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Internalisation of Short-Chain Isoprenyl Diphosphates by Chromaffin Cells from Bovine Adrenal Medulla

G. Van Dessel,*,† H. De Busser,† and A. Lagrou†
**UIA-Laboratory for Pathological Biochemistry and †RUCA-Laboratory for Human Biochemistry, University of Antwerp, Groenenborgerlaan 171, B2020 Antwerp, Belgium*

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The uptake of FPP and GGPP was studied using primary cultures of chromaffin cells as an experimental model. The translocation of the isoprenyl diphosphates was shown to be both dose- and time-dependent. After incubation and a series of thorough washing cycles the residual radioactivity could only be released by either detergent or alkali treatment endorsing true internalisation rather than sorption onto the external cell surface. Preliminary experiments suggest that the isoprenyl diphosphates are internalised - at least for the greater part - without a preceeding dephosphorylation step. The translocation process does not seem to follow regular Michaelis-Menten kinetics. Upon varying pH (pH-range 6-8) or temperature (0°–40°C) of the incubation medium only moderate changes in internalisation rate were observed. The metabolic poisons (CN^-, F^-, chloroquine) administered were without any significant effect, suggesting the transport phenomenon not to be energy dependent nor to proceed via receptor-mediated endocytosis. Upon inclusion of metallic ions in the test medium a pronounced increase in uptake was registered for Zn^{2+}-ions, while most other cations did not cause any significant alterations. From the amino acid modifying agents screened sulphydryl reagents (NEM, PCMB, IA) resulted into a moderate unexpected elevation of uptake. An interesting feature was the activatory effect on the uptake of the diphosphorylated derivatives elicited by the corresponding free alcohols farnesol and geranylgeraniol; shorter and longer chain alcohols were without effect. In order to assess the specificity of the transport system a number of (pyro)phosphorylated compounds were included in the incubation medium indicating dolichylmonophosphate and isopentenylpyrophosphate to promote the uptake. © 1997 Academic Press

Abbreviations: C:M : chloroform-methanol mixtures; FPP : farnesylpyrophosphate; GGPP : geranylgeranylpyrophosphate; IA : iodoacetate; NEM : N-ethylmaleimide; PCMB : p-chloromercuribenzoate; PE : phosphatidylethanolamine; PI : phosphatidylinositol; PS : phosphatidylserine; SPH : sphingomyelin.

The classical strategy for labelling isoprenylated proteins involves incubation of cells or tissue slices with radioactive mevalonate and inclusion of a HMG CoA reductase inhibitor, such as mevinolin, in the culture medium (1). Another biochemical approach consists of suppression of the endogenous mevalonate synthesis accompanied by supplementation of the cell culture medium with either [^{3}H] farnesyl - or [^{3}H] geranylgeranylpyrophosphate (2). The success of the latter strategy implies the occurrence of a transport system translocating these phosphorylated prenoids across the cell membranes. Moreover, in view of the multisubcellular localisation of some parts of the mevalonate pathway e.g. the biosynthesis of FPP, the occurrence of a transmembranous transport mechanism for the phosphorylated precursors must be assumed.

In view of our research interest aiming to link protein isoprenylation with regulated exocytosis, the uptake of [^{3}H] FPP and [^{3}H] GGPP was studied using primary cultures of chromaffin cells as an experimental model. Upon stimulation these adrenal medulla cells secrete catecholamines from intracellular granules via a regulated exocytotic secretion process (3). Studies on the permeability of membranes for isoprenyldiphosphates are scarce. In plants Soler *et al.* (4) have reported on the uptake of IPP by plastids isolated from *Vitis vinifera L.* Cell suspensions. Recently Danesi *et al.* (2) have hypothetised the occurrence of a transport system of phosphorylated prenoids across the cell membranes of PC-3 cells originating from a human prostate cancer line.

EXPERIMENTAL PROCEDURES

Chemicals. [^{3}H] FPP (17.3 Ci/mmol) and [^{3}H] GGPP (18.7 Ci/mmol) were purchased from Amersham (UK). All other isoprenoids were a gift from the Kuraray Co. (Japan). Wheat germ pyrophospha-

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tase was purchased from Sigma. Culture flasks, plastic petri dishes were from NUNC. All other reagents were analytical grade or of the highest purity available from either Merck or Sigma.

Cell preparation. Primary cultures of bovine chromaffin cells were prepared and cultivated as described by Livett *et al.* (5). They were seeded at a concentration of 10^6 cells/dish (30 mm petri dish) in Dulbecco's modified Eagle's medium, containing 25mM Hepes, 4mM L-glutamine, 1 g/l glucose, 10 % foetal calf serum, 2.5 μg/ml each of fluorodeoxyuridine and cytosine arabinoside; 50 μg/ml gentamycin, 100 units/ml penicillin and 100 μg/ml streptomycin.

Uptake protocol. Prior to incubation the primary chromaffin cell cultures (10^6 cells/3 cm petri dish) were washed with serum free medium (2 $\times$ 0.5 ml) whereafter the cells were supplemented with 2 ml of this medium as well as with the appropriate amounts of [^{3}H] FPP and [^{3}H] GGPP. In the standard assay the final concentration was 10 nM. After 60 min. incubation at 37°C, the serum free medium was removed and the cells exhaustively washed (5 $\times$ 0.5 ml Locke's buffer). Subsequently the cells were disrupted with 0.1M NaOH (2 $\times$ 0.5 ml). Aliquots of the lysate were taken for protein determination and measurement of the radioactivity (Lumagel). Effectors were added just before the administration of the labelled pyrophosphorylated precursor or after an appropriate preincubation time as mentioned in the legend of the corresponding figures.

Lipid analysis. For lipid identification the cells were extracted either with n-butanol (isoprenyl diphosphates) (6) or several C:M:W mixtures (7) (total lipids) as mentioned in the corresponding table. TLC of these lipids was performed on precoated silica gel Si60 plates (E. Merck) with two eluent systems: eluent A: di-isobutylketon-acetic acid-water 8/5/1 (v/v/v), eluent B: toluene-ethylacetate 4/1 (v/v).

Assay. Protein concent was measured by the method of Lowry *et al.* (8) using bovine serum albumin as standard. Dephosphorylation of pyrophosphorylated prenoids was achieved by treatment with wheat germ phosphatase (9).

RESULTS

Evidence for a True Internalization Process

In order to differentiate between true internalization and simple sorption onto the external cell surface the cells, after incubation and removal of the assay medium, were washed successively with respectively 5 $\times$ 0.5 ml Locke's buffer, 1 % DMSO, 5 % DMSO, 1 % Triton X100 and 5 % Triton X100. Analyses, performed on the combined washes (2.5 ml each), indicate that the radioactivity mobilized by the cells could only be released after detergent or alkaline treatment (data not shown). In the experiments further described cells were washed as a routine with 5 $\times$ 0.5 ml Locke's buffer, whereafter the internalized radioactivity was released with 2 $\times$ 0.5 ml 0.1M NaOH.

Chemical Characterization of the Internalized Radioactivity

Cells were extracted with n-butanol (4 $\times$ 1 ml) as described in Materials and Methods. After TLC (eluent A) the radioactivity in the butanol extract comigrated with FPP or GGPP standards. No significant activity was found at the level of the migration distance of the free alcohols. Dephosphorylation of the butanol extracted material using wheat germ phosphatase yielded compounds comigrating on TLC (eluent B) with respectively free farnesol and free geranylgeraniol. In the C:M 2/1 (v/v) extract + 8 % of the FPP derived radioactivity was recovered; for GGPP derived activity + 15 % was measured. Much minor amounts (< 1 %) were found in the C:M:W (10/10/3 v/v/v) extracts as well as in the residual delipidated protein pellet (Table I).

TABLE I

Distribution of FPP or GGPP Derived Radioactivity after Solvent Extraction of the Incubated Cells

	FPP (%)	GGPP (%)*
Cells	1*	0.7*
C:M 2/1	7.4**	14**
C:M:W 10/10/3	0.5**	0.8**
Protein residu	0.4**	0.7**
n-Butanol	92**	85**

* vs. 100% radioactivity present in the incubation mixture.
** vs. 100% cell associated radioactivity.

Subcellular Fate of the Internalized Radioactivity

After incubation with the respective isoprenyl diphosphates and an exhaustive washing procedure the cells were homogenized (2 min sonication Branson 2000, 4°C) and the ensuing homogenate subfractionated in a membranous and a supernatant fraction (140,000*g* $\times$ 60 min). About 20 % of the radioactivity could be recovered from the membranous fraction, while the major part was residing in the soluble fraction. The partition behaviour of FPP and GGPP is quite different in an artificial octanol-water biphasic system where FPP is found over 90 % in the aqueous phase, while only 10 % of GGPP is present in this phase. On the other hand when using even more apolar phases such as heptane or octane nearly all radioactivity (> 97 %) for both FPP and GGPP can be traced in the aqueous phase. In this respect a number of experiments with egg lecithin liposomes as artificial membrane acceptor system were performed. With 100 % egg lecithin liposomes and also after varying the chemical composition (including 10 % of respectively PE, PI, PS and SPH) no uptake nor incorporation of FPP or GGPP could be observed (data not shown).

Uptake Kinetics

The translocation of the isoprenyl diphosphates was shown to be both time and dose dependent (Fig. 1 A,B). After 30 min incubation the uptake time course curve levelled off. This phenomenon was not observed for the dose-dependency curve. Even at a 100 molar excess of both FPP and GGPP this curve did not reach a plateau (Fig. 1c) as such not allowing calculation of a K_m value

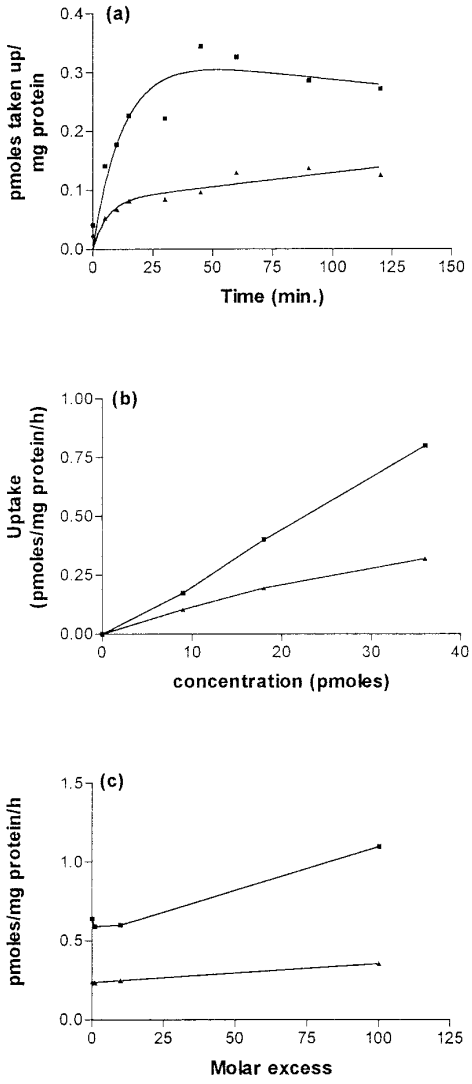

FIG. 1. Kinetics of the isoprenyldiphosphate uptake by chromaffin cells. Cells were incubated as described in Materials and Methods. ■ : FPP uptake; ▲ : GGPP uptake. (a) Time course of the uptake of [^{3}H] FPP and [^{3}H] GGPP by chromaffin cells. (b) Dose dependent uptake of [^{3}H] FPP and [^{3}H] GGPP by chromaffin cells. (c) Influence of excess of cold FPP and GGPP on the uptake of the corresponding labelled isoprenyl diphosphates.

for the uptake process. From the experiments it is also clear that FPP is more efficiently taken up by these cells than the longer chain GGPP.

Upon varying the pH (pH range 6-8) and temperature (0°C, 24°C, 37°C) of the incubation medium no statistically significant changes in internalization rates were noticed (data not shown).

Energy Dependency

The dependency of the membrane transport on energy supply was checked by including a series of drugs (NaCN,NaF, chloroquine, glucose; all at 10μM)) in the culture medium. These metabolic blockers were without any significant inhibitory effect.

Specificity of the Uptake

In order to assess the specificity of the transport a number of (pyro)phosphorylated compounds which could interfere with FPP and GGPP uptake were included in the incubation medium. None of them manifested convincing inhibitory effects on FPP or GGPP transport. Contrary Dol-P and IPP (at elevated concentrations) even promoted uptake especially for FPP (Fig. 2A).

Effects of Free Isoprenols

An interesting unexpected feature was the activatory effect elicited by the corresponding free alcohols farnesol and geranylgeraniol, FPP transport being most effectively stimulated (Fig. 2B). Moreover this activatory effect of farnesol and geraniol was in a limited way dose dependent, within the concentration range investigated (10-20μM). Trimethylundecadien-1-ol, a structural analogue of farnesol displaying 1 carbon and 1 desaturation less than farnesol, and t-nerolidol, a geraniol isomer, stimulated in the same way. The primary alcohol function was not essential as farnesal (also farnesylbromide; data not shown) resulted into similar enhancing effects. The shorter chain geraniol and the long chain dolichol were much less effective. Un-

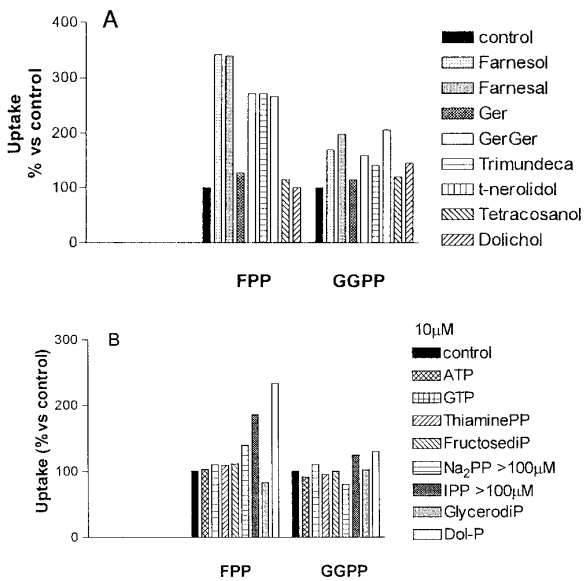

FIG. 2. (A) Influence of (pyro)phosphorylated compounds on the uptake of [^{3}H] FPP and [^{3}H] GGPP. Cells were incubated as described under Materials and Methods in the presence of 10μM of the indicated (pyro)phosphorylated compound. (B) Modulation of the uptake of [^{3}H] FPP and [^{3}H] GGPP by free isoprenols. Effect of free alcohols (10μM) on the uptake of FPP and GGPP. Cells were incubated as described under Material and Methods.

branched short and long chain alcohols (octanol, tetracosanol) were without any effect on the translocation.

Effect of Cations

FPP and GGPP are anionic species and as such the uptake may be hampered as the result of electrostatic exclusion by the negative cell surface potential. In this respect a number of mono- and divalent-metal ions were supplemented to the incubation medium in order to couple a less negative value to the membrane potential. From the mono- and divalent cations tested only in the presence of Zn^{2+} ions a pronounced increase of uptake was perceived. In presence of 2mM Zn^{2+} ions the FPP uptake was increased to upto 3 times while for the GGPP uptake a much more significant activation was noted ($\times$ 10-20).

Effect of Amino Acids Modifying Agents

The possible involvement of a protein in the uptake of pyrophosphorylated isoprenoids was investigated by preincubation with several amino acid modifying agents. Both non-permeant (p-chloromercuribenzoate and Hg^{2+}-ions) and permeant (NEM) sulphydryl reagents seem to stimulate the uptake up to twofold or more. Alternatively dansylchloride (free amino groups) and the Woodward reagent (free carboxylate groups) did not alter the uptake process.

DISCUSSION

The data in this paper provide for the first time experimental evidence for the occurrence of a transport system for FPP and GGPP across biological membranes as earlier hypothetised by Danesi *et al.* (2). The observation that the radioactivity after uptake by the chromaffin cells could only be released by either detergent or alkali treatment endorses true internalization rather than sorption onto the external cell surface. From extraction and TLC experiments it can be concluded that both FPP and GGPP are taken up by the cells in their unmodified phosphorylated form. This was affirmed by the lack of inhibition from the corresponding free alcohols. On the contrary inclusion of farnesol and geranylgeraniol in the incubation mixture resulted in an enhanced uptake of the pyrophosphorylated compounds. At this moment we have no clearcut explanation at hand for this phenomenon. The most pronounced activatory effects were found with isoprenoids of similar chain length; shorter and longer chain alcohols were much less effective. The divergent results obtained when including geraniol or t-nerolidol indicate that both chain length and steric configuration are determinant. Further as farnesal and farnesylbromide elicited the same stimulatory effect as farnesol it is evident that the specificity of the effect must reside within the hydrophobic tail of these prenoids.

At this moment some of the experimental data are hard to reconcile *e.g.* the uptake time course curve (levelling off) and the uptake concentration dependency curve (not reaching a plateau, even at 100 molar excess). The lack of saturation does not indicate a protein mediated uptake process. On the other hand from (i) the negative results obtained with the liposomal systems, (ii) the effects elicited by sulphydryl-reagents, and (iii) the specific activation by Zn^{2+} ions one could speculate that protein carriers might be involved. As the other cations tested did not cause any significant alteration, one may conclude that a local depolarization of the membranes is not obligatory as found for IPP transport in plastids (4).

The finding that a limited amount of radioactivity was also recovered in the C:M extracts as well as in the residual protein pellet suggests that after uptake FPP and GGPP are partially metabolized. It is a well established biochemical fact that protein prenyltransferases are cytosolic enzymes. In agreement, from the subcellular data it can be concluded that after internalization the greater part of both FPP and GGPP reside in the same aqueous compartment. This finding is also reflected in the partition behavior of FPP and GGPP in an artificial octane-water system. On the other hand, FPP and GGPP behave quite differently in the biphasic system octanol-water.

From the experiments with metabolic blockers and in agreement with Soler *et al.* (4) no energizable phenomenon seems to be responsable for the uptake; the failure of chloroquine to inhibit the transport implies that receptor mediated endocytosis is not involved.

Finally, from the lack of inhibition by a series of pyrophosphorylated compounds one can conclude that FPP and GGPP are not transported through the cell membrane via a general transport of anionic species. Most remarkable was the activatory effect from the long chain dolichylmonophosphate and IPP (at elevated concentrations) especially on the uptake of FPP.

As a conclusion the experimental data submitted undoubtedly reveal the occurrence of a translocation system for FPP and GGPP across the cell barrier of chromaffin cells. The mechanism of this most intriguing transport remains to be elucidated as the present data neither support single diffusion nor protein mediated uptake.

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