Low level of sarcolemmal phosphatidylinositol 4,5-bisphosphate in cardiomyopathic hamster (UM-X7.1) heart

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Abstract

Objective: Phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P₂) is not only a precursor to inositol 1,4,5-trisphosphate (Ins 1,4,5-P₃) and sn-1,2 diacylglycerol, but also essential for the function of several membrane proteins. The aim of this study was to evaluate the changes in the level of this phospholipid in the cell plasma membrane (sarcolemma, SL) of cardiomyopathic hamster (CMPH) heart.

Methods: We examined the cardiac SL PtdIns 4,5-P₂ mass and the activities of the enzymes responsible for its synthesis and hydrolysis in 250-day-old UM-X7.1 CMPH at a severe stage of congestive heart failure (CHF) and in age-matched controls (Syrian Golden hamsters).

Results: The SL PtdIns 4,5-P₂ mass in CMPH was reduced by 72% of the control value. The activities of PtdIns 4 kinase and PtdIns 4-P₂5 kinase were depressed by 69 and 50% of control values, respectively. Although, the total phospholipase C (PLC) activity was moderately, although significantly, decreased (by 18% of control), PLCβ₁ isoenzyme activity in the SL membrane was elevated, with a concomitant increase in its protein content, whereas PLCβ₂ and PLCγ₁ isoenzyme activities were depressed despite the increase in their protein levels. A 2-fold increase in the Ins 1,4,5-P₃ concentration in the cytosol of the failing heart of CMPH was also observed.

Conclusions: Reduced SL level of PtdIns 4,5-P₂ may severely jeopardize cardiac cell function in this hamster model of CHF. In addition, the profound changes in the profile of heart SL PLC isoenzyme could alter the complex second messenger responses of these isoenzymes, and elevated Ins 1,4,5-P₃ levels may contribute to intracellular Ca²⁺ overload in the failing cardiomyocyte. © 2001 Elsevier Science BV. All rights reserved.

Keywords: Cardiomyopathy; Heart failure; Myocytes; Sarcolemma; Second messenger; Signal transduction

1. Introduction

Phosphatidylinositol 4,5 bisphosphate (PtdIns 4,5-P₂) directly regulates diverse bioprocesses occurring at the cardiac cell membrane (sarcolemma, SL) as, for example, the function of the inward rectifier K⁺ channels [1] as well as the Na⁺/Ca²⁺ exchange and Ca²⁺-pump activities [2,3]. In addition, PtdIns 4,5-P₂ is a precursor of second messengers (see below) and is a distinct signalling molecule per se [4,5] in that it serves as a membrane attachment site and activity regulator for important signalling-related proteins containing pleckstrin-homology domains [6]. Notable examples of these proteins are protein kinases such as β-adrenoceptor kinase, phospholipase D1, cytosolic phospholipase A₂ and phosphoinositide-phospholipase C δ₁ isoenzyme [6–10].

Our recent studies evidenced significant defects in SL PtdIns 4,5-P₂ level, synthesis and hydrolysis during overt
CHF secondary to large transmural myocardial infarcts (MI) in rats [11]. These abnormalities could have a relevant impact on the complex PtdIns 4,5-P₂-related signalling and may constitute an important mechanism underlying the defective cardiac performance in CHF. Thus, we conducted the present study on an etiologically different model of CHF i.e. the UM-X7.1 subline of cardiomyopathic hamster at the age of 250 days, when the animals are in overt CHF [12]. The aim of our study was to verify the possibility that the dysfunction of SL PtdIns 4,5-P₂ and of its synthesis and hydrolysis already observed in post-MI CHF [11], could also occur in the failing cardiomyopathic hamster, which would suggest that these defects are intrinsic to the state of ventricular failure in CHF.

2. Methods

2.1. Experimental model

Male UM-X7.1 cardiomyopathic hamsters at the age of 250 days (from the laboratory of Dr. G. Jasmin, Department of Pathology, University of Montreal, Canada) were employed in this study as a model of congestive heart failure [13]. UM-X7.1 is a cardiomyopathic subline of the Syrian Golden hamster [14–16]. Thus, as in past studies [12,16–18], age-matched, healthy Syrian Golden hamsters (Mesocricetus auratus) (outbred, from Charles River, Canada) were considered true controls and were used as control group. The animals were maintained on normal diet and water ad libitum. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

2.2. Preparation of cardiac sarcolemmal and cytosolic fractions

The animals were sacrificed by decapitation, and the hearts were quickly excised and immersed in ice-cold 0.6 M sucrose, 10 mM imidazole, pH 7.0 (buffer A). The ventricular tissue from three to five hearts was pooled to prepare cytosolic and SL fractions according to the method of Pitts [19] as previously described [11]. The tissue was washed, minced and homogenized in 3.5 ml of buffer A/g tissue with a Polytron (6×10 s, setting 5). Large cellular particles were removed by centrifugation at 12 000 g (30 min, 4°C). A small aliquot of the first supernatant was centrifuged at 110 000 g (60 min, 4°C), and the resulting supernatant was frozen and stored (−80°C) as the soluble cytosolic fraction. The rest of the first supernatant was diluted with 300 mM KCl-buffer to solubilize myofibrillar proteins [20], and further processed for the preparation of SL according to the method used previously [11] as shown in Fig. 1. The resultant SL-enriched pellet was resuspended in 0.25 M sucrose, 10 mM histidine (pH 7.4), frozen in liquid N₂ and stored at −80°C until assayed. Marker enzymes were assessed in this SL preparation (n=4). In particular, the relative specific activity (specific activity in SL/specific activity in homogenate) of K⁺-p-nitrophenol phosphatase (SL marker) was similar in control and cardiomyopathic hamster (CMPH) preparations (9.2±0.8 and 8.6±0.6 in control and CMPH, respectively). This indicated an equal degree of enrichment of the SL membrane in control and experimental preparations. The relative specific activity of rotenone-insensitive NADPH-cytochrome C reductase (SR marker) was 0.49±0.07 and 0.54±0.08 and that of cytochrome c oxidase (mitochondrial marker) was 0.63±0.06 and 0.68±0.07 in control and CMPH preparations. These results appear to indicate that the SL fractions under study were relatively pure and had only a minimal, but equal amount of, contamination from other subcellular organelles.

All the above steps were carried out at 0–4°C. Protein concentrations were determined by the Lowry method as indicated elsewhere [11].

2.3. PtdIns 4 kinase and PtdIns 4-phosphate 5 kinase assay

The activities of PtdIns 4 kinase and PtdIns 4-phosphate 5 kinase were assayed as described previously [11]. The SL preparation (30 µg protein) was preincubated in a solution mixture containing 40 mM HEPES–Tris, pH 7.4, 5 mM MgCl₂, 2 mM EGTA, 1 mM dithiothreitol and 30 mg alamethicin for 30 min at 30°C. PtdIns 4 kinase and PtdIns 4-phosphate 5 kinase were assayed in the absence or presence of 25 µM PtdIns and PtdIns 4-P, respectively. The phosphorylation was started by adding [γ-³²P]-ATP in a final concentration of 1 mM (0.16 Ci/mmol; this specific activity should not have been appreciably influenced by possible residues of inorganic phosphate noncovalently bound to the SL membranes). The reaction, which was linear up to 1 min of incubation for both kinases [Mesaeei et al., unpublished observations] was terminated 1 min later by adding methanol–10 M HCl (100:1, v/v) followed by the addition of 2.5 M HCl and chloroform. After centrifugation, the aqueous phase was discarded and the organic phase was washed once with chloroform–methanol–0.6 M HCl (3:48:47, v/v/v). Aliquots of the combined organic phases were used for the analysis of phosphoinositides by thin-layer chromatography. The solvent for the separation of phosphoinositide species contained chloroform–acetone–methanol–glacial acetic acid–water (40:15:13:12:8, v/v/v). The phospholipid migration was monitored using authentic unlabelled PtdIns 4-P and PtdIns 4,5-P₂ phospholipid standards. The ³²P-labeled phospholipid spots were visualized by overnight autoradiography using X-Omat-R X-ray film. PtdIns 4-P and PtdIns 4,5-P₂ were scraped from the plates and the
radioactivity in each fraction was determined by liquid scintillation counting.

In order to rule out the possibility that changes in phosphatase activity in the CMPH heart may affect the results reported here, some experiments in which the SL membrane fractionation as well as the phosphorylation assay were carried out in the presence of inhibitors of phosphatase activity (10 nM microcystin-LR and 10 nM sodium pyrophosphate) [21]. However, the results in the presence of phosphatase inhibitors were similar to those reported here; this indicated that changes in phosphatase activity in the CMPH heart did not influence the results reported here.

2.4. Total phospholipase C assay

The total PLC activity associated with the SL membrane was determined as previously reported [11,22]. Briefly, the substrate was prepared by mixing an aliquot of [3H]-PtdIns 4,5-P2, with an aliquot of unlabeled PtdIns 4,5-P2. The mixture was dried under a stream of N2 and redissolved in 0.1 g/ml (232 mM) sodium cholate. The substrate solution was kept under N2 gas overnight at 4°C and was diluted to 112 mM sodium cholate shortly before addition to the incubation mixture. Typically, reactions were carried out at 37°C in a mixture containing 30 mM HEPES–Tris (pH 7.0), 100 mM NaCl, 2 mM EGTA, 3.13 mM CaCl2 [to generate a free Ca2+ concentration of 1.13 mM [22], which was calculated according to the MAXCHELATOR computer program [23]]. 15 µg SL protein, 14 mM sodium cholate and 20 µM [3H]-PtdIns 4,5-P2 (20–30 dpm/pmol). The reactions were terminated after 2.5 min by the addition of 144 µl ice-cold chloroform–methanol–HCl (1:2:0.2, v/v) followed by 48 µl of 2 M KCl and 48 µl chloroform. Blanks were carried out under identical conditions except that protein was added after the reaction was stopped. Phase separation was facilitated by mixing and centrifugation, and the resulting aqueous phase was aspirated and applied to a 500-µl Dowex AG1-X8 microcolumn (formate form, 100–200 mesh). After the column was rinsed with water and with borax in sodium formate, inositol mono-, bis- and trisphosphates were eluted each with 1 ml of 0.1 M formic acid containing 0.2, 0.4 and 1 M ammonium formate, respectively. The radioactivity in each eluate was quantitated by liquid scintillation counting in 10 volumes of CytoScint™. Ins 1,3,5-P3 was the primary product of PtdIns 4,5-P2 hydrolysis as already indicated [11].

2.5. Immunoprecipitation of PLCβ1, γ, and δ, and assay for their activity

These procedures have been already reported [11]. Briefly, sarcolemmal membrane proteins were extracted.
Precipitates were removed by centrifugation at 10,000 g, by rotation for 2 h at 4°C. The samples were then centrifuged (280,000 g for 25 min) and the supernatant recovered as the solubilized membrane fraction. The membrane extract was incubated overnight at 4°C (rotation) with monoclonal antibodies to PLCs (anti-bovine PLC-β₁, mixed monoclonal antibodies (No. 05-164); anti-bovine PLC-γ₁, mixed monoclonal IgG antibodies (No. 05-163); anti-bovine PLC-δ₁, mouse monoclonal antibodies (No. 05-343); all from UpState Biotechnology, Lake Placid, NY, USA) and 20 μl packed beads) at 4°C overnight (5 s) at 10,000 g and assayed for the activity of PLC isoenzymes as described previously [11]. Briefly, the reaction was performed in the presence of 30 mM HEPES, pH 6.8, 70 mM KCl, 100 mM NaCl, 0.8 mM EGTA, 0.8 mM CaCl₂. To generate a free Ca²⁺ concentration of 23.3 μM [24], which was calculated according to the computer program [23], 20 μM [³H]-PtdIns 4,5-P₂ (20–30 dpm/pmol) dissolved in 14 mM sodium cholate overnight and an aliquot (10 μl) of immunoprecipitate suspension. The reaction was carried out at 37°C for 2.5 min, after which it was stopped by trichloroacetic acid precipitation. Precipitates were removed by centrifugation at 10,000 g for 5 min, and the supernatant was collected for quantification of inositol phosphates by liquid scintillation counting. The efficiency of the immunoprecipitation of each isoenzyme was ascertained by determining any residual PLC isoenzyme activity in the 10,000 g supernatant after subjecting this supernatant to a further immunoprecipitation. For control experiments, immunoprecipitation and subsequent activity measurements were conducted with non-immune mouse IgG.

The activities of each PLC isoenzyme have been measured basically according to the procedure established by Wahl et al. [26], it should be pointed out that the activities of these isoenzymes as reported here represent the relative activities since the antibodies used for immunoprecipitation are known to inhibit the PLC isoenzyme activities [24,25]. Furthermore, only a small proportion of each PLC isoenzyme in the SL membrane fraction was solubilized for immunoprecipitation. Thus some caution should be exercised while interpreting the results on PLC isoenzyme activities reported here.

2.6. Determination of cytosolic Ins 1,4,5-P₃ and SL PtdIns 4,5-P₂ content

Cytosolic Ins 1,4,5-P₃ level was determined using the Biotrak radioimmunoassay kit (Amersham Life Science, Canada). The manufacturer’s instructions modified according to the method of Chilvers et al. [27] were followed. Sarcolemmal PtdIns 4,5-P₂ content was quantified by conversion of PtdIns 4,5-P₂ in lipid extracts into Ins 1,4,5-P₃ by alkaline hydrolysis. Extracts were then neutralized and assayed for Ins1,4,5-P₃ as already indicated [11].

2.7. Western blot of PLC isoenzymes

High-molecular-weight markers (Bio-Rad, Hercules, CA, USA) and 20 μg of SL or cytosolic proteins were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Separated proteins were transferred on to 0.45 μm polyvinylidene difluoride (PVDF) membrane. PVDF membrane was blocked overnight at 4°C in Tris-buffered saline with 0.1% (v/v) Tween-20 (TBS–T) containing 5% (w/v) skim milk and probed with primary PLC isoenzyme antibodies. Primary antibodies were diluted in TBS–T (1:2000). Horseradish peroxidase (HRP)-labeled anti-rabbit IgG (Bio-Rad) was diluted 1:3000 in TBS–T and used as secondary antibody. PLCβ₁, γ₁, and δ₁ were visualized by enhanced chemiluminescence (ECL) according to the manufacturer’s instructions (Boehringer Mannheim, Laval, Quebec, Canada). Band intensities of the Western blot were quantified using a CCD camera imaging densitometer (Bio-Rad GS 670).

2.8. Statistical analysis

All values are expressed as mean±SEM. The differences between two groups were evaluated by Student’s t-test. A probability of 95% or more (P<0.05) was considered significant.

3. Results

3.1. General characteristics of the experimental animals

The heart underwent a significant increase in mass as indicated by an increase in the ventricular weight and by the augmented ratio of ventricular weight to body weight compared to control values. The presence of ascites in CMPH indicated the occurrence of congestive heart failure in these animals (Table 1).
Table 1
General characteristics of control and cardiomyopathic hamsters

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cardiomyopathic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt (g)</td>
<td>225±16.5</td>
<td>258±19.3</td>
</tr>
<tr>
<td>Ventricular wt (mg)</td>
<td>875±8.5</td>
<td>1234±6.5*</td>
</tr>
<tr>
<td>Ventricular/body wt ratio (mg/g)</td>
<td>3.9±0.1</td>
<td>4.8±0.1*</td>
</tr>
<tr>
<td>Ascites (ml)</td>
<td>N.D.</td>
<td>7.9±0.5</td>
</tr>
<tr>
<td>Sarcolemmal yield (mg protein/g heart)</td>
<td>0.330±0.090</td>
<td>0.397±0.050</td>
</tr>
</tbody>
</table>

* Values are means±SEM of three to five experiments. *, P<0.05 vs. controls. N.D., not detectable.

3.2. Sarcolemmal phosphoinositide kinase and total phospholipase C activities

The activities of PtdIns 4 kinase and PtdIns 4-phosphate 5 kinase were significantly depressed in the failing heart as compared to control (Fig. 2A and B). Of note, the activity of PtdIns 4-kinase, which is considered as the rate-limiting step in the PtdIns 4,5-P₂ biosynthetic pathway, was diminished by 69% of control. A moderate, but significant decrease (18% of control) of the total SL phospholipase C activity was also observed in the failing heart (Fig. 3).

3.3. Sarcolemmal PLC isoenzyme protein content and activities

The rank order of the PLC isoenzymes’ catalytic activities towards PtdIns 4,5-P₂ was PLCγ₃>PLCβ₂>PLCδ₁ (Fig. 4). Western blot analysis with monoclonal antibodies that discriminate among the PLC isoenzymes under study was used to determine the immunoreactive PLCβ₁, γ₁, and δ₁ protein bands. It was revealed that the three forms are present in the CMPH heart SL with typical molecular masses for PLCβ₁, γ₁ and δ₁ (Fig. 5A and B) [11]. The rank order of the PLC isoenzymes’ immunoreactivity in control SL was PLCγ₃>PLCδ₁>PLCβ₁ (Fig. 5Aa and B). In the failing heart of the CMPH, an increase in the activity and content of PLCδ₁ vs. controls was observed (Figs. 4C, 5A and B). However, in contrast, a decrease in PLCβ₁ and γ₁ activities (Fig. 4A and B) was evident, even though the SL amounts of these isoenzymes were increased (Fig. 5A and B). Therefore, profound changes in the profile of SL PLC isoenzymes occur in the CMPH heart at an overt stage of CHF, in that PLCδ₁ displays an elevated activity while PLCβ₁ and γ₁ are diminished.

The cytosolic PLC isoenzymes in vivo must migrate to the membranes, where their lipid substrate resides, to catalyze the production of second messengers in stimulated cells [28]. Therefore, we assessed the amount of cytosolic PLC isoforms which could be available for binding to membranes in response to stimuli. Densitometric analysis of the band intensities for PLCβ₁, γ₁ and δ₁ proteins were:
displays a form of hypertrophic cardiomyopathy [16] resembling that occurring in humans [12,29] and constitutes a good model of naturally occurring congestive heart failure [13]. In the present pathophysiological study, we have investigated the sarcolemmal PtdIns 4,5-P level, synthesis and hydrolysis at a stage of overt CHF in UM-X7.1 cardiomyopathic hamsters. Our findings show for the first time that intrinsic to the pathophysiology of CHF in these animals, are: (1) a severe reduction of SL PtdIns 4,5-P mass; (2) decreased activities of SL PtdIns 4 kinase and PtdIns 4-phosphate 5 kinase; (3) changes in protein mass/activity profile of SL PLC\_\beta\_1, \gamma_1 and \delta_1 isoenzymes.

The low PtdIns 4,5-P\_2 mass seems to be caused mainly by its decreased synthesis by PtdIns 4 kinase and PtdIns 4-P 5 kinase. To replenish the SL PtdIns 4,5-P\_2 pool, its PtdIns precursor, which is the substrate for PtdIns 4 kinase, has to be maintained at a certain level in the membrane. We have shown an increase in the SL content of PtdIns in this model of heart failure [17]. This would imply that the translocation of newly synthesized PtdIns from sarcoplasmic reticulum to the plasma membrane by the cytosolic PtdIns-transfer protein is normal [30,31]. Therefore, it is plausible that the severe reduction of PtdIns 4 kinase activity, the rate-limiting step in PtdIns 4,5-P biosynthetic pathway, is one of the key defects responsible for the drastic deficit of PtdIns 4,5-P\_2 in the SL membrane of the failing CMPH heart.

The potential functional relevance of the observed deficit in SL Ptd Ins 4,5-P\_2 mass in failing cardiomyopathic hearts arises from the diverse biochemical and signalling events that could be affected by the altered concentration of this polyphosphoinositide in the membrane [1±10,32,33]. Specifically, the lack of PtdIns 4,5-P\_2 substrate can attenuate the PLC-dependent generation of PtdIns 4,5-P and DAG and the formation of phosphatidylinositol 3,4,5-trisphosphate by the competent D3 kinase. The decreased number of PtdIns 4,5-P\_2 molecules [(n = 3) in controls and 0.3±0.1, 18.3±2.54 and 13.1±1.3, densitometric units (n=3) in failing CMPH hearts, respectively. Thus, PLC\_\gamma_1 was the most abundant from both control and failing cytosol.

3.4. Sarcolemmal PtdIns 4,5-P\_2 mass and cytosolic Ins 1,4,5-P\_3 level

Measurement of the SL PtdIns 4,5-P\_2 mass revealed an intense decrease in the failing heart of CMPH (Table 2). Ins 1,4,5-P\_3 is a downstream signal molecule generated by PLC activities. A two-fold increase in the cytosolic level of Ins 1,4,5-P\_3 was observed in CMPH hearts (Table 2).

4. Discussion

The hamster cardiomyopathic subline (UM-X7.1) employed in this and other studies from our laboratory [17,18]
Fig. 5. Western blot of sarcolemmal PLC isoenzymes from cardiomyopathic hamster hearts. (A) Representative Western blots showing (arrows) 145-kDa PLCβ1, and γ1 and 97-kDa PLCδ1; molecular weight markers are shown on left (relative molecular weight (M) expressed as $10^3$). (B) Quantified data of PLC isoenzyme protein concentration. Data are means±SEM of three experiments. CM, cardiomyopathic. *, $P<0.05$ vs. control.

a cofactor [39]. Although the specific role played by each PLD isoenzyme in cardiac cell function is still undefined, PLDs’ importance is shown by our studies that relate their hydrolysis product, phosphatidic acid, to the increase in intracellular Ca$^{2+}$ and cardiac performance [40], and by the possible involvement of SL PLD2 in the reorganization of the actin cytoskeleton [41].

In post-infarct CHF we found overabundance and hyperactivity of the SL PLCβ1, with a drastic reduction of PLCγ1 and δ1 protein mass and activity [11]. Instead, in failing cardiomyopathic heart, the depression of total SL PLC activity was associated with hyperactivity of the δ1 isoform. PLCβ1 and γ1 were hypoactive and their protein levels did not correlate with the measured activities. Discrepancies between protein mass and activity were already observed by us in the case of SL PLCs [11] and reported by others in the case of human heart transglutaminase II [42]. It may be possible that the cardiomyopathy had induced defects in the protein structure of the PLC isoenzymes, and this could have affected their catalytic activity and/or their binding to the substrate. Alterations of the physicochemical characteristics of the membrane environment of the isoenzymes may have also contributed to the discrepancies between the mass and activity of SL PLCβ1 and γ1 [17,28]. Specific studies should examine these possibilities. The diminished PLCβ1 activity in failing CMPH suggests that the signalling via the $\alpha_1$-adrenoceptor/Gq/PLCβ1 pathway could be compromised under in vivo conditions. Consequently, unlike post-infarct CHF [11], this pathway may not serve as a source of positive inotropy to compensate the defective β-adrenergic response seen in the genetic cardiomyopathy model of CHF [38].

However, only a detailed examination of all components of the pathway and of the inotropic response to $\alpha_1$-adrenoceptor stimulation in failing CMPH may clarify this issue. A downgrade of the biological functions of angiotensin II and of the other agonists that operate mainly (if not exclusively) via Gqα/PLCβ1 [43] may also be expected.

Diverse functions may be impaired in the cardiomyopathic model of CHF as a direct consequence of PLCγ1 and δ1 abnormalities. In fact (1) a significant attenuation of the myocardial responsiveness to polypeptide growth factors, which activate downstream PLCγ1 as a
specific effector enzyme [43], may be expected; (2) the stimulation of PLCγ1 by intramembranous signaling lipid molecules [e.g. phosphatidic acid, arachidonic acid (released by phospholipase A2) and phosphatidylinositol 3,4,5-trisphosphate [43]] would be limited; (3) the increase in PLCδ1, which is also stimulated by phosphatidic acid [44], may favor the interactions between SL phospholipase D and C pathways [45].

In conclusion, it seems that the occurrence of a severe reduction in the synthesis and mass of SL PtdIns 4,5-P2 and of abnormalities in PLC isoenzymes is a common feature of CHF in the two models examined thus far, e.g. genetic cardiomyopathy and post-infarct CHF [11]. However, the specific changes in protein mass/activity profiles of PLC isoenzymes are dissimilar depending on the etiology of CHF. This indicates that the subcellular mechanisms underlying congestive heart failure of different etiology are not the same and implicate the need for different therapeutic strategies [46–48].

Acknowledgements

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