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Role of Calcium Ions in Rapid Effects of L-Thyroxine on Phosphoinositide Metabolism in Rat Liver Cells

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Abstract—The role of calcium ions in the L-thyroxine-induced initiation of hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) and also the course of releasing individual fractions of inositol phosphates and diacylglycerides (DAG) were studied in liver cells during early stages of the hormone effect. L-Thyroxine stimulated a rapid hydrolysis in hepatocytes of $PtdInsP_2$ labeled with [^{14}C]linoleic acid and [^{3}H]inositol mediated by phosphoinositide-specific phospholipase C. This was associated with accumulation of [^{14}C]DAG, total inositol phosphates, [^{3}H]inositol 1,4,5-trisphosphate ($Ins1,4,5P_3$) and [^{3}H]inositol 1,4-bisphosphate ($Ins1,4P_2$). Elimination of calcium ions from the incubation medium of hepatocytes did not abolish the effect of thyroxine on the accumulation of [^{14}C]DAG and total [^{3}H]inositol phosphates. Preincubation of liver cells with TMB-8 increased the stimulatory effect of L-thyroxine on the accumulation of [^{14}C]DAG. During the incubation of hepatocytes in the presence of the hormone the content of ^{14}C-labeled fatty acids did not change. The L-thyroxine-induced accumulation of [^{3}H]$Ins1,4,5P_3$ and [^{3}H]$Ins1,4P_2$ did not depend on the presence of calcium ions in the incubation medium of the cells.

Key words: thyroxine, hepatocytes, phospholipase C, phosphatidylinositol 4,5-bisphosphate, diacylglycerol, inositol 1,4,5-trisphosphate, calcium

Various extracellular stimuli including hormones and neurotransmitters are known to cause hydrolysis of $PtdInsP_2$ under the influence of PtdIns-specific phospholipase C (PLC), and this hydrolysis is accompanied by generation of secondary messengers, such as diacylglycerols (DAG) and inositol 1,4,5-trisphosphate ($Ins1,4,5P_3$), mobilization of calcium ions from intracellular depots, and activation of protein kinase C in the cell [1].

Thyroid hormones are known to play an important role in the regulation of activity of the PtdIns-specific PLC. The involvement of the PtdIns-specific PLC in rapid effects of thyroxine was indirectly shown on HeLa cells. Thus, introduction of thyroxine in physiological concentrations into the incubation medium of HeLa cells pretreated with a recombinant human interferon INF-γ significantly increased antiviral and immunomodulatory effects of INF-γ [2]. A specific inhibitor of PLC, U-73122, completely abolished this effect.

We have earlier shown that L-thyroxine induces a rapid, during the first 15 sec, accumulation of [^{14}C]DAG in hepatocytes prelabeled with [^{14}C]oleic acid [3]. This accumulation of DAG coincided in time with the decrease in the level of [^{14}C]$PtdInsP_2$ and generation of $Ins1,4,5P_3$ in the cells prelabeled with [^{3}H]inositol. A specific inhibitor of PtdIns-specific PLC, neomycin, abolished the effect of L-thyroxine.

Injection of high, above-physiological doses of L-thyroxine resulting in hyperthrophy of the cardiac muscle significantly decreased the activity of PtdIns-specific PLC in the plasma membranes of cardiomyocytes [4].

The activity of PtdIns-specific PLC of liver cells increased in thyroidectomized rats [5]. A single injection of thyroxine to thyroidectomized rats decreased the activity of the enzyme, and the effect of the hormone was mediated by mobilization of calcium ions from intracellular stores.

On the perfused liver both L-triiodothyronine and L-thyroxine were shown to stimulate a rapid entrance of Ca^{2+} into the cell and increase the concentration of free calcium ions in the cytoplasm [6]. The concentration of Ca^{2+} in the cytoplasm and the enzymatic activity of PtdIns-specific PLC were in close correlation. Thus, the activity of the PtdIns-specific PLC was stimulated in the presence of micromolar concentrations of Ca^{2+} [7], whereas higher concentrations of calcium in the cytoplasm inhibited the activity of the enzyme [8].

However, the mechanism of regulation by thyroid hormone of PtdIns-specific PLC in liver cells and the role

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of Ca^{2+} in this process are far from being elucidated. Therefore, the purpose of the present work was to determine the role of calcium ions in the initiation of hydrolysis of $PtdInsP_2$ under the influence of L-thyroxine with the involvement of PtdIns-specific PLC and also to study the course of releasing of individual fractions of inositol phosphates in liver cells during early stages of the effect of L-thyroxine.

MATERIALS AND METHODS

HEPES and Trypan Blue were from Serva (Germany), L-thyroxine was from Reanal (Hungary), standard PtdIns, PtdInsP, and $PtdInsP_2$ were from Sigma (USA), the mixture of [^{3}H]InsP, [^{3}H]$InsP_2$, [^{3}H]$InsP_3$, and [^{14}C]linoleic acid (58 mCi/mmol) was from Amersham (England), silica gel was from Woelm (Germany), TMB-8 (8-N,N-diethylaminooctyl-3,4,5-trimethoxybenzoate) was from Fluka (Switzerland), and other reagents of chemical purity were of domestic production.

Ninety-day-old male Wistar rats were used. Before opening the abdominal cavity animals were anesthetized with diethyl ether. Hepatocytes were isolated as described in [9]. The cell viability was assessed with Trypan Blue, and viable cells were 90-95% of the total cell number. Freshly isolated hepatocytes were resuspended in the medium which contained 118 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 0.2% $NaHCO_3$, 0.1% BSA, penicillin (61 mg/liter), and streptomycin (100 mg/liter) (medium A), and [^{14}C]linoleic acid (2 μCi/ml) (pH 7.5) and incubated at 37°C for 15-240 min. The cell concentration in this case was 10^7 per 1 ml. After the incubation, the hepatocytes were washed twice with excess of the same buffer cooled to 4°C. Afterwards the hepatocytes were diluted in medium A to the concentration of 10^6 cells per 1 ml.

Neutral lipids were extracted as described in [10], and inositol-containing phospholipids were extracted as described in [11]. Phospholipids were fractionated by thin-layer chromatography on Woelm silica gel in the solvent systems as follows: hexane–diethyl ether–AcOH (73 : 25 : 2 v/v) for diacylglycerols [12], $CHCl_3$–CH_3OH–acetone–AcOH–H_2O (30 : 10 : 11.5 : 9 : 6 v/v) for phosphoinositides [11]. Lipids were developed in iodine vapors and identified by comparison to standards.

To determine the production in the cells of water-soluble inositol phosphates, the freshly isolated hepatocytes were resuspended in Eagle's medium supplemented with [^{3}H]myoinositol (1 μCi/ml), 25 mM HEPES, 25 mM glutamine, penicillin (61 mg/liter), streptomycin (100 mg/liter), 10% fetal calf serum (pH 7.5) and incubated for 24 h at 37°C. The cell concentration in this case was 10^6 per 1 ml. After the incubation, the hepatocytes were washed twice in phosphate buffer and resuspended for 10 min at 37°C in medium which contained 0.1% BSA and 20 mM LiCl. Then the hepatocyte suspension was supplemented with L-thyroxine (10^{-8} M) and incubated for 0-2 min. In a special case, cells were resuspended in Ca^{2+}-free medium in the presence of 2 mM EDTA.

The reaction was stopped by addition of 1 ml of 10 mM cooled formic acid, and the samples were kept on ice for 30 min. Then 3 ml of 5 mM NH_4OH was added (final pH 8-9) and the samples were centrifuged for 5 min at 3000*g*.

[^{3}H]Inositol phosphates were analyzed in 2.5 ml of the supernatant fluid. Water-soluble inositol phosphates were fractionated by ion-exchange chromatography as described in [13]. [^{3}H]InsP, [^{3}H]$InsP_2$, and [^{3}H]$InsP_3$ were used as standards.

Radioactivity of samples containing ^{14}C-labeled lipids or ^{3}H-labeled inositol phosphates was determined using ZhS-8 scintillator with a BETA radioactivity counter.

RESULTS

The time course of incorporation of [^{14}C]linoleic acid into freshly isolated hepatocytes is presented in Fig. 1.

Addition of 10^{-8} M thyroxine into the incubation medium of hepatocytes prelabeled with [^{14}C]linoleic acid during the first 15 sec of the experiment decreased by 12% the content of labeled [^{14}C]$PtdInsP_2$ (Table 1). And the contents of [^{14}C]phosphatidylinositol (PtdIns) and [^{14}C]phosphatidylinositol 4-phosphate (PtdInsP) did not change significantly. After 30 sec of the cell incubation with the hormone the content of [^{14}C]$PtdInsP_2$ became

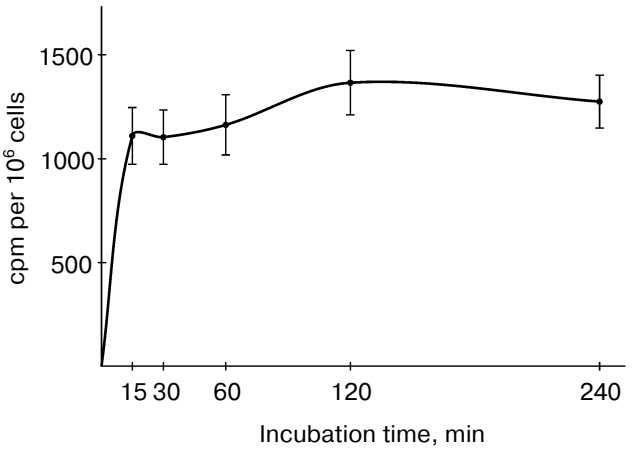

Fig. 1. Incorporation of [^{14}C]linoleic acid into total phospholipids of freshly isolated hepatocytes (cpm per 10^6 cells, $n = 5$).

Table 1. The *in vitro* effect of L-thyroxine (10^{-8} M)[a] on contents of [^{14}C]PtdIns, [^{14}C]PtdInsP, and [^{14}C]PtdInsP$_2$ in hepatocytes prelabeled with [^{14}C]linoleic acid during 90 min (data in % of the control, $n = 5$)

Lipid	Incubation time with the hormone, sec			
	15	30	60	120
PtdIns	102.75 ± 10.6	103.5 ± 11.1	87.7 ± 6.58	95.87 ± 6.65
PtdInsP	92.85 ± 9.49	79.9 ± 12.07[b]	92.7 ± 8.74	96.07 ± 21.63
PtdInsP$_2$	88.3 ± 2.53[b]	95.2 ± 4.09	96.8 ± 8.85	93.67 ± 10.21

[a] Control samples contained 10^{-7} M NaOH.
[b] $p_{\text{control-thyroxine}} < 0.05$.

Table 2. The *in vitro* effect of L-thyroxine (10^{-8} M)[a] on content of [^{14}C]DAG in hepatocytes prelabeled with [^{14}C]linoleic acid during 90 min (data in % of the incorporation into neutral lipids, $n = 5$)

Experiment conditions	Incubation time with the hormone, sec			
	15	30	60	120
Control	6.592 ± 0.977	10.058 ± 2.258	9.66 ± 1.683	7.506 ± 1.683
L-Thyroxine	11.51 ± 2.037[b]	10.072 ± 2.592	9.21 ± 2.419	8.645 ± 2.696

[a] Control samples contained 10^{-7} M NaOH.
[b] $p_{\text{control-thyroxine}} < 0.05$.

normal and the content of [^{14}C]PtdInsP decreased. During the subsequent incubation in the presence of L-thyroxine contents of [^{14}C]PtdIns, [^{14}C]PtdInsP, and [^{14}C]PtdInsP$_2$ did not differ from the control values.

The level of [^{14}C]PtdInsP$_2$ in hepatocytes under the influence of L-thyroxine decreased concurrently with increase in the production of [^{14}C]DAG (Table 2). The time course and quantitative characteristics of the DAG accumulation along with the decrease in PtdInsP$_2$ content in hepatocytes prelabeled with [^{14}C]linoleic acid completely agreed with our earlier findings for the cells preincubated with [^{14}C]oleic acid [3]. The release of ^{14}C-labeled free fatty acids in hepatocytes labeled with [^{14}C]linoleic acid by 15, 30, 60, and 120 sec of contact with the hormone was 95.7 ± 3.8, 99.5 ± 2.6, 104.9 ± 2.3, and 103.6 ± 2.9% of the control value, respectively.

Introduction of physiological doses of L-thyroxine into the calcium-free incubation medium of hepatocytes in the presence of 2 mM EDTA increased by 133% the content of [^{14}C]DAG as compared to the control (Table

Table 3. Effect of calcium ions[a] on the L-thyroxine-induced[b] generation of [^{14}C]DAG in hepatocytes (data are presented in cpm per 10^7 cells, $n = 5$)

Experiment conditions	[^{14}C]DAG
Control	1831 ± 337
L-Thyroxine	4272 ± 780[d]
Control + TMB-8[c]	1610 ± 349
L-Thyroxine + TMB-8	7537 ± 1139[d]

[a] Hepatocytes preincubated with ^{14}C-labeled fatty acid were washed free from the label and resuspended in the medium A (see "Materials and Methods") without calcium ions but with 2 mM EDTA.
[b] The incubation medium contained L-thyroxine (10^{-8} M). Concentration of NaOH in the control samples was 10^{-7} M. The cells were incubated with the hormone for 15 sec.
[c] A chelator of the intracellular calcium, TMB-8 (20 μM), was introduced 15 min before the addition of the hormone.
[d] $p_{\text{control-thyroxine}} < 0.05$.

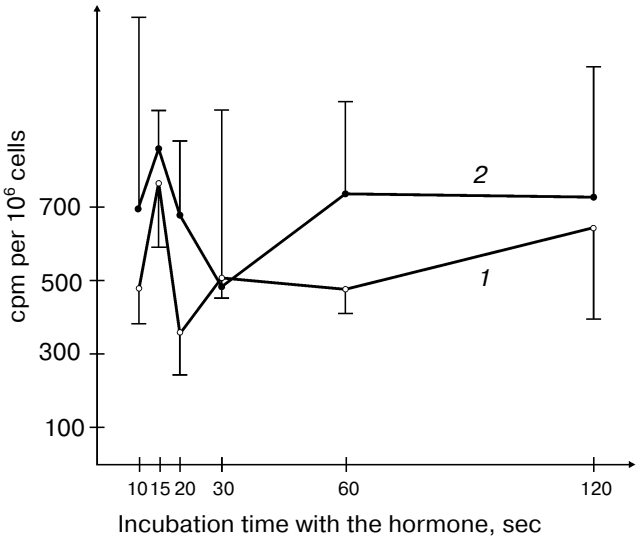

Fig. 2. Effect of L-thyroxine (10^{-8} M) on the release of inositol phosphates in hepatocytes prelabeled with [^{3}H]inositol (cpm per 10^6 cells): *1*) L-thyroxine; *2*) L-thyroxine + EDTA. The control samples contained 10^{-7} M NaOH, and the preincubation time of cells in the presence of [^{3}H]inositol was 24 h. For the conditions of labeling, see "Materials and Methods".

3). Incubation of rat liver cells with L-thyroxine in the presence of a chelator of intracellular calcium, TMB-8, was accompanied by 368% accumulation of [^{14}C]DAG by the 15th second of the hormone action, as compared to the control.

Introduction of L-thyroxine into the incubation medium of hepatocytes prelabeled with [^{3}H]inositol for 24 h stimulated the accumulation of total water-soluble [^{3}H]inositol phosphates during the first 10 sec, with the maximum by the 15th second of the cell exposure to the hormone (Fig. 2). This was associated with increase in amounts of [^{3}H]Ins1,4P_2 and [^{3}H]Ins1,4,5P_3 under the influence of L-thyroxine (Fig. 3c, Table 4). The hormone effect on hepatocytes resuspended in the Ca^{2+}-free medium was associated with a similar course of accumulation of both total water-soluble [^{3}H]inositol phosphates and their individual fractions (Figs. 2 and 3d). Quantitative characteristics of accumulation of individual fractions of water-soluble [^{3}H]inositol phosphates are presented in Table 4.

DISCUSSION

The results of the present work and earlier studies [3] have shown that L-thyroxine causes a rapid but transient activation of PtdIns-specific PLC in hepatocytes prelabeled with ^{14}C-labeled unsaturated fatty acids or with [^{3}H]inositol. This activation is accompanied by accumu-

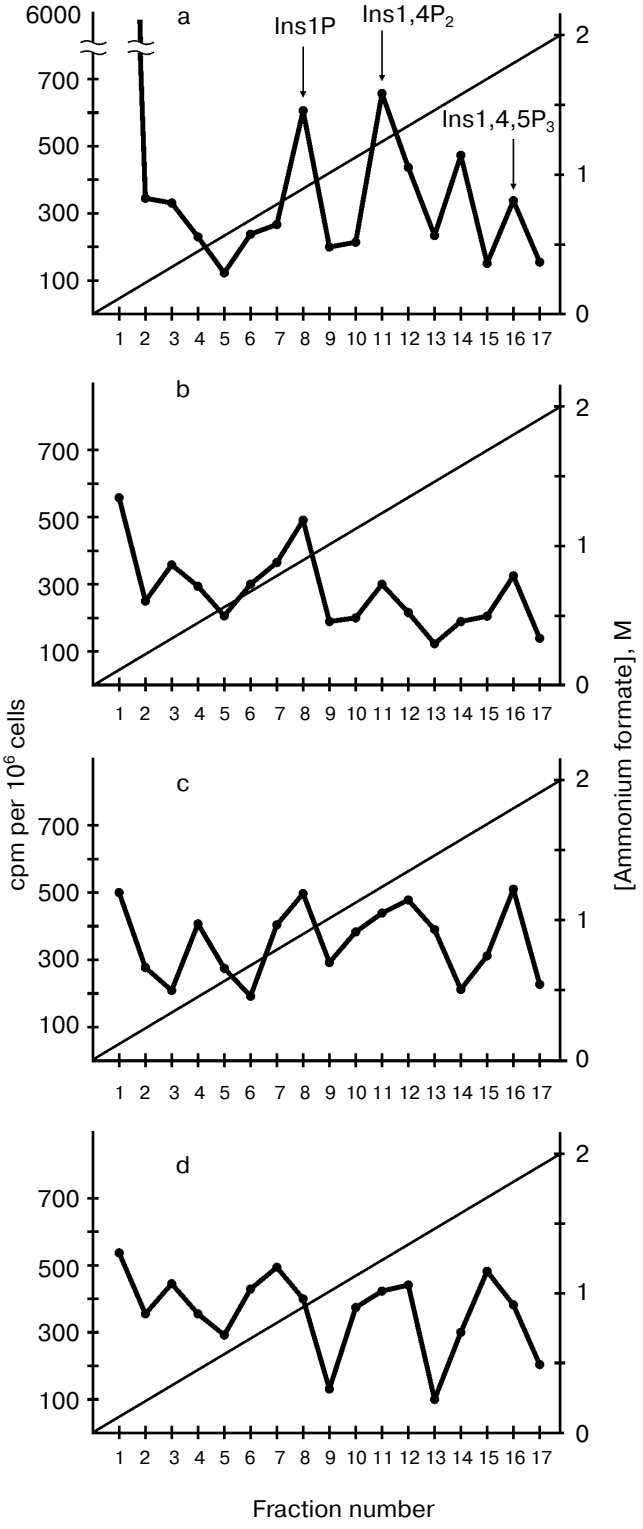

Fig. 3. Effect of L-thyroxine (10^{-8} M) on generation of [^{3}H]Ins1,4,5P_3, [^{3}H]Ins1,4P_2, and [^{3}H]Ins1P in rat hepatocytes prelabeled with [^{3}H]inositol for 24 h as described in "Materials and Methods" (control samples contained 10^{-7} M NaOH): a) standard solutions of [^{3}H]Ins1P, [^{3}H]Ins1,4P_2, [^{3}H]Ins1,4,5P_3; b) control; c) L-thyroxine; d) L-thyroxine was introduced into the incubation medium of hepatocytes without Ca^{2+} but with 2 mM EDTA.

Table 4. Effect of L-thyroxine (10^{-8} M) on production of individual fractions of inositol phosphates in hepatocytes prelabeled with [^{3}H]inositol (data are presented in cpm per 10^7 cells, $n = 5$)

Inositol phosphates	Control[a]	L-Thyroxine	L-Thyroxine + EDTA[b]
Ins1P	1360 ± 182	1483 ± 95	1538 ± 207
Ins1,4P	901 ± 132	1856 ± 125[c]	1620 ± 150[c]
Ins1,4,5P	632 ± 32	1086 ± 37[c]	1091 ± 25[c]

[a] Control samples contained 10^{-7} M NaOH.
[b] Cells were incubated in the presence of L-thyroxine in the Ca^{2+}-free medium.
[c] $p_{\text{control-thyroxine}} < 0.05$.

lation of degradation products of [^{14}C]PtdInsP_2—[^{14}C]DAG and [^{3}H]Ins1,4,5P_3.

To date, cells have been found to contain three types of PLC—β, γ, δ [14]. In liver cells different isoforms of all PLC types are found [15]. Calcium ions play an important role in the regulation of activities of all known subtypes of PtdIns-specific PLC. Thus, for hydrolysis of PtdInsP_2 with involvement of PtdIns-specific PLC the presence of calcium ions in the medium is required, which activate the enzyme by binding to its catalytic domain [16]. Moreover, Ca^{2+} induces conformational changes in PLC which provides for a necessary spatial orientation of the catalytic domain relatively to the substrate in the plasma membrane [17].

On liver cells, thymocytes, and cardiomyocytes thyroid hormones were shown to cause a rapid, within 30 sec, and a transient entrance of Ca^{2+} into the cell [6, 18]. It was suggested that Ca^{2+} could play the role of a signal messenger responsible for regulation of transient effects of thyroxine on the cells.

We have shown that elimination of calcium ions from the incubation medium did not abolish the accumulation of [^{3}H]Ins1,4,5P_3 and also of total [^{3}H]inositol phosphates. Our data suggested that PtdIns-specific PLC should be activated in hepatocytes by thyroxine by a mechanism unassociated with a rapid entrance of Ca^{2+} into the cell.

At present, another mechanism for transient effects of thyroid hormones has been found, and the initial stage of this mechanism is the binding of the hormone to a specific site on the plasma membrane surface, namely, to a specific receptor associated with the heterotrimeric G_i-protein, and the βγ-subunit of this protein regulates the activity of PtdIns-specific PLC [2]. This mechanism of transient effects of thyroid hormones was earlier found for HeLa and CV-1 cell lines which have no nuclear receptors to thyroid hormones; however, a similar action mechanism was recently shown also for BG-9 skin fibroblasts which had nuclear receptors to thyroid hormones [19].

Plasma membranes of liver cells were earlier shown to possess binding sites for thyroxine and triiodothyronine, which were supposed to be receptors mediating non-genomic effects of thyroid hormones [20].

Therefore, the thyroxine-caused activation of PtdIns-specific PLC of hepatocytes observed in the present work is suggested to be mediated by interaction of thyroxine with a specific binding site on the plasma membrane surface of hepatocytes.

The binding of Ins1,4,5P_3 to specific receptors on the membrane surface of endoplasmic reticulum is known to result in the release of Ca^{2+} from intracellular depots [21]. And the agonist-dependent generation of Ins1,4,5P_3 is a marker of the Ca^{2+} release in the cell [22]. In their turn, DAG and Ca^{2+} induce the translocation of protein kinase C from the cytosol into membranes and activate it [23]. It is shown that on exposure of isolated hepatocytes to angiotensin II, epinephrine, and some other hormones the cell content of Ca^{2+} increases during the first seconds of action of the agonists, and this increase is accompanied by activation of protein kinase C [24]. L-Thyroxine was earlier shown to have a rapid but transient stimulatory effect on the accumulation of DAG in isolated hepatocytes the lipids of which were prelabeled with [^{14}C]oleic acid, and this stimulation occurred concurrently with the translocation of protein kinase C from the cytosol into the membrane fraction and manifold activation of the membrane-bound enzyme [25].

Utilization of metabolically active second messengers DAG and Ins1,4,5P_3 is an important stage in the termination of the hormonal signal transmission and cell return into the initial state.

DAG kinase plays a determining role in the utilization of DAG. Increase in the DAG level as a result of the agonist-dependent hydrolysis of PtdInsP_2 with involvement of PtdIns-specific PLC initiates the translocation of DAG kinase from the cytosol into membranes [26]. The Ins1,4,5P_3-induced increase in the Ca^{2+} concentration in the cytoplasm stimulates the activity of DAG kinase [27]. However, DAG lipase can also be involved in the utilization of DAG because a calcium ionophore A23187 stimulates the release of arachidonic acid from DAG under the influence of DAG lipase [28]. However, under conditions of our experiments thyroxine failed to stimulate the release of fatty acids in hepatocytes labeled with [^{14}C]linoleic acid. Thus, the contribution of lipase to the decrease in the DAG level by the 30th second of the experiment can be excluded.

The exposure to L-thyroxine of hepatocytes preincubated with a chelator of intracellular calcium, TMB-8, resulted in a twofold increase in the level of [^{14}C]DAG as

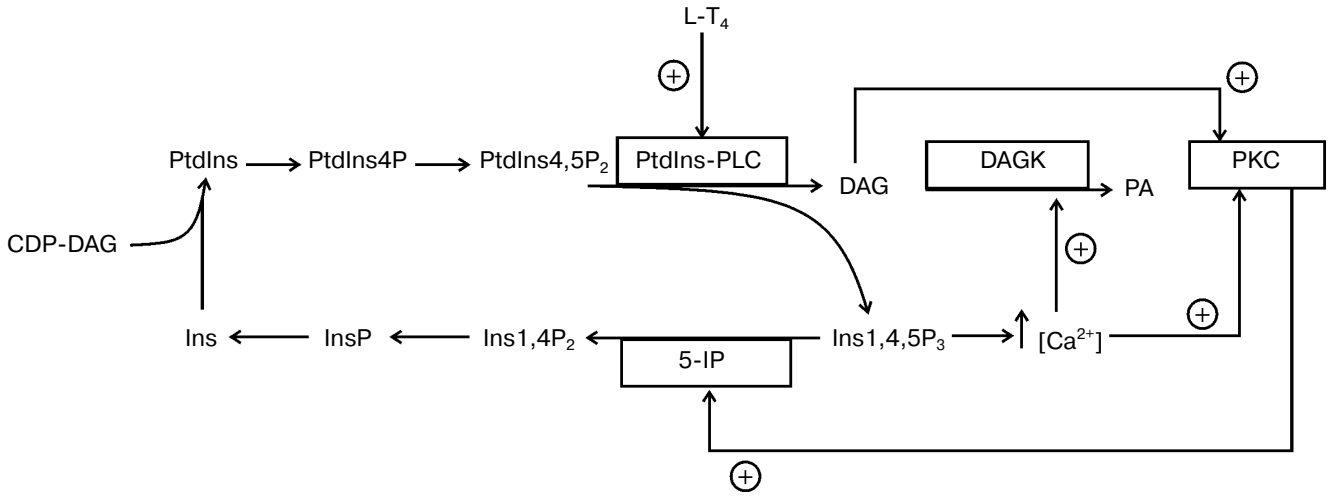

Fig. 4. Role of calcium ions in the rapid effects of L-thyroxine. Abbreviations: PtdIns-PLC) PtdIns-specific PLC; PKC) protein kinase C; 5-IP) the type I inositol phosphatase; DAGK) diacylglycerol kinase; PA) phosphatidic acid; L-T_4) L-thyroxine; CDP-DAG) CDP-diacylglycerol.

compared to data on accumulation of [^{14}C]DAG on the exposure of intact hepatocytes to the hormone (Table 3). The concentration of calcium ions in the cytoplasm increased by production of Ins1,4,5P_3, and by binding calcium ions TMB-8 seemed to prevent the activation of DAG kinase of liver cells and utilization of newly synthesized [^{14}C]DAG.

Two pathways of Ins1,4,5P_3 transformation are now known: phosphorylation under the influence of inositol triphosphate 3-kinase with production of Ins1,3,4,5P_4, which is also a secondary messenger and induces the entrance of Ca^{2+} into the cell [29], and hydrolysis of Ins1,4,5P_3 under the influence of the type I 5-inositol phosphatase [21]. Ins1,4P_2 produced during this reaction cannot mobilize Ca^{2+}.

Activity of the type I 5-inositol phosphatase was found in various subcellular fractions of rat liver [30]. The amino acid analysis of type I 5-inositol phosphatase has shown it to be in a stoichiometric complex with a plextrine-homologous domain. According to concepts of [31], the plextrine-homologous domain is a possible site for phosphorylation with protein kinase C. The interaction of thrombin with specific receptors on the platelet surface is shown to activate protein kinase C which in its turn phosphorylates the type I 5-inositol phosphatase of platelets and thus regulates the termination of the signal transmission [32]. These data and also our finding that during the early stages of thyroxine influence on liver cells the accumulation of [H^3]Ins1,4P_2 was concurrent to generation of [H^3]Ins1,4,5P_3 (Fig. 3) suggest that thyroxine stimulates not only a rapid accumulation of Ins1,4,5P_3, but also the termination of this process, probably due to activation of the type I 5-inositol phosphatase.

Moreover, thyroxine can induce a rapid activation of resynthesis of polyphosphoinositides. This hypothesis is supported by data of the present work on the rapid recovery of the content of labeled PtdInsP_2 in liver cells and also by earlier studies on a protein kinase C-dependent rapid activation of synthesis of PtdInsP_2 in isolated hepatocytes under the influence of thyroxine [33]. Thus, thyroxine is likely to cause a rapid degradation of polyphosphoinositides and the termination of this process due to increase in the metabolism of [H^3]Ins1,4,5P_3 and DAG and resynthesis of PtdInsP_2.

Based on the findings, a scheme is suggested for the effect of thyroxine on metabolism of phosphoinositides and generation of their degradation products, inositol phosphates and DAG (Fig. 4). A rapid hydrolysis of PtdInsP_2 with involvement of PtdIns-specific PLC accompanied by production of [^{14}C]DAG and Ins1,4,5P_3 is one of early events in the realization of rapid effects of thyroxine on rat hepatocytes. The accumulation of DAG and increase in the cytoplasmic concentration of calcium induced by Ins1,4,5P_3 under the influence of L-thyroxine cause afterwards the translocation of protein kinase C and its activation [25]. The increase in the calcium ion concentration in the cytoplasm under the influence of Ins1,4,5P_3 seems to play an important role in utilization of the newly synthesized DAG, possibly due to activation of DAG kinase. Thyroxine initiates not only the rapid stimulation of production of Ins1,4,5P_3 but also a rapid termination of this process due to activation of phosphatases responsible for production of Ins1,4P_2 and Ins1P.

The observed activation of the PtdIns-specific PLC is not a result of increase in the intracellular concentration of Ca^{2+} due to entrance of Ca^{2+} into the cell during the first seconds of the hormone action but seems to occur during the interaction of thyroxine with a specific receptor on the plasma membrane surface.

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