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MODIFICATION OF THE INHIBITORY EFFECTS OF CCl_4 ON PHOSPHOLIPID AND PROTEIN BIOSYNTHESIS BY PROSTACYCLIN

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Abstract—CCl_4 induced cellular injury and its modification by prostacyclin (PGI_2) was studied in cultured rat hepatocytes. Biosynthesis of both intracellular and serum proteins and that of phospholipids decreased upon CCl_4 treatments (IC_{50} 7.0, 2.5 and 3.2 mM, respectively). After 1 hr exposure of the cells to CCl_4, the reductions in the biosynthesis increased further with time. PGI_2 treatments (10^{-5}–10^{-9} M) of the hepatocytes subsequent to CCl_4 poisoning resulted in partial recovery from the cell injury evaluated at the fifth hour of the experiment. Optimal effects of PGI_2 were found at a concentration of 10^{-7}–10^{-8} M, while higher and lower concentrations offered less protection. Upon the addition of CCl_4 a higher catabolic rate of PIP_2 and an increased formation of inositol phosphates were observed. This alteration was shown to precede the defects in the labelling of the major phospholipid components. Furthermore, these changes were circumvented in the presence of PGI_2. Thus, PIP_2 metabolism appears to be a critical process in the mechanism of this type of cellular injury and its protection by PGI_2.

Since the discovery of the cytoprotective effects of several prostanoids by Robert [1], the effects of prostacyclin (PGI_2)† and its derivatives on liver injuries including CCl_4 induced acute damage was extensively investigated [2–5]. Protection against the CCl_4 induced liver cell injury was also demonstrated *in vitro* using isolated liver cells or primary hepatocyte cultures [5–7]. To detect cell injury and its reversal, measurements of dye exclusion and release of intracellular enzymes are the most commonly used tests in these studies [8]. Although these methods allow the monitoring of "cell viability" they do not make possible the more detailed characterization of the injury elicited by a given agent. Accordingly, only a limited information is available on the possible molecular mechanisms implicated in the cytoprotection offered by prostacyclin.

We reported [5] that PGI_2 ameliorated the CCl_4 induced cytoplasmic enzyme release. Furthermore PGI_2 ameliorated the decrease in gluconeogenic activity of isolated hepatocytes and PGI_2 also restored the impaired catabolism of triglycerides [9, 10]. The fact that all these alteractions could be equally circumvented by PGI_2 suggested that these metabolic changes of the injured cells may be related to a common critical molecular mechanism.

In the present work, phospholipid metabolism and protein biosynthesis of cultured hepatocytes were studied in CCl_4 induced cell injury and during cytoprotection offered by PGI_2. It was assumed that measurements of the biosynthesis of serum proteins might give an insight into the alterations of an important hepatocyte function. The investigation of the metabolism of phospholipids allowed us to study processes, related to the functional integrity of cellular membranes which is a prerequisite for cell viability.

MATERIALS AND METHODS

Hepatocyte cultures. Rat liver hepatocytes were isolated by the method of Seglen [11] using female Fisher-344 rats of 220–240 g weight after an 18 hr withdrawal of food (LATI, Gödöllö, Hungary). Hepatocyte suspensions of viability higher than 90%, as determined by the trypan blue exclusion test, were plated in collagen coated Petri dishes [12] at a density of 8×10^4 cell/cm^2. Hanks-MEM culture medium (Flow Laboratories Ltd, Irvine, U.K.) supplemented with 5% calf fetal serum (Flow Laboratories Ltd), 0.1% glucose, 10^{-6} M insulin, 10^{-6} M dexamethasone (Richter Pharmacy Works, Budapest, Hungary) and 0.05 mg/mL gentamycin (Serva, Heidelberg, F.R.G.) was used for seeding (3 hr in CO_2:O_2:N_2; 4.8:21:74.2 vol.% atmosphere) then changed for fresh medium without the additives for further 18 hr.

Treatments. For CCl_4 treatments, a stock of 20% CCl_4 (Reanal Fine Chemicals, Budapest, Hungary) in 96% ethanol was freshly dissolved in Hanks-MEM medium and sonicated for 10 sec. Ethanol in the applied concentrations had no effects on the labelling of phospholipids and proteins. PGI_2 was synthesized at Chinoin Pharmacy Works (Budapest, Hungary) and was also freshly dissolved in Hanks-MEM medium before use. To determine cellular viability

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†Abbreviations: PGI_2, prostacyclin; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PC, phosphatidyl choline; PE, phosphatidylethanolamine; PA, phosphatidic acid; PL, phospholipid; IP_3, inositol-1,4,5-triphosphate; IP_2, inositol-1,4-bisphosphate; IP, inositol-1-phosphate; P_i, inorganic phosphate; IC_{50}, concentration resulting 50% inhibition; TLC, thin layer chromatography; PCA, perchloric acid.

hepatocytes were stained with 0.1% trypan blue in phosphate buffer (pH 7.4) and the average percentage of stained (non-viable) cells was calculated using light-microscopy. Treatments of hepatocytes were carried out in 2 mL Hanks MEM according to two experimental protocols. For studying metabolic changes the injured cells were exposed to 3 mM CCl_4 for 1 hr, followed by a 4 hr incubation in fresh medium in the presence or absence of PGI_2 (10^{-5}–10^{-9} M). Protein and phospholipid biosynthesis were measured by adding labelled precursors in the final (1 or 2 hr) period of the incubation. In the other series of experiments, hepatocytes were labelled first with $^{32}P_i$ and after 60 min, 3 mM CCl_4 or 3 mM CCl_4 plus 10^{-7} M PGI_2 were added for an additional 2 or 60 min.

Assays. For studying protein biosynthesis hepatocyte culture were labelled with 40 kBq/mL [^{14}C]-amino acid mixture (CB59, 8 MBq/mL, U-^{14}C-amino acid mixture UVVVR, Prague, Czechoslovakia) and the incorporated radioactivity determined in the acid insoluble materials of the cells, (i.e. intracellular proteins). Biosynthesis of secreted serum proteins were measured by processing aliquots of the culture medium to which 5 μg/mL unlabelled rat serum and antibody against rat serum in 1:100 dilution (RSP-antibody, Human Vaccine Institute, Budapest, Hungary) was added. The immunocomplex was prepared from the immunoprecipitation mixture according to Tran-Thi *et al.* [13]. Phospholipid biosynthesis was studied after 1 hr labelling with 1,6 MBq/mL $^{32}P_i$ (800 MBq/mL [^{32}P]-H_3PO_4, Izinta, Budapest, Hungary). The phospholipids were isolated from the cells as described by Enyedi *et al.* [14] and also by Griffin and Hawthorne [15].

Biosynthesis of the nucleotide triphosphates was measured in the PCA soluble fraction of $^{32}P_i$ labelled cells. PCA was removed by potassium carbonate, samples diluted to 1:10 with distilled water and submitted to ion exchange chromatography on Dowex-1 × 8 (formate form, 5 × 1.5 cm) columns [16]. Fractions eluted between 0.4 and 1.0 M ammonium formate were lyophilized and the radioactivity counted. Predominance of nucleotide triphosphates among the labelled compounds was confirmed by HPLC analysis [17].

Separation of $^{32}P_i$-labelled phospholipids was performed by TLC on 10 × 20 cm silica plates (DC Alufolien, Merck, Darmstadt. F.R.G.) pretreated by 1% potassium oxalate and 2 mM EDTA in 0.5 M boric acid. Developing mixture consisted of chloroform:methanol:2 M ammonia (7:7:2). A second dimension run in chloroform:methanol:acetic acid:H_2O (500:30:8:2) was occasionally made for the identification of the phospholipid components [14]. PA and PIP_2 standards were purchased from the Sigma Chemical Co. (St Louis, MO); PI from Serva (Heidelberg, F.R.G.); PE and lyso-PI were gifts of Dr Pick (National Institute of Haematology, Budapest, Hungary). For detection of the phospholipids, either the molybdenate reagent of Waskowsky [18] or exposure to iodine vapour in combination with autoradiography of the labelled chromatograms were used. For the latter, Medifort RP X-ray films (Forte, Vác, Hungary) were exposed for 24 or 72 hr.

Table 1. Percentage of viable hepatocytes after exposure to CCl_4 and treated with prostacycline (PGI_2)

Treatment CCl_4	PGI_2	Viable cells (%)
—	—	96.1 ± 1.27
—	10^{-5} M	97.1 ± 0.9
—	10^{-7} M	96.6 ± 1.7
—	10^{-9} M	96.0 ± 2.2
3 mM	—	85.3 ± 3.1*
3 mM	10^{-5} M	93.6 ± 1.0†
3 mM	10^{-7} M	97.0 ± 1.7†
3 mM	10^{-9} M	82.4 ± 2.9
5 mM	—	35.9 ± 17.6*
5 mM	10^{-7} M	73.8 ± 2.9†

Primary hepatocyte cultures 21 hr after seeding in Hanks-MEM medium (supplemented with 10% fetal calf serum, 10^{-6} M insulin, 3×10^{-6} M dexamethasone, 12.5 mM glucose and 200 μg/mL gentamycin) were treated with CCl_4 for 1 hr. Then CCl_4 was removed, fresh culture medium was added, and the indicated samples were treated with PGI_2 for 3 hr. At the end of the incubation the viability of the hepatocytes was determined by *in situ* trypan blue dye exclusion test. Results represent the mean ± SE (N = 4).

* Indicates significant decrease in viability in comparison to controls.

† Indicates significant protection in comparison to the corresponding CCl_4 sample.

Identified spots were scraped from the plates and their radioactivity measured. The recovery determined in the total phospholipid fraction was 85–90%.

The formation of inositol phosphates from the phosphoinositides was studied after a 1 hr prelabelling period using 1.2 MBq/mL [2-^{3}H-*myo*]-inositol (TRK317 455 GBq/mmol, Amersham International, Amersham U.K.). Treatments with 3 mM CCl_4 and 10^{-7} M PGI_2 were carried out in fresh medium containing 1 mM of unlabelled myoinositol and 10 mM LiCl. Macromolecules and lipids were precipitated in the presence of 0.5 M PCA, centrifuges and the supernatants freed from PCA by potassium carbonate, then chromatographed on Dowex-1 × 8 (formate form, 6 × 1.5 cm) columns according to Berridge [19]. Fractions containing inositol phosphates were lyophilized and their radioactivities counted. All radioactivity measurements were carried out in a Beckman LS 100 liquid scintillation counter after solubilization of the samples in soluene.

Results were expressed as mean ± SE from three experiments each comprised of triplicate samples. To judge the significance of the differences between experimental groups the Student's *t*-test was used at $P < 0.05$ level.

RESULTS

Table 1 shows the cellular viability of the hepatocyte culture by using the trypan blue dye exclusion method. Administrating CCl_4 for 1 hr at 3 mM concentration caused a modest, but at 5 mM significant

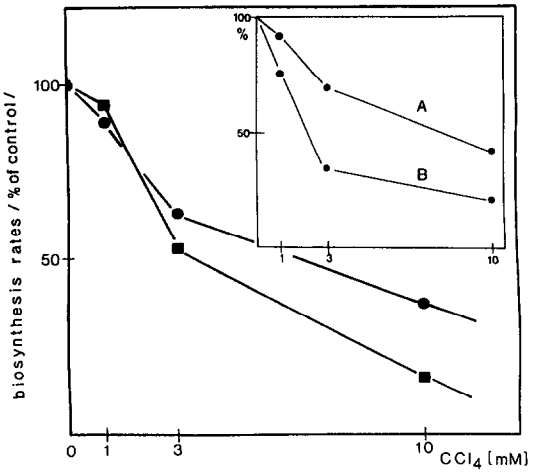

Fig. 1. Effect of CCl_4 (1–10 mM) on the protein and phospholipid biosynthesis of cultured hepatocytes. Inset: CCl_4 induced effects on the synthesis of intracellular proteins (A) and secreted serum proteins (B). Hepatocytes were treated with CCl_4 for 1 hr followed by a reincubation for 4 hr in the absence of the toxin. The biosynthesis of phospholipids was measured between 4–5 hr of the experiment by $^{32}P_i$ incorporation into the phospholipid fraction (—■—). The biosynthesis of proteins was measured between the 3–5 hr of the experiment by ^{14}C-amino acid incorporation into the total protein fraction (—●—) or proteins of the cell layer (A) and RSP-antibody precipitable proteins in the medium of the cultured cells (B). Data represent 3 experiments, the individual assays were carried out in triplicate.

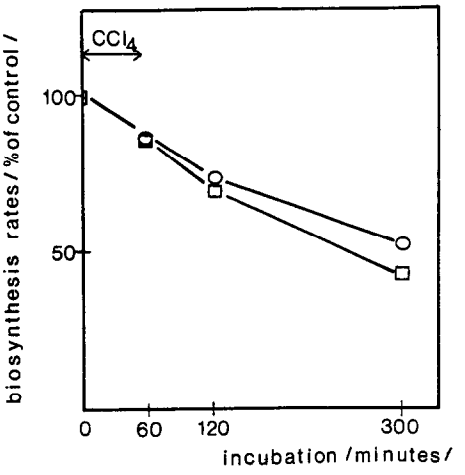

Fig. 2. Time course of the development of hepatocellular injury upon 3 mM CCl_4 treatment. Hepatocytes in cultures were treated with CCl_4 for 1 hr, then fresh medium containing no CCl_4 was added and the biosynthesis of phospholipids (—□—) and proteins (—○—) were measured between 0–1, 1–2 and 4–5 hr of the experiment. Data represent 3 experiments, the individual assays were carried out in triplicate.

loss in viability 4 hr after removing CCl_4. PGI_2 administered after removal of CCl_4, was able to restore the cell viability at concentration of 10^{-5} and 10^{-7} M. These data also imply that using 3 mM CCl_4 protein and phospholipid biosynthesis were investigated in cultures containing 85% viable hepatocytes. As this viability test is associated with the integrity of cell membranes, it does not seem probable that the biochemical changes at this concentration are due to direct (i.e. the solvents) effects of CCl_4 on cell membrane. Nevertheless the drastic toxic action of CCl_4 was detected upon applying 5 mM concentration. It is noteworthy that the cytoprotective action of PGI_2 could be also demonstrated at this high CCl_4 concentration, when PGI_2 was administered after CCl_4.

Treatment of cultured rat hepatocytes with CCl_4 resulted in the impairment of both protein and phospholipid biosynthesis in a dose-related fashion. Figure 1 shows that the synthesis of secreted serum proteins is more sensitive to CCl_4 actions than that of intracellular proteins or phospholipids. IC_{50} was 3.2, 6.0, 2.5 and 7.0 mM for phospholipid biosynthesis, biosynthesis of total proteins, secreted serum proteins and intracellular proteins, respectively. The time course of the hepatocyte injury is shown in Fig. 2. The slight decrease in the protein and phospholipid biosynthesis found upon a 1 hr treatment with 3 mM CCl_4 was followed by marked reductions of these values, although CCl_4 was no longer present in the culture medium. The effects of PGI_2 showed an optimum between 10^{-7}–10^{-8} M, which concentrations almost abolished the CCl_4 induced reductions in the biosynthesis of proteins and phospholipids. It is noteworthy that a slight (10–30%) enhancing effect of PGI_2 on normal cells was regularly detected in our experiments but this did not follow a bell-shaped dose–response curve (Figs. 3 and 4).

Measurement of the incorporations of $^{32}P_i$ into the nucleotide triphosphate pool was also determined. It was found that CCl_4 caused a reduction of the labelling of this fraction which was partially circumvented by treatments with 10^{-7} M PGI_2 (Fig. 4).

The results obtained on the thin-layer chromatographic separation of labeled phospholipids showed that phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA) and also phosphatidylinositol-4-phosphate (PIP) were labelled in parallel with total phospholipids. CCl_4 caused a 50–60% decrease in the $^{32}P_i$ labelling of these phospholipids, which changes, however, were restored by PGI_2 at similar rate as of total phospholipids. On the contrary, phosphatidylinositol (PI) and phosphatidylinositol-4,5-bisphosphate (PIP_2) behaved quite differently as PI was much less but PIP_2 more extensively affected by CCl_4. It appears as an important finding that PGI_2 at the concentration of 10^{-7} completely reversed the substantial reduction (90%) of the PIP_2 labelling induced by CCl_4 (Fig. 5).

In the next series of experiments the hepatocytes were first labelled with $^{32}P_i$ and then poisoned with CCl_4. As shown in Fig. 6, the incorporation of $^{32}P_i$ into PC or PI was only slightly affected even by a 60 min exposure of the cells to 3 mM CCl_4. At the same time CCl_4 blocked the increase in the labelling of PIP and PIP_2 even at 2 min after CCl_4 treatment. In samples with simultaneous PGI_2 (10^{-7} M) treatments these changes were significantly ameliorated.

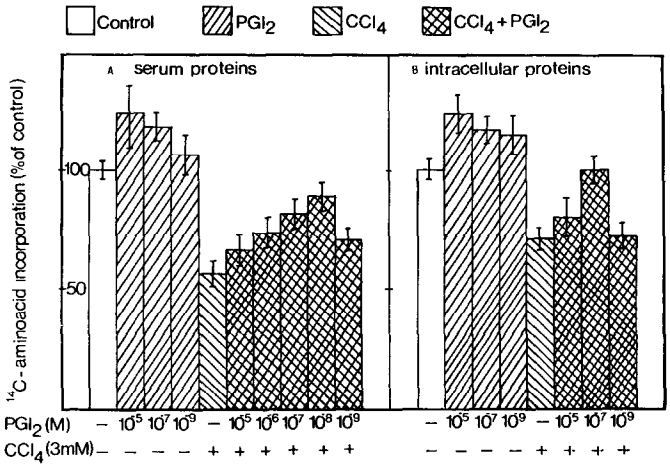

Fig. 3. Effect of PGI_2 on the biosynthesis of serum proteins and intracellular proteins in hepatocytes poisoned by CCl_4. Cells were treated with 3 mM CCl_4 for 1 hr then fresh medium added and PGI_2 was applied in the indicated concentrations. Control cells and PGI_2 treated controls were incubated simultaneously. Biosynthesis of secreted serum proteins (A) and intracellular proteins (B) were measured by ^{14}C-amino acid incorporations into the corresponding fractions between 3–5 hr of the experiment. Each bar is mean ± SE for 4 experiments.

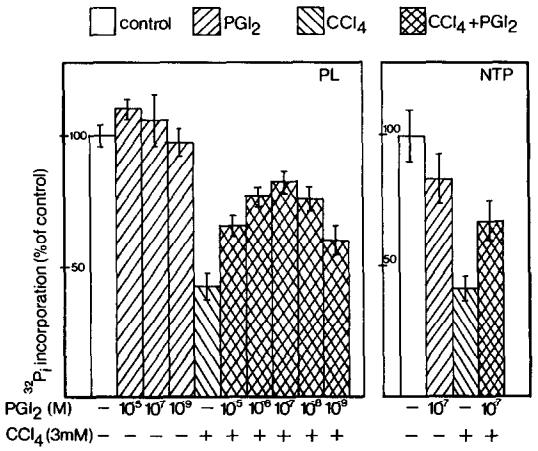

Fig. 4. Effect of PGI_2 on the incorporation of $^{32}P_i$ into phospholipids (PL) and nucleotide triphosphates (NIP) of CCl_4 treated and control hepatocytes. Cells were poisoned by 3 mM CCl_4 for 1 hr followed by a reincubation in the absence or presence of PGI_2 at the concentrations indicated. Unpoisoned cells and PGI_2 treated controls were incubated simultaneously. $^{32}P_i$ was added between 4–5 hr of the experiment and its incorporation into both phospholipid and nucleotide triphosphate fraction of the cells were determined. Each bar is mean ± SE for three experiments; each assay was carried out in triplicate.

Since it has been well documented that the metabolic degradation of PIP_2 generates inositol–phosphates the action of CCl_4 and PGI_2 was investigated on the formation of these phosphoinositide metabolites (IP_3, IP_2, IP) in [^{3}H]-myoinositol labelled hepatocytes. In accordance with the higher rate of PIP_2 degradation, an elevated level of inositol phosphates were measured after CCl_4 poisoning. This increase was almost completely abolished by PGI_2 (Fig. 7).

DISCUSSION

CCl_4 is known to induce a wide array of toxic events in the liver (lipid peroxidation, formation of adducts between its metabolites and macromolecules, imbalance in ionic homeostasis etc.) leading to the dysfunction of several cellular processes and finally to cell death [20, 21]. It has been generally accepted that the endoplasmic reticulum itself is an immediate site of attack by the CCl_4 free radicals formed by the cytochrome P450. Since endoplasmic reticulum is the site of protein and phospholipid biosynthesis it is not surprising that these biopolymers are drastically affected by CCl_4 [22]. The critical metabolic events in the development of the injury may be elucidated by applying cytoprotective agents. For the evaluation of cellular injury, protein and phospholipid biosynthesis was measured which, in addition to gluconeogenesis [5] could also be reduced after CCl_4 treatment and all these changes could be ameliorated by PGI_2. It was noteworthy that the addition of 10^{-7} M PGI_2 to cells after the removal of the hepatotoxic chemical almost completely prevented further decreases in both protein and phospholipid biosynthesis measured 5 hr after the initiation of the injury. Application of various doses of PGI_2 resulted in a bell shape dose–response curve similar to that found by Guarner *et al.* (7).

Our finding that the formation of nucleotide triphosphates from labelled phosphate changes simultaneously with the biosynthesis of proteins and phospholipids suggested that the dysfunction of these cellular processes may be caused by the shortage of ATP supply. However, the marked difference between the sensitivity to CCl_4 of the biosynthesis of intracellular vs secreted serum proteins indicated that the secretory processes were more affected. An impaired integrity of cellular membranes may underlie the higher CCl_4 sensitivity of the secretory processes measured herein. Membrane integrity was

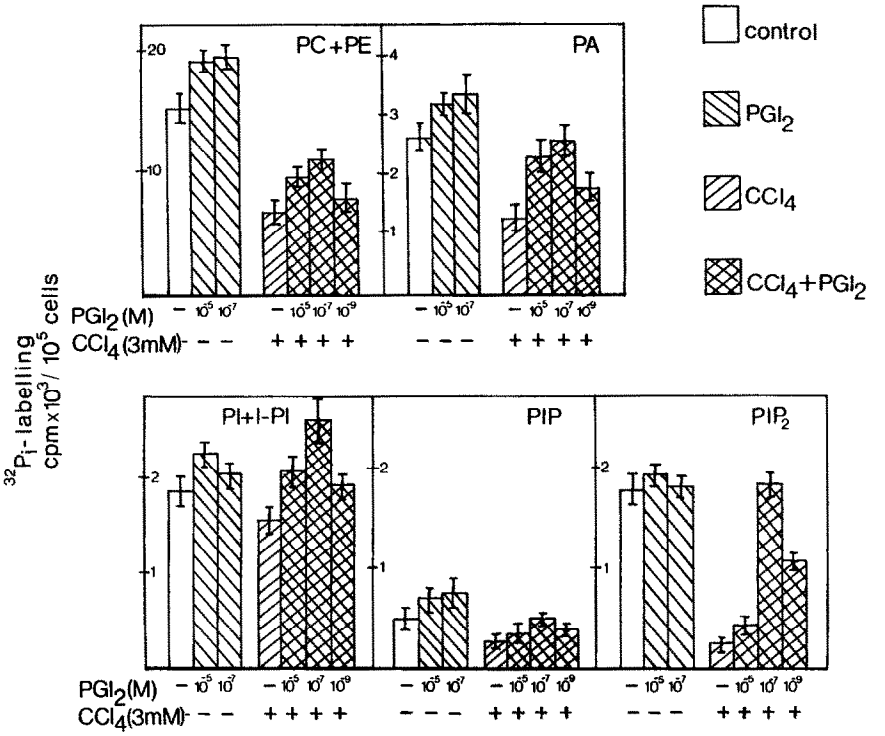

Fig. 5. Influence of PGI_2 on the incorporation of $^{32}P_i$ into the different phospholipids in CCl_4 treated and control hepatocytes. Cells were treated with CCl_4 for 1 hr. After CCl_4 removal cells were incubated further in the absence or presence of PGI_2 at the concentrations indicated. Phospholipid labelling took place between 4–5 hr of the experiment. Cellular phospholipids were isolated and separated by TLC on silica plates. Incorporation of $^{32}P_i$ into the different phospholipid spots were determined as described in Materials and Methods. Each bar is mean ± SE for 2 experiments. Each assay was carried out in triplicate.

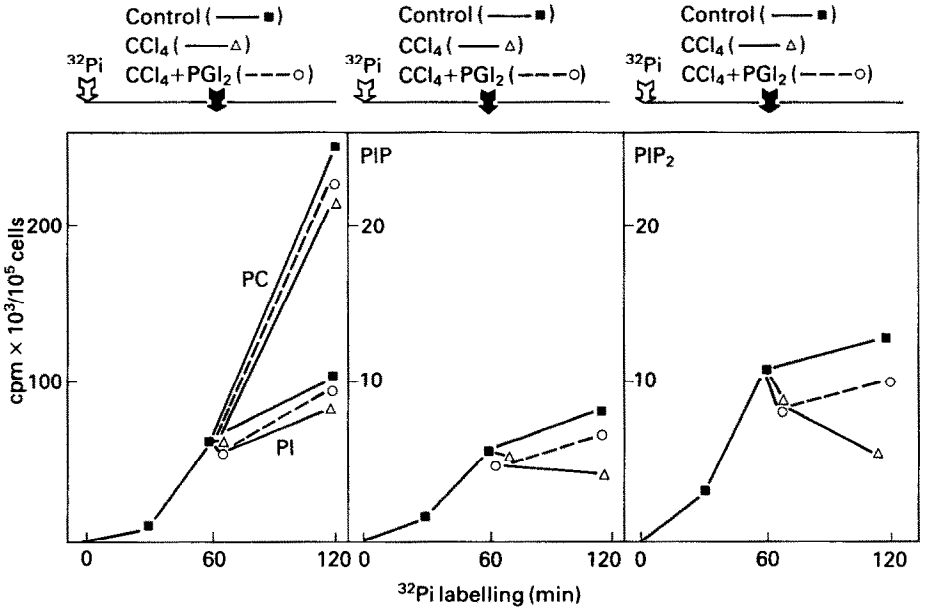

Fig. 6. Effects of CCl_4 on the labelling of PC, PI, PIP and PIP_2 in cells prelabelled with $^{32}P_i$ and its modification by PGI_2. After labelling of the cells, for 60 min 3 mM CCl_4 or 3 mM CCl_4 plus 10^{-7} M PGI_2 was added. Reactions were stopped 2 and 60 min later, respectively, followed by the preparation of phospholipids and separation by TLC. Data represent 3 experiments for each point. The individual assays were carried out in triplicate.

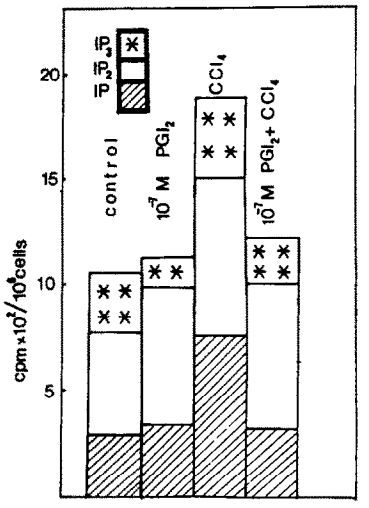

Fig. 7. Formation of inositol phosphates in hepatocytes treated with CCl_4 and its modification by PGI_2. Cells were prelabelled with [2-^{3}H-*myo*]inositol for 60 min, medium was replaced by fresh, containing 1 mM unlabelled *myo*-inositol, 10 mM LiCl and subsequently treated with 3 mM CCl_4 or 3 mM CCl_4 plus 10^{-7}M PGI_2 for 60 min. Analysis of the inositol phosphates were carried out by the use of ion exchange chromatography according to Berridge [19]. Data represent 2 experiments for each bar. The individual assays were carried out in triplicate.

studied by metabolic labelling of phospholipids both before and after the induction of the injury and a highly sensitive response to CCl_4 in the metabolism of PIP_2 was observed. The labelling of PIP_2 in $^{32}P_i$ preincubated cells substantially declined upon 60 min treatment with CCl_4 while the major phospholipid components (PC, PE or PI) showed no marked differences from controls up to this time. The elevation of the production of labelled inositol phosphates after CCl_4 treatment in [^{3}H-*myo*]inositol prelabelled cells may indicate that a phosphatidylinositol diphosphate specific phospholipase C was simultaneously activated i.e. a pathway by which PIP_2 can be metabolized. The decreased labelling of PIP_2 may be interpreted either in terms of an increased catabolism or a decreased synthesis rate, but the lack of accumulation in the radioactivity of PIP, —the metabolic precursor of PIP_2— argues for the former possibility. The fact that upon CCl_4 application a change in the metabolism of PIP_2 precedes the reductions in the labelling of major phospholipids and dominates also in later stages of the injury suggests that PIP_2 catabolism may represent a critical event in CCl_4 induced cell injury as was also reported by Lamb and Schwertz [23]. It was concluded by Lamb *et al.* [24] that both ischemia and CCl_4 exposure cause increases in hepatic phospholipase C. The present observation that this metabolic shift could be effectively controlled by PGI_2 seems to indicate that the key mechanism for cytoprotection in this experimental system may operate also at this level. This however, does not exclude other possible mechanisms PGI_2; such as the reduction of lipid peroxidation [25]. The potential role of these events in the development of hepatocyte injury may be appreciated by the fact that PIP_2 is a minor but electrically strongly charged constituent of the cell membranes [26]. The elevated decomposition of PIP_2 could alter locally the physicochemical properties of the membrane affecting its functions e.g. in ionic transports. In fact, PIP_2 and inositol phosphates have been suggested to regulate plasma membrane Ca^{2+}-pump and impairment of this Ca^{2+}-ATP-ase was also documented to ensue during CCl_4 induced injury [27, 28] resulting in the progression of the ionic imbalance in the cell. On the other hand, the increased generation of IP_3 as a calcium trigger signal molecule may also contribute to the redistribution of the intracellular calcium from endoplasmic reticulum stores to the cytosol [29, 30] as has been reported for hepatotoxins [31]. Therefore the shift in the metabolism of PIP_2 could be related to the rise of cytosolic calcium which is regarded as an important factor in the mechanism of cellular injury [32–35]. It is conceivable that some of the numerous alterations related to various metabolic pathways— e.g. reduced gluconeogenesis, and protein synthesis, ATP depletion etc. —in CCl_4 induced cell injury are elicited by the uncontrolled and higher rate of PIP_2 conversion. Since it has been well documented that changes in phosphatidyl inositol metabolism have a variety of consequences for cell metabolism the presented data showing that PGI_2 acts at this level may elucidate the mechanism for the modification of the different metabolic alterations in the injured cell by PGI_2.

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