapidly during the first 6 hours and after 24 hours its S.A. decreases only half the value found after 6 hours. After administration of ^{3}H-myo-inositol, the S.A. rises in all three phosphoinositides with identical rates.

Key words: Phosphoinositide turnover — Flight muscle — Membrane phosphorylations

Introduction

There is now ample evidence for the great importance of phosphoinositides, namely phosphatidyl-myo-inositol (PI) and its phosphorylated derivatives (polyphosphoinositides — PPI) phosphatidyl-myo-inositol-4-phosphate (DPI) and phosphatidyl-myo-inositol-4,5-bis-phosphate (TPI) in biological membranes. For PI, it has been known that its polar head-group has a rapid basal turnover and that the last can be increased rather specifically by stimulation in a wide variety of tissues (Michell 1979), the precise physiological implication of this phenomenon is, however, still a matter of discussion. In this laboratory a similar effect was found to be evoked in insect muscle by flight, but in this material an increase of ^{32}P incorporation occurs immediately after cessation of the flight (Strunecká et al. 1981). DPI and especially its phosphorylated product, TPI, also appear to be very

closely connected with the functional changes of tissue. The very rapid turnover of the phosphate in position 5 of TPI (Hawthorne and White 1975) and its high energy of hydrolysis (Kiselev 1977) together with the ability of TPI to form complex with divalent cations (Hendrickson and Reinertsen 1969) suggest that they may be involved in rapid membrane processes, for instance in those accompanying excitation. Increase of the turnover rate of this phosphate after stimulation (Birnberger et al. 1971; White et al. 1974; Tretyak and Limarenko 1978) appears to confirm this concept. However, the presence and high turnover of these substances in non-exitable tissue e.g. in erythrocytes (Schneider and Kirschner 1970) or in a structure as inert as myelin (Desmukh et al. 1978) show that they may participate in more general functions of biological membranes.

Presence of DPI and TPI in the insect muscle was reported by Kilian and Schacht (1979). The purpose of the present paper was to verify their presence and to study the time course of their labelling in the flight muscle of *Periplaneta americana*.

Methods

Male *Periplaneta americana*, 2 months after adult ecdysis were used in these experiments. 750—1850 kBq of ^{32}P-orthophosphate (UVVVR Praha) or 75 kBq of ^{3}H-myo-inositol (Amersham) was injected intraabdominally to each insect in 20 µl of isotonic saline. After 2—24 hours of injection the pterothoracic muscles were excised, rapidly fixed by liquid nitrogen and pulverized. The material from 30 animals was pooled and used for one determination. Phospholipids (except PPI) were extracted three times successively by chloroform-methanol mixtures (1 : 1, 2 : 1, 2 : 1 v/v) using volumes 20, 10 and 10 ml respectively per ten animals. Calcium chloride 60 µmoles per g wet weight of the tissue (Hauser and Eichberg 1973) was added to the extraction medium to prevent splitting of PPI during extraction. The pellet remaining after phospholipid extraction was treated twice with 4 ml of chloroform-methanol (2 : 1 v/v) containing 15 µl of conc. HCl. Extraction was carried out for 20 min. at 30°C under continuous stirring. The combined extracts were washed twice by shaking with 0.2 vol. of 1N HCl and finally with 0.2 vol. of distilled water. The extracts were then immediately concentrated by evaporation in the atmosphere of N_2 and applied on thin-layer chromatography plates with Silikagel H (Merck). The solvent system used was similar to that described by Jolles et al. (1979). PPI was identified by cochromatography with the respective standarts. Phospholipids were separated according to Rouser et al. (1969) by thin-layer chromatography on Silikagel H (Merck) and Magnon (Kavalier Votice).

For determination the specific radioactivity of individual phosphates in DPI and TPI use was made of their spontaneous decomposition. Chromatographic plates with the applied samples were allowed to stand at room temperature for 12—14 hours. The spots of DPI and TPI were scraped out, eluted with the same solvent and rechromatographied. As breakdown products of TPI and DPI, DP plus PI and PI respectively were identified.

Phosphorus content in the spots was estimated according to Rouser et al. (1969) and/or Duck-Chong (1979). An Isocap 300 (Nuclear Chicago) liquid scintilation counter was used for the radioactivity measurements.

Haemolymph was obtained with the aid of a microsyringe (about 20 µl from each animal) and the analysis of its phosphorus content was performed according to Strunecká et al. (1974). The soluble phosphates of muscle tissue were also extracted and determined according to Strunecká et al. (1974).

All reagents were products of analytical grade. Solvents were purified by distillation. Standards of TPI were prepared from ox brain using the method of Dittmer and Dawson (1961). The purity of the preparations thus obtained was checked by determination of fatty acids phosphate ratio.

Results

1. Content of DPI and TPI in the tissue

According to our analysis the pterothoracic musculature of *Periplaneta americana* contains 30.5 ± 0.6 nmol g^{-1} wet weight DPI and 6.5 ± 0.5 nmol g^{-1} wet weight TPI. However, the real values of these substances in vivo will be probably higher since the obtained results might be lowered by hydrolysis during the isolation procedure. In the aim to minimalize the possible influence of hydrolysis experimental conditions were optimalised on the basis of preliminary observations to give maximal yield of polyphosphoinositides.

2. Incorporation of radioactive phosphate

Injection of ^{32}P-orthophosphate into the body cavity of the cockroach leads to considerable increase of radioactivity of the phosphate in the haemolymph followed by a gradual decrease during the time of observation (Table 1 and Fig. 1). On the other hand, the S.A. of P_i and phosphoarginine in the tissue reaches a rather high value within two hours after application of the labelled phosphate and this level does not change significantly during the next 22 hours of observation. These results are in agreement with earlier findings of Strunecká and Kubišta (1974). The S.A. of PI and PC increases during the whole time of observation, the S.A. of PI being considerably higher than that of PC, especially at short time of incubation (Strunecká et al. 1981). The time course of labeling of DPI and TPI is rather surprising. The S.A. of DPI is lower than that of PI at all three time-intervals of observation; that of TPI is always higher, however unlike all other phosphorus compounds of insect muscle hitherto examined, it does not exhibits an ascending course during the whole period of 24 hours. After 6 hours the S.A. of TPI is distinctly higher than that found after 24 hours.

Values of the S.A. of individual phosphates, given in the lower part of Table 1 allow an insight into the behaviour of PPI during phosphate incorporation. The S.A. of the diesteric phosphates of DPI and TPI are considerably lower than those of PI probably due to a distinct lag in their labelling curve especially during the first two hours of incorporation (see inset in Fig. 1). This lag may also be demonstrated by the ratio of label incorporated in the diesteric phosphate of DPI to that of PI amounting 0.11, 0.18, 0.39 for 2, 6 and 24 hours of incorporation respectively. The absolute differences in S.A. for the same time intervals were found to amount 459, 1130 and 1525 Bq $nmol^{-1}$ of phosphate. The relatively small change in the

Table 1. Incorporation of [^{32}P] into acid-soluble phosphorus compounds and phospholipids of the flight musculature of *Periplaneta americana* after parenteral administration of [^{32}P]-orthophosphate. Means from 6 experiments ± S.E.M.

Substance	Specific radioactivity Bq µmol ^{1}P after incorporation		
	2 hours	6 hours	24 hours
Inorganic phosphate (haemolymph)	54 275 ± 1 505	34 882 ± 1 308	20 575 ± 1 548
Inorganic phosphate (tissue)	15 522 ± 728	15 620 ± 648	15 585 ± 753
Phosphoarginine	14 250 ± 1 008	14 335 ± 537	14 418 ± 522
Phosphatidylcholine	104 ± 22	553 ± 47	1 422 ± 120
Phosphatidylinositol	532 ± 42	1 358 ± 70	2 458 ± 123
Phosphatidylinositol phosphate (DPI)	313 ± 45	1 108 ± 47	2 042 ± 83
Phosphatidylinositol-bis-phosphate (TPI)	1 507 ± 202	4 155 ± 292	3 002 ± 170
Diesteric phosphate of DPI	63 ± 18	416 ± 108	1 163 ± 155
Diesteric phosphate of TPI	47 ± 21	249 ± 132	970 ± 185
4′-phosphate of DPI	563 ± 47	1 801 ± 87	2 920 ± 75
4′-phosphate of TPI	500 ± 68	1 496 ± 318	2 795 ± 88
5′-phosphate of TPI	3 973 ± 325	10 720 ± 498	5 240 ± 422

Table 2. Incorporation of ^{3}H-myo-inositol into phosphoinositides of the flight muscles of *Periplaneta americana*. Means from 6 experiments ± S.E.M.

Substance	Specific radioactivity Bqµmol^{-1} phospholipid after incorporation		
Phosphatidylinositol	40 100 ± 13 800	122 100 ± 20 100	259 400 ± 20 100
Phosphatidylinositol phosphate	34 900 ± 7 800	125 400 ± 17 900	264 700 ± 25 200
Phosphatidylinositol-bis-phosphate	30 700 ± 9 300	110 000 ± 23 100	250 800 ± 19 800

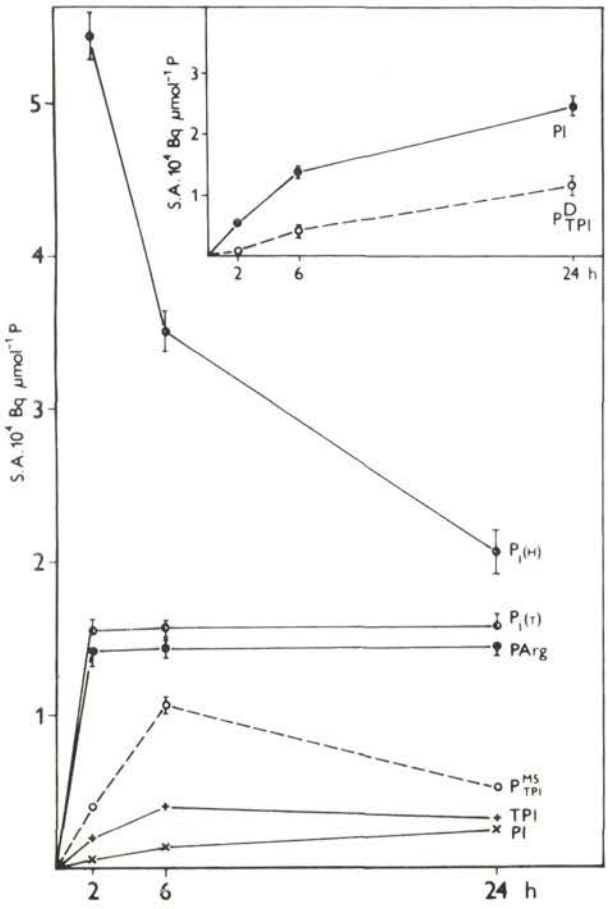

Fig. 1. The values of S.A. of selected phosphorus compounds of insect muscle 2, 6 and 24 hours after administration of [^{32}P]-orthophosphate. P_i(H) — haemolymph orthophosphate, P_i(T) tissue orthophosphate, PArg — phosphoarginine, PI — phosphatidylinositol, TPI — triphosphoinositide, P^{M5}_{TPI} — the M5-phosphate group of TPI. Inset: Incorporation of [^{32}P] — orthophosphate into diesteric group of PI and of TPI.

absolute differences in S.A. between 6 and 24 hours of incorporation points to the fact that after the lag period the relative incorporation rate into the diesteric phosphates of both TPI and for DPI and PI as well is about equal.

The phosphate incorporation in the position 4 of DPI and TPI do not exhibit such a lag. The S.A. of the above compounds increased during the whole time of observation being in all points a little higher than that of PI. The phosphate in the position 5 of TPI incorporates much more rapidly than all other lipid phosphates

observed, showing a pattern similar to incorporation into the TPI "in toto". Nevertheless after 24 hours the amounts of phosphate incorporated into the position 5 of TPI have decreased to about a half of the value recorded for 6 hours of incorporation (Fig. 1) i.e. a little more than in the case of TPI. Thus, while the low total S.A. of DPI reflected the low phosphate incorporation into its diesteric phosphate, bounds the characteristic course of changes in the S.A. of TPI may be predominantly prescribed to the incorporation of phosphate into the position 5 on the myo-inositol ring.

3. Incorporation of ^{3}H-myo-inositol

As may be seen in Table 2, the incorporation of ^{3}H-myo-inositol proceeds at an equal rate into all three phosphoinositides examined. The differences among values found at all three investigated time intervals are not statistically significant.

Discussion

The content of DPI in the flight musculature of *Periplaneta americana* is near to the values given for *Agrotis* ypsilon muscle, while that of TPI is lower (Kilian and Schacht 1979). In comparison with nervous tissue (Hawthorne and White 1975) the level of PPI in insect flight muscle is low.

To explain the behaviour of phosphoinositides in labelling experiments with ^{32}P-orthophosphate and ^{3}H-inositol, the following findings seem to be of great importance: i) the labels of the diesteric phosphate in DPI and TPI are equal, but considerably lower than those in PI, because the rate of increase of their S.A. is very slow during the first two hours following the addition of the label. Nevertheless, after 24 hours, the rates of DPI and TPI labelling appears to reach the rate of PI labelling, ii) the incorporation curves for ^{3}H-myo-inositol are in fact identical for all three phosphoinositides, iii) the phosphate in the position 4 in both DPI and TPI has a similar pattern of incorporation, iv) the phosphate incorporation into the position 5 of TPI exhibits a unique behaviour by reaching maximum value after 6 hours and infrequently sinking to a considerably lower value after 24 hours of experiment. The tissue arginine phosphate remains constant for the whole time of observation.

To explain the specific features of time-course of labelling of the diesteric phosphate of DPI and TPI they should be assumed two pools of PI: a fast exchangeable one, which is readily accesible for newly synthetized PI and a slow exchangeable one which may be reached by freshly formed PI only after a considerable delay. This second pool seems to be only substrate for DPI and TPI formation and an important component of the plasma membrane. Both of these PI pools should exchange the inositol moiety with free inositol; the possibility of such

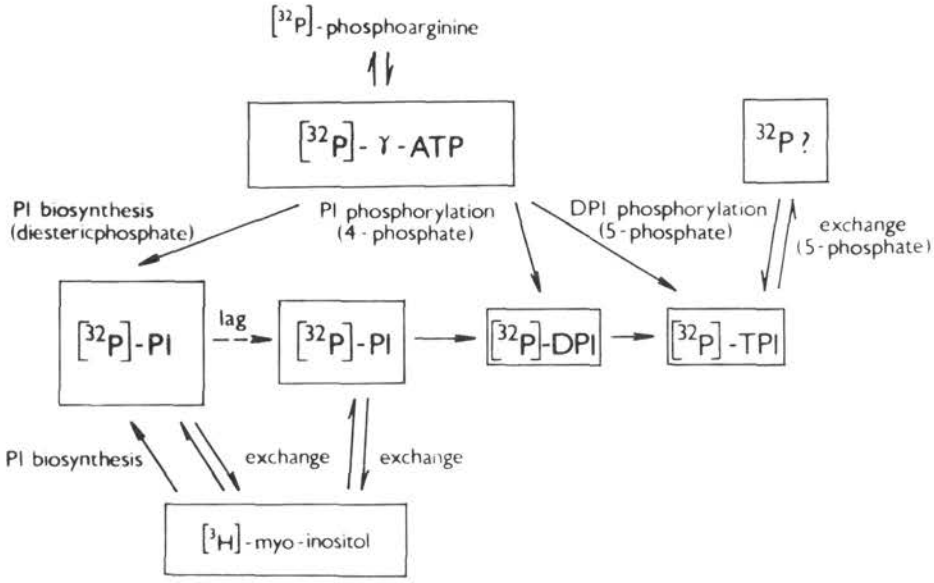

Fig. 2. A scheme of the compartmentation of the metabolism of inositol phospholipids in insect muscle. The primary source of all phosphate moieties is the γ-phosphate of ATP, which equilibrates rapidly with phosphoarginine (Strunecká and Kubišta 1974); by the determination of the S.A. of phosphoarginine the S.A. of γ-phosphate is determined as well. The phosphate pool exchanging with TPI (the rectangle at right) may be extracellular phosphate. Other aspects of the scheme are explained in the text.

exchange is well documented in literature (Takenawa and Egawa 1980). This appears to be only explanation, since the ^{3}H-myo-inositol incorporation, in contrast to ^{32}P incorporation to diesteric phosphates, follows a similar course in all phosphoinositides. The assumption is supported that all phosphoinositides in the tissue are ultimately derived from one pool.

DPI appears to be formed from PI by phosphorylation utilizing the high-energy phosphates available in the tissue. The above reaction seems to proceed at rate allowing DPI to exceed slightly the mean specific radiactivity of the diesteric phosphate of DPI.

The incorporation of phosphate into position 5 of TPI occurs very rapidly. After 6 hours it has a value corresponding to two thirds of the S.A. of phosphoarginine. In contrast to all other intracellular phosphates of insect muscle hitherto studied (Strunecká and Kubišta 1974; Helm et al. 1977) the S.A. of 5′-phosphate of TPI has been found to be lower 24 hours after administration of radioactive orthophosphate to experimental animals than 6 hours.

This unique behaviour of 5′-phosphate of TPI can be explained only by assumption that it equilibrates with some phosphate pool the specific activity of

which decreases after 6 hours of incorporation. The only phosphate pool available which might meet the above demands is the extracellular phosphate; however, a number of hypothetical assumptions must be made to explain its exchange with 5-phosphate of TPI.

The scheme in Fig. 3 summarizes our ideas about the compartmentation of phospoinositides in insect muscle cells with respect to the exchangeability of their inositol and phosphate groups. We assume that the 5-phosphate of TPI may be derived primarily but not exclusively from intracellular ATP, hence to its radioactivity could considerably contribute also some other phosphate pool.

Whatever the case of the observed labelling pattern may be, two points stand out clearly:

1. Determination of the total specific radiactivity of phosphate in phosphoinosititides may be misleading unless the S.A. of individual phosphates of DPI and TPI are determined at the same time.

2. The very high incorporation rate of the 5-phosphate of TPI and its peculiar time course strongly confirms some important physiological function of this compound in bio-membranes, a function essentially different from that of other phospholipids. The distinct time lag in the labelling of diesteric phosphate of TPI is not in accordance with the hypothesis of Michell (1979) assuming that this process involves a splitting of the above substance to diglyceride followed by resynthesis of the whole polar head of the molecule.

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Received January 25, 1982 / Accepted July 19, 1982