Effects of hypoxia, glucose deprivation and acidosis on phosphatidylcholine synthesis in HL-1 cardiomyocytes. CTP:phosphocholine cytidyltransferase activity correlates with sarcolemmal disruption

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A decrease in [³H]Cho (choline) incorporation in to PtdCho (phosphatidylcholine) preceded the onset of LDH (lactate dehydrogenase) release in HL-1 cardiomyocytes submitted to simulated ischaemia. This observation led us to examine the role of PtdCho synthesis in sarcolemmal disruption in HL-1 cardiomyocytes. To address this objective we analysed the individual effects of hypoxia, glucose deprivation and acidosis, three prominent components of ischaemia, on the different steps of the Kennedy pathway for the synthesis of PtdCho. Pulse and pulse-chase experiments with [³H]Cho, performed in whole HL-1 cells submitted to hypoxia or normoxia, in the presence or absence of glucose at different pHs indicated first, that CK (choline kinase) was inhibited by hypoxia and acidosis, whereas glucose deprivation exacerbated the inhibition caused by hypoxia. Second, the rate-limiting reaction in PtdCho synthesis, catalysed by CCT (CTP:phosphocholine cytidyltransferase), was inhibited by hypoxia and glu-

cose deprivation, but unexpectedly activated by acidosis. In cell-free system assays, acidosis inhibited both CK and CCT. In experiments performed in whole cells, the effect of acidosis was likely to be direct on CK, but indirect or intact-cell-dependent on CCT. Since hypoxia and glucose deprivation favoured membrane disruption, but acidosis prevented it, we hypothesized that the modulation of CCT could be an important determinant of cell survival. Supporting this hypothesis, we show that CCT activity in whole-cell experiments clearly correlated with LDH release, but not with ATP concentration. Altogether our results suggest a significant role for CCT activity in sarcolemmal disruption during ischaemia.

Key words: cardiomyocytes, CTP:phosphocholine cytidyltransferase (CTP), ischaemia, necrosis, phosphatidylcholine (PtdCho), phospholipid bilayer.

INTRODUCTION

The contribution of phospholipid metabolism to plasma membrane disruption in necrotic cell death induced by hypoxia or ischaemia has been classically attributed to the action of phospholipases, loss of asymmetry or the accumulation of bilayer-disrupting amphiphilic lipids, such as lysophospholipids [1–3]. The involvement of phospholipid synthesis in the necrosis of heart tissue undergoing ischaemia or hypoxia; however, has hardly been addressed [4,5].

PtdCho (phosphatidylcholine) is a major phospholipid component in cell membranes, which accounts for 40 % of total phospholipids in cardiomyocytes [1]. The pathway for the *de novo* synthesis of PtdCho was described by Kennedy and Weiss in 1956 [6]. Cho (choline) enters the cell, where it is phosphorylated to PCho (phosphocholine) by the action of CK (choline kinase). Then, PCho is activated by CTP to CDP-Cho in the reaction catalysed by CCT (CTP:phosphocholine cytidyltransferase). Finally, the phosphocholine moieties of CDP-Cho and DAG (diacylglycerol) condense to produce PtdCho in the last step catalysed by CPT (CDP-choline:sn-1,2-diacylglycerol choline-phosphotransferase) [7]. Due to the big pool-size of PCho as compared with CDP-Cho, it has been established that CCT catalyses the rate-limiting step for PtdCho synthesis. CCT can be regulated by different mechanisms, such as phosphorylation [8], membrane translocation [9,10] which is favoured by certain fatty acids [11], or calpain-mediated degradation [12]. Nevertheless, the rest of the enzymes may also contribute to modulate the overall rate of PtdCho synthesis [13].

For several tissues other than myocardium, there is increasing evidence suggesting the involvement of the inhibition of PtdCho synthesis in the molecular mechanisms leading to cell death triggered by different insults [4], such as ischaemia or glutamate excitotoxicity in brain [14], TNF (tumour necrosis factor)α [15], exogenous ceramides [16–18] or anti-tumour treatments [19–21]. Both necrotic and apoptotic cell-death involve inhibition of PtdCho synthesis [22], and the different enzymes of the Kennedy pathway have been found to be targeted differently by these insults [4]. In cells with a thermo-sensitive CCT, cell death was inducible by switching the culture to the restrictive temperature [23]. It has also been demonstrated that overexpression of CCT, or supplementation of lysoPtdCho or PtdCho prevents cell death [16,20,24,25].

In cardiac tissue, previous studies reported the inhibition of PtdCho synthesis during hypoxia or ischaemia. Hatch and Choy described the inhibition of PtdCho synthesis in perfused hearts undergoing hypoxia [26], PtdCho synthesis was also impaired by hypoxia in isolated rat ventricular myocytes [28], and a net loss of choline after global ischaemia has been recently demonstrated in reperfused rat hearts [29]. In this context, several authors suggested that the depletion of ATP and CTP were the cause for the observed inhibition of PtdCho synthesis [5,27].

The molecular mechanism that may underlie involvement of the inhibition of PtdCho synthesis in cell death remains obscure. On one hand, inhibiting phospholipid synthesis could decrease the total amount of phospholipids, making the sarcolemma fragile and facilitating its rupture. Nonetheless, the magnitude of the decrease in phospholipid content after ischaemia or hypoxia is

Abbreviations used: CCT, CTP:phosphocholine cytidyltransferase; CDP-Cho, CDP-choline; Cho, choline; CK, choline kinase; CPT, CDP-Cho:sn-1,2-diacylglycerol phosphotransferase; DAG, diacylglycerol; LDH, lactate dehydrogenase; PCho, phosphocholine; PtdCho, phosphatidylcholine.

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controversial, with observations ranging from no change at all to a 40 % decrease in rat hearts submitted to ischaemia for 2 h [1]. Indirect metabolic consequences of the inhibition of PtdCho synthesis, such as the decrease in sphingomyelin and increases in ceramide or DAG (diacylglycerol) [30,31], may also have a role to play in cell death processes. On the other hand, the close relationship between phospholipid synthesis and cell cycle regulation [32], together with the nuclear localization of the regulatory enzyme CCT [33], allows speculation that turning on and off the synthesis of PtdCho may have critical consequences for the cell's fate.

The main aim of this work was to analyse the involvement of PtdCho synthesis in membrane disruption in HL-1 cardiomyocytes submitted to simulated ischaemia. This objective was based on the evidence and observations argued above, together with our interest in investigating the individual contribution of glucose deprivation or acidosis, two prominent consequences of ischaemia, on the modulation of PtdCho synthesis. For this purpose we monitored [^3H]Cho labelling of the intermediate metabolites of PtdCho synthesis in pulse, and pulse-chase experiments, as well as LDH (lactate dehydrogenase) release and ATP levels in HL-1 cells under different conditions of pH and glucose supplementation during normoxia or hypoxia (0.1 % O_2). Taking advantage of the different effects elicited by acidosis, glucose deprivation and hypoxia on the individual steps of PtdCho synthesis and on membrane disruption, we analysed the putative role of PtdCho synthesis in necrotic cell death in HL-1 cardiomyocytes undergoing simulated ischaemia.

EXPERIMENTAL

Materials

[Methyl- ^3H] Cho chloride (82 Ci/mmol) and [^{14}C]phosphorylcholine were purchased from Amersham Biosciences; silica gel-60 TLC plates were from Merck; ATP bioluminescent somatic cell assay kit and Protease Inhibitor Cocktail were from Sigma; Claycomb culture media was from JRH Biosciences Ltd.; and cell culture supplements were from GIBCO. The rest of the reagents used were of analytical grade.

HL-1 cell culture

The HL-1 cell line, a tumoral atrial cardiomyocyte cell line, was a gift from Dr W. C. Claycomb (Louisiana State University Medical Centre, LA, U.S.A.). HL-1 cells were grown in Claycomb media [34] supplemented with 10 % foetal bovine serum, 4 mM L-glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin and 10 μM norepinephrine at 37 °C in a humid atmosphere of 5 % CO_2 . Cells were plated at 30 000 cells $\cdot\text{cm}^{-2}$ on to 0.02 % gelatin pre-treated multiwell plates and cultured to 80–90 % confluence, which was reached after 3 days. For all the assays, HL-1 cells were incubated in Hepes buffer, containing (in mM): 140 NaCl, 3.6 KCl, 1.2 MgSO_4 , 1.3 CaCl_2 , 20 Hepes (acid form), with or without 5 mM glucose, and adjusted to the desired pH with NaOH.

Hypoxia

Hypoxia was achieved by placing the cells in to a hypoxic chamber (Microaerophylic workstation, model: INVIVO2, Ruskinn Technologies) with a Gas Mixing Module with O_2 set at 0.1 % and N_2 for the balance of the mixture. The temperature was set at 37 °C and humidity roughly controlled at 80 %. After placing the cells in to the hypoxic chamber from a normoxic atmosphere, the O_2 concentration in the incubation media dropped sharply to 1 % O_2 within 10 min, and reached equilibrium with the chamber atmosphere (0.1 % O_2) within 30 min (results not shown). The

zero-time for hypoxia was the time when cells were introduced in to the hypoxic chamber.

LDH measurement

LDH activity released in to the incubation media was used as an index of cell membrane disruption. LDH was assayed against saturating concentrations of pyruvate and NADH, which was monitored by its absorbance at 340 nm. The released LDH was always expressed as a percentage of total LDH in the well, which was measured after lysing the cells in a well from the same plate with 0.05 % Triton X-100.

Pulse-labelling with [^3H]Cho

[^3H]Cho (0.5 $\mu\text{Ci/ml}$) was present in the incubation medium of HL-1 cells for the times indicated in the different treatments.

Pulse-chase experiments with [^3H]Cho

HL-1 cells were prelabelled for 2 h by adding 0.5 $\mu\text{Ci/ml}$ [^3H]Cho in to the culture medium. Then, the medium was removed and the radioactive label washed out. The chase with 10 μM of non-radioactive Cho was started simultaneously with the desired treatment.

Extraction, separation and counting of radiolabelled intermediate metabolites of PtdCho synthesis

At the end of the protocol, the incubation medium was removed, the cells washed, and metabolites extracted by adding 600 μl of chloroform/methanol (1:2, v/v) to the well, the cells were then rapidly transferred to Eppendorff tubes. The two phases were separated by adding 250 μl of chloroform and 375 μl of H_2O , and tubes were shaken vigorously and centrifuged at 2000 g . To separate the water-soluble Cho-metabolites from the sample, the upper aqueous phase was evaporated under a stream of N_2 , re-suspended in 20 μl of H_2O /methanol (3:1, v/v), and spotted on to TLC silica gel-60 plates developed with the mobile phase; 95 % ethanol/2 % NH_4OH (1:1, v/v). Identification of radiolabelled Cho, PCho and CDP-Cho was made by comigration with authentic standards. The radiolabelled lipid in the lower organic phase was > 95 % PtdCho at the conditions used in this study, as verified by TLC on silica gel-60 plates developed in chloroform/acetic acid/methanol/water (5:2:4:1, by vol) (results not shown). Quantitative determination was performed by scraping the silica gel in to liquid scintillation vials from areas corresponding to migration of the standards.

Subcellular fractionation

HL-1 cells grown to confluence were trypsinized, washed, pelleted and lysed by sonication (5 bursts for 5 s), in a hypotonic lysis buffer, containing 10 mM Tris/HCl (pH 7.4), 1 mM EDTA, 0.1 mM dithiothreitol and Protease Inhibitor Cocktail (original mixture provided by SIGMA was diluted to 1:100). The homogenate was centrifuged at 3000 g for 5 min and the resulting supernatant was centrifuged at 100 000 g for a further 45 min. The final pellet, which was resuspended in lysis buffer, and the final supernatants, containing membranes and cytosol respectively, were kept in aliquots at -80°C for up to one month before use.

CK and CCT determination in cell-free systems

Both reactions were carried out in a 100 μl final volume with 150 mM Tris/HCl and 10 mM MgCl_2 and 3–20 μg of protein fraction per assay. CK was assayed with 10 mM ATP and 0.2 μM [^3H]Cho (1 Ci/mmol) as substrates. CCT was assayed with 4 mM CTP and 0.2 mM [^{14}C]PCho (12.5 mCi/mmol) as substrates. The

reaction took place at 37°C for 15 min and was stopped by adding 600 µl of chloroform/methanol (1:2, v/v). Radiolabelled metabolites were extracted, separated and counted as described above.

Determination of ATP concentration

Cells were incubated for 2 or 3 h under different conditions, as indicated. At the end of the protocol the incubation medium was removed, and cells were rapidly frozen in liquid N₂. Cells were kept at -80°C for up to one week. ATP concentration was determined with the aid of the Bioluminescent Somatic Cell Assay Kit from Sigma. This kit provides some lysis buffer, standard ATP and a reaction mixture containing luciferin and luciferase. The ATP released from the cells by means of lysis buffer reacts with luciferin and after a two-step reaction (the first one catalysed by luciferase), it produces an amount of light, which is measured with a luminometer, proportional to the concentration of ATP, provided that ATP is the limiting factor.

Statistics

Statistical analysis was performed with the aid of commercially available software (SPSS-PC version 11.0). The effect of the different treatments (hypoxia, glucose deprivation and acidosis) was studied by 2 way (2 × 2) or 3 way (2 × 2 × 2) ANOVA, as indicated in Figure legends. Values are shown as mean ± S.E.M., unless indicated. Significance level was established at $P < 0.05$.

RESULTS

Inhibition of [³H]Cho incorporation in to PtdCho precedes LDH release in cells undergoing hypoxia and glucose deprivation

Figure 1 shows the time-course of LDH release (A) and [³H]Cho incorporation in to PtdCho (B) in HL-1 cells submitted to hypoxia in the absence of glucose. At 1 h 30 min the rate of [³H]Cho incorporation in to PtdCho was decreased to approx. 50 %, while LDH release was not significantly higher in hypoxic and glucose-deprived cells as compared with control cells. At 30 min later [³H]Cho incorporation in to PtdCho was almost totally blocked, while released LDH accounted for only 5 % of total cellular LDH in hypoxic and glucose-deprived cells as compared with control cells. These data indicate that the inhibition of the *de novo* synthesis of PtdCho by hypoxia in the absence of glucose precedes the onset of LDH release, which reflects the membrane disruption of HL-1 cardiomyocytes.

Acidosis decreases [³H]Cho incorporation in to PtdCho and LDH release

Acidosis, together with oxygen depletion and glucose deprivation, occurs during ischaemia. Incubation at pH 6.4, in the absence of glucose, decreased [³H]Cho incorporation in to PtdCho in both hypoxic and control HL-1 cells (Figure 2B), whereas it drastically blocked LDH release in hypoxic cells (Figure 2A). The observation that both hypoxia and acidosis inhibited [³H]Cho incorporation in to PtdCho, but only hypoxia at pH 7.4 triggered cell rupture that was prevented by acidosis, led us to perform a more detailed analysis of their effect on the pathway of PtdCho synthesis.

Analysis of PtdCho synthesis in pulse-labelling experiments with [³H]Cho

The next series of experiments were planned to measure the particular effects elicited by hypoxia, glucose deprivation and acidosis on the individual steps of the *de novo* synthesis of PtdCho

Figure 1 Time-course of LDH release and [³H]Cho incorporation in to PtdCho in HL-1 cells undergoing hypoxia and glucose deprivation

HL-1 cells were incubated with Hepes buffer at pH 7.4 in the presence of glucose during normoxia (○) or hypoxia (0.1 % O₂) in the absence of glucose (●) for 4 h. [³H]Cho (0.5 µCi/ml) was added at zero-time. LDH release (A) and [³H]Cho incorporation in to PtdCho (B) were determined at different times. Results are means ± S.D. for two experiments performed in triplicate.

Figure 2 Effect of acidosis on LDH release and [³H]Cho incorporation in to PtdCho

HL-1 cells were incubated in Hepes buffer at pHs 7.4 or 6.4 in the absence of glucose during normoxia (white bars) or hypoxia (0.1 % O₂, grey bars) for 3 h. [³H]Cho (0.5 µCi/ml) was added at zero-time. At the end of the protocol, LDH release (A) and [³H]Cho incorporation in to PtdCho (B) were determined. Hx, hypoxia; Ac, acidosis; *, interaction. Results are means ± S.E.M. for three experiments performed in triplicate. Statistical analysis: 2 way (2 × 2) ANOVA. Panel A: Hx $P = 0.000$; Ac $P = 0.000$; Hx*Ac $P = 0.001$. Panel B: Hx $P = 0.000$; Ac $P = 0.000$; Hx*Ac $P = 0.000$.

in an intact cell system. For this purpose we first determined the time-course for the incorporation of [³H]Cho in to the intermediate metabolites under control conditions (Figure 3). [³H]Cho labelling of PCho increased during the first hour, declined thereafter and reached a plateau (isotopic equilibrium) after 2 h of incubation. The larger amount of label in PCho, as compared with the radiolabel incorporated in to the other intermediate metabolites, reflects a large pool of PCho and indicates that in these conditions CCT

Figure 3 $[^3\text{H}]\text{Cho}$ incorporation in to the intermediate metabolites of PtdCho synthesis

HL-1 cardiomyocytes were incubated in Hepes buffer at pH 7.4 under normoxic conditions in the presence of glucose for 3 h. $[^3\text{H}]\text{Cho}$ (0.5 $\mu\text{Ci}/\text{ml}$) was added at zero-time. At different times $[^3\text{H}]\text{PtdCho}$ and soluble $[^3\text{H}]\text{Cho}$ -metabolites were extracted, separated by TLC and counted for radioactivity. Results are representative of three experiments performed in triplicate with similar results. *, refers to the y-axis scale.

catalyses the rate-limiting step. This observation confirms for this cellular model what has already been described in other tissues [7]. The radioactive labelling of CDP-Cho and intracellular Cho were already at isotopic equilibrium after 1 h of incubation. The incorporation of $[^3\text{H}]\text{Cho}$ in to PtdCho, by contrast, was not at isotopic equilibrium at up to 4 h (see also Figure 1), which reflects the large amount of cellular PtdCho present. We therefore monitored the incorporation of $[^3\text{H}]\text{Cho}$ in to PCho and PtdCho before they reached the isotopic equilibrium, in experiments using a 1 h pulse of $[^3\text{H}]\text{Cho}$ at different intervals of the protocol. Under these conditions, the labelling in PCho reflects the rate of Cho uptake and subsequent phosphorylation by CK, whereas the labelling in PtdCho gives an idea of the global conversion rate from Cho to PtdCho. The results obtained are shown in Figure 4. Several observations can be highlighted from all these data: first, 2 h of hypoxia at pH 7.4 in the absence of glucose decreased $[^3\text{H}]\text{Cho}$ incorporation in to PtdCho (Figure 4B), most likely due to the inhibition of $[^3\text{H}]\text{Cho}$ incorporation in to PCho (Figure 4A). Second, 3 h of hypoxia at pH 7.4 in the presence of glucose decreased $[^3\text{H}]\text{Cho}$ incorporation in to PtdCho (Figure 4D). This effect could not be entirely explained by the slight decrease in PCho labelling (Figure 4C), revealing an inhibitory effect of hypoxia at some point between PCho and PtdCho synthesis. Third, acidosis drastically decreased the labelling of PCho (Figures 4A and 4C) and in turn the labelling of PtdCho (Figures 4B and 4D). Fourth, glucose deprivation decreased PtdCho labelling (Figures 4B and 4D) without affecting PCho labelling under all conditions tested, indicating an inhibitory effect for glucose deprivation beyond Pcho synthesis. And fifth, glucose deprivation exacerbated the inhibitory effect of hypoxia on $[^3\text{H}]\text{Cho}$ incorporation in to PCho and PtdCho. Of note, the effects of acidosis and glucose deprivation were already apparent after 2 h of incubation. We also observed a slight decrease in $[^3\text{H}]\text{Cho}$ incorporation in to PtdCho, but not in to PCho during the third hour of the protocol, as compared with the second. However, it did not affect the observations already mentioned.

Effect of acidosis on Cho uptake

The drastic decrease in $[^3\text{H}]\text{Cho}$ incorporation in to PCho induced by acidosis could reflect either a blockade of Cho uptake, an inhi-

bition of CK, or both. To investigate this, we monitored the uptake of $[^3\text{H}]\text{Cho}$ and its phosphorylation with a pulse of $[^3\text{H}]\text{Cho}$ in a minute time-scale, after incubating HL-1 cells at pHs 7.4 or 6.4 for 1 h. Acidosis did not modify the initial rate of $[^3\text{H}]\text{Cho}$ uptake (Figure 5A) but decreased the subsequent incorporation of label in to PCho (Figure 5B), indicating that the effect of acidosis was on CK activity, rather than on Cho uptake.

Analysis of PtdCho synthesis in pulse-chase experiments with $[^3\text{H}]\text{Cho}$

To further analyse the individual effects induced by hypoxia, glucose deprivation and acidosis on the different steps of PtdCho synthesis we monitored the labelling of PCho and PtdCho in pulse-chase experiments, where cells were prelabelled for 2 h with $[^3\text{H}]\text{Cho}$. At this point, the highest amount of radioactivity is in the PCho pool (see Figure 3). Then, the radioactive label was chased by incubating the cells in the presence of non-radioactive Cho for 3 h under different conditions. In this experimental approach, the $[^3\text{H}]\text{Cho}$ label that disappears from PCho, appears in PtdCho, which mostly reflects the rate of the CCT-catalysed step. In Figure 6 we show the decrease in $[^3\text{H}]\text{PCho}$ (A) and the increase in $[^3\text{H}]\text{PtdCho}$ (B) during the chase, after subtracting their respective labelling at zero-time of the chase. The data in panels (A) and (B) are fairly complementary, confirming that the main bulk of radioactive label in PCho is transferred to PtdCho during the chase. Both the increase in $[^3\text{H}]\text{PtdCho}$ and the decrease in $[^3\text{H}]\text{PCho}$ were slowed down in cells undergoing hypoxia or glucose deprivation, as compared with their respective controls at any pH, indicating an inhibition of the CCT-catalysed step. Acidosis, by contrast, accelerated the increase in $[^3\text{H}]\text{PtdCho}$ labelling and the decrease in $[^3\text{H}]\text{PCho}$ labelling during the chase, which revealed an activation of CCT, that was masked by the CK inhibition in pulse-labelling experiments.

Effect of switching to acidosis in pulse-labelling experiments with $[^3\text{H}]\text{Cho}$

The opposite effects of acidosis on different steps of the PtdCho synthesis pathway, i.e. an inhibition of CK and activation of CCT, led us to perform additional experiments in an attempt to understand the net effect of acidosis. HL-1 cells were labelled for different durations at pH 7.4 in the presence of glucose. Then, at 20, 40 and 60 min of incubation the pH of the medium was switched to pH 6.4, while the pulse of $[^3\text{H}]\text{Cho}$ was maintained for 20 additional minutes. The labelling of all the intermediate metabolites of PtdCho synthesis was monitored before the pH switch and for a further 20 min at pH 6.4. The results in Figure 7 show how the switch to acidic buffer enhances intracellular $[^3\text{H}]\text{Cho}$, which most likely reflects the inhibition of CK, decreases $[^3\text{H}]\text{PCho}$ and increases $[^3\text{H}]\text{CDP-Cho}$, which is in agreement with an acceleration of the CCT-catalysed step. Interestingly, the amount of labelling in PtdCho was increased at the expense of labelled PCho.

Effect of acidosis on CK and CCT in a cell-free system

In order to test whether the effects of acidosis were direct or indirect, we monitored the activities of CK and CCT in subcellular fractions. CK activity was assayed at different pHs in the cytosolic fraction from HL-1 cell lysates, using $[^3\text{H}]\text{Cho}$ and ATP as substrates. The results obtained coincided with those from whole-cell experiments, with lower CK activity at lower pHs (Figure 8). CCT was assayed in cytosolic and membrane fractions from HL-1 cell lysates with $[^{14}\text{C}]\text{PCho}$ and CTP as substrates, and with or without micelles of PtdCho and oleate as lipid activators. The CCT

Figure 4 Pulse-labelling experiments with [3 H]Cho

HL-1 cells were incubated in Hepes buffer under different conditions, as indicated, during a maximum 3 h protocol. Cells were labelled with [3 H]Cho for 1 h, which lasted from the 1st to 2nd hour of the protocol (**A, C**) or from the 2nd to 3rd hour of the protocol (**B, D**). At the end of the protocol [3 H]Cho-metabolites were extracted, separated by TLC and counted for radioactivity. The amount of labelling incorporated in to PCho is shown in (**A**) and (**B**), and in to PtdCho, in (**C**) and (**D**). Hx, hypoxia; GD, glucose deprivation; Ac, acidosis; *, interaction. Results are means $\pm$ S.E.M. for three experiments performed in triplicate. Statistical analyses: 3 way ($2 \times 2 \times 2$) ANOVA. Panel A: Hx $P = 0.010$; GD $P = \text{NS}$; Ac $P = 0.000$; Hx*GD $P = 0.015$; Hx*Ac $P = 0.030$. Panel B: Hx $P = 0.048$; GD $P = 0.000$; Ac $P = 0.000$; Hx*Ac $P = 0.009$; GD*Ac $P = 0.001$. Panel C: Hx $P = 0.003$; GD $P = 0.040$; Ac $P = 0.000$; Hx*Ac $P = 0.005$. Panel D: Hx $P = 0.031$; GD $P = 0.002$; Ac $P = 0.000$; Hx*Ac $P = 0.044$; GD*Ac $P = 0.036$.

activity was higher in membranes than in cytosol (Figure 9A). In both fractions, and in contrast with what was observed in whole-cell experiments, acidosis drastically inhibited CCT activity. To test a putative effect of acidosis on the subcellular localization of CCT, we incubated HL-1 cells at pHs 7.4 or 6.4 for 1 h, separated the cells in to cytosolic and membrane fractions, and measured the CCT activity in each fraction. Pre-incubation of cells at pH 6.4 slightly increased, although not significantly, the amount of CCT activity in membranes, without modifying CCT activity in the cytosolic fraction (Figure 9B). Interestingly, the lipid mixture of PtdCho and oleate was not able to further increase CCT activity in membranes from cells incubated at pH 6.4, as occurred in membranes from cells incubated at pH 7.4.

The scheme shown in Figure 10 summarizes the modulation of PtdCho synthesis by hypoxia, glucose deprivation and acidosis,

as deduced from radioactive labelling in whole-cell experiments. Hypoxia inhibited both CK and CCT-catalysed reactions, glucose deprivation inhibited the CCT-catalysed reaction and exacerbated the effect of hypoxia on the CK-catalysed reaction, and acidosis elicited two opposing effects on PtdCho synthesis: it inhibited CK, but enhanced the CCT-catalysed reaction, the first most likely being a direct effect, and the second, an indirect or whole-cell-dependent effect.

Correlation between CCT activity and LDH release

Cell death, as measured by LDH release, triggered by the different experimental conditions tested in [3 H]Cho-labelling experiments is shown in Figure 11. Hypoxia was found to induce LDH release, an effect that was enhanced by glucose deprivation and abolished by acidosis.

Figure 5 Effect of pH on $[^3\text{H}]\text{Cho}$ uptake

HL-1 cells were incubated in Hepes buffer with 5 mM glucose at pHs 7.4 or 6.4 for 1 h. Then $0.2 \mu\text{M}$ $[^3\text{H}]\text{Cho}$ (0.25 Ci/mmol) was added. At 5 and 10 min at room temperature cells were washed with cold buffer. $[^3\text{H}]\text{Cho}$ and $[^3\text{H}]\text{PCho}$ were extracted, separated by TLC and counted for radioactivity. Results are means $\pm$ S.E.M. for two experiments performed in triplicate.

We next analysed the putative correlation between the activity of CCT and the release of LDH. For this purpose we plotted the data presented in Figure 6(B) against that in Figure 11, together with additional data points obtained from similar experiments, in which HL-1 cells were exposed to normoxic or hypoxic conditions in the absence of glucose at different pHs (7.4, 7.1, 6.8 and 6.4; Figure 8). In some experiments performed at pH 7.4 in the absence of glucose, 10 mM glycine was added. Glycine has been previously shown to decrease cell death in HL-1 cells submitted to metabolic inhibition and recovery [35]. In our experimental model glycine prevented LDH release and enhanced CCT activity, as did acidosis. Under all these conditions the percentage of LDH release correlated linearly ($R = 0.85$) with the logarithm of the increase in radioactive labelling of PtdCho during the chase, which reflects the rate of the CCT-catalysed step (Figure 12).

Correlation between CCT activity and ATP concentration

Since CTP, which depends on ATP concentration [36], is required to convert PCho in to CDP-Cho through the reaction catalysed by CCT, the correlation between LDH release and CCT activity could simply be a reflection of the concentration of ATP affecting both parameters. To test this hypothesis we measured ATP concentration after incubating the cells for 3 h during normoxia or hypoxia, in the absence or presence of glucose at different pHs (7.4 or 6.4). Hypoxia decreased ATP levels in all conditions, the

Figure 6 Pulse-chase experiments with $[^3\text{H}]\text{Cho}$

HL-1 cells were prelabelled in culture media with $[^3\text{H}]\text{Cho}$ ($0.5 \mu\text{Ci/ml}$) for 2 h. Then, $[^3\text{H}]\text{Cho}$ was washed and chased in Hepes buffer with $10 \mu\text{M}$ Cho for 3 h under different conditions, as indicated. At the end of the protocol $[^3\text{H}]\text{Cho}$ -metabolites were extracted, separated by TLC and counted for radioactivity. The net amount of labelling that disappeared from PCho (A) and was incorporated in to PtdCho (B) during the chase is shown. Results are means $\pm$ S.E.M. for three experiments performed in triplicate. Hx, hypoxia; GD, glucose deprivation; Ac, acidosis; *, interaction. Statistical analysis: 3 way ($2 \times 2 \times 2$) ANOVA Panel A: Hx $P = 0.000$; GD $P = 0.000$; Ac $P = 0.000$; Hx*Ac $P = 0.000$. Panel B: Hx $P = 0.000$; GD $P = 0.000$; Ac $P = 0.000$; Hx*Ac $P = 0.000$.

most drastic decrease being observed at pH 7.4 in the absence of glucose, a condition associated with the largest LDH release. Interestingly, acidosis decreased ATP levels in normoxic cells, but increased them in hypoxic cells. When the accumulation of $[^3\text{H}]\text{PtdCho}$ during the chase (i.e. CCT activity) was plotted against the concentration of ATP for the conditions indicated (Figure 13), it became apparent that there was no general correlation between ATP and the CCT-catalysed reaction rate.

DISCUSSION

In this work we demonstrate that the rate of the CCT-catalysed reaction in PtdCho synthesis in a whole-cell system correlates with membrane disruption in cultured HL-1 cardiomyocytes. Our results also indicate that ATP levels are not likely to be the unique rationale for this correlation. We suggest that CCT activity may be involved in necrotic cell death of cardiomyocytes subjected to hypoxia and glucose deprivation.

Figure 7 Effect of a switch to acidosis in pulse-labelling experiments with $[^3\text{H}]\text{Cho}$

HL-1 cells were incubated in Hepes buffer in the presence of 5 mM glucose at pH 7.4 and pulse-labelled with $[^3\text{H}]\text{Cho}$ ($0.5 \mu\text{Ci}/\text{ml}$) for the times indicated ($\circ$). At the times indicated by the arrows the pH of the media was switched to 6.4 and the pulse-labelling continued for 20 min (grey to $\bullet$). At the end of the protocol $[^3\text{H}]\text{Cho}$ -metabolites were extracted, separated by TLC and counted for radioactivity. $[^3\text{H}]\text{Cho}$ (A), $[^3\text{H}]\text{PCho}$ (B), $[^3\text{H}]\text{CDP-Cho}$ (C), and $[^3\text{H}]\text{PtdCho}$ (D). Results are representative of two experiments performed in triplicate with similar results.

Figure 8 Effect of pH on CK activity in the cytosolic fraction

The cytosolic fraction ($3 \mu\text{g}$ of protein/assay) from HL-1 cells was incubated in the presence of $0.2 \mu\text{M}$ $[^3\text{H}]\text{Cho}$ ($1 \text{ Ci}/\text{mmol}$) and 10 mM ATP at different pHs for 15 min at 37°C . The reaction was stopped with chloroform/methanol (1:2, v/v). $[^3\text{H}]\text{PCho}$ was extracted, separated by TLC and counted for radioactivity. Results are means $\pm$ S.D. for two experiments performed in triplicate.

We show that $[^3\text{H}]\text{Cho}$ incorporation in to PtdCho is already inhibited by hypoxia and glucose deprivation before the onset of membrane disruption in HL-1 cells. This is in accordance with previous work from other authors using isolated rat hearts, in which $[^3\text{H}]\text{Cho}$ incorporation in to PtdCho was inhibited by 60 min of hypoxia [26], or 10 min of low-flow perfusion [5], two insults not strong or long-lasting enough to induce cell death. These results led us to formulate the hypothesis that inhibition

Figure 9 Effect of pH on CCT activity in the membrane and cytosolic fractions

The effect of pH on CCT activity was measured directly in the membrane and cytosolic fractions from HL-1 cells without any treatment (A) or in the membrane and cytosolic fractions from HL-1 cells that were incubated at different pHs for 1 h before sucullular fractionation (B). In (A), $20 \mu\text{g}$ of the protein fraction was incubated in the presence of $[^{14}\text{C}]\text{PCho}$ ($12.5 \mu\text{Ci}/\text{mmol}$) and 4 mM CTP at different pHs for 15 min at 37°C . In (B), $20 \mu\text{g}$ of the protein fraction from cells pre-incubated at pHs 6.4 or 7.4 for 1 h was incubated at pH 7.4 in the presence of $[^{14}\text{C}]\text{PCho}$ ($12.5 \mu\text{Ci}/\text{mmol}$) and 4 mM CTP. All the assays were performed in the presence (grey bars) or absence (white bars) of a PtdCho/oleate mixture. Results are means $\pm$ S.E.M. for three (A) and two (B) experiments performed in triplicate.

of PtdCho synthesis could contribute to triggering sarcolemmal rupture in cardiomyocytes subjected to hypoxia and glucose deprivation. However, acidosis, which has been consistently found to be protective against myocardial ischaemia/reperfusion injury [37], and indeed abolished the release of LDH elicited by hypoxia and glucose deprivation in our cell model, was also able to decrease the net $[^3\text{H}]\text{Cho}$ incorporation in to PtdCho.

The puzzling observation in relation to the effect of acidosis, led us to further investigate the effects elicited by the three phenomena that occur simultaneously during coronary occlusion, i.e. hypoxia, glucose deprivation and acidosis, on the individual reactions of the Kennedy pathway for the *de novo* synthesis of PtdCho. This was achieved by means of an exhaustive analysis of pulse and pulse-chase labelling experiments with $[^3\text{H}]\text{Cho}$. The pulse-labelling experiments allowed us to observe the inhibitory effects on the CK-catalysed steps of acidosis and hypoxia, as well as the exacerbation of the effects of hypoxia by glucose deprivation.

Figure 10 Schematic representation of the effects of hypoxia, glucose deprivation and acidosis on the individual reactions of the *de novo* synthesis of PtdCho in whole-cell experiments

The scheme shows a summary of the results obtained from pulse, and pulse-chase experiments using [3 H]Cho. Arrows indicate stimulatory effects. Dashed arrow indicates an indirect stimulatory effect. Truncated lines indicate inhibitory effects. Cho, choline; PCho, phosphocholine; CDP-Cho, CDP-choline; PtdCho, phosphatidylcholine; CK, choline kinase (EC 2.7.1.32); CCT, CTP:phosphocholine cytidyltransferase (EC 2.7.7.15); CPT, CDP-choline:sn-7,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2)

Figure 11 LDH release

HL-1 cells were incubated in HEPES buffer under different conditions for 3 h, as indicated. At the end of the protocol, the amount of LDH released in to the incubation media was determined. Results are means $\pm$ S.E.M. for at least three experiments performed in triplicate. Hx, hypoxia; GD, glucose deprivation; Ac, acidosis; *, interaction. Statistical analysis: 3 way ($2 \times 2 \times 2$) ANOVA. Hx $P = 0.000$; GD $P = 0.005$; Ac $P = 0.000$; Hx*GD $P = 0.003$; Hx*Ac $P = 0.000$; GD*Ac $P = 0.014$; Hx*GD*Ac $P = 0.014$.

The pulse-chase experimental approach allowed us to evaluate the modulation of the PtdCho synthesis beyond that of PCho. As is shown in the scheme presented in Figure 10, hypoxia and glucose deprivation, two deleterious treatments for HL-1 cells, inhibited the CCT-catalysed step, but acidosis, a protective treatment, enhanced it. Of note, glycine, a treatment known to protect against cell death induced by hypoxia, ischaemia, or metabolic inhibition [35], also stimulated the CCT-catalysed step.

Although we clearly show that hypoxia and glucose deprivation inhibited CCT activity, which was basically demonstrated by their effect in slowing down the decrease in [3 H]PCho and accelerating the increase in [3 H]PtdCho in pulse-chase experiments, any putative effect of these two treatments on the last step of PtdCho synthesis catalysed by CPT, could not be ruled out.

The opposite effects of acidosis on CK (inhibition) and CCT (activation) deserved some more attention. We first confirmed that

Figure 12 Correlation between CCT-catalysed reaction rate and LDH release

Data from experiments presented in Figures 6 and 8 were pooled with additional data obtained from cells subjected at pHs 7.1 and 6.8, to hypoxia or normoxia, in the absence of glucose. All data points are plotted with the CCT activity on the x-axis, and LDH release on the y-axis. Open and filled symbols are data points corresponding to normoxic or hypoxic cells respectively. Triangles are data points obtained in the presence of 10 mM glycine, at pH 7.4 in the absence of glucose. Circles are data points obtained at different pHs in the presence or absence of 5 mM glucose. For experimental protocol details see the legends in Figures 6 and 8. Data for both parameters were previously normalized to the values obtained at pH 7.4 in the presence of glucose for each experiment. Results are means $\pm$ S.E.M. for at least three experiments performed in triplicate.

Figure 13 Correlation between the CCT-catalysed reaction rate and ATP concentration

HL-1 cells were incubated for 3 h under different conditions, as indicated, and subsequently frozen in liquid N_2 . ATP was extracted and the concentration was determined by luminescence assay. The ATP concentrations obtained and the data corresponding to CCT activity presented in Figure 6 are plotted together. Data for both parameters were previously normalized to the values obtained at pH 7.4 in the presence of glucose for each experiment. Open and filled symbols are data points corresponding to normoxic or hypoxic cells respectively. Triangles correspond to pH 7.4 (large symbol) or pH 6.4 (small symbol) in the absence of glucose; circles correspond to pH 7.4 (large symbol) or pH 6.4 (small symbol) in the presence of glucose. Results are means $\pm$ S.E.M. for at least three experiments performed in triplicate.

the decrease in [3 H]Cho incorporation in to PCho was mainly a reflection of CK inhibition, rather than a decrease in [3 H]Cho uptake. Moreover, CK activity in the cytosolic fraction of HL-1 cells was inhibited by low pH, which is in agreement with previous work on CK characterization [38]. We then reassessed the activation of CCT by switching the pH from 7.4 to 6.4 during the pulse with [3 H]Cho in whole cells. In these experiments, even though CK was inhibited, acidosis induced a small but significant increase in labelling in to PtdCho at the expense of [3 H]PCho.

In a cellular context, the activation of CCT by acidosis could counteract an inhibition of PtdCho synthesis. However, this cannot be a long-lasting effect in the absence of PCho sources other than Cho phosphorylation. The results obtained in experiments performed in cell-free systems strongly contrast with those obtained in whole cells. Acidosis clearly inhibited CCT activity in membrane, as well as in cytosolic fractions both in the presence or absence of lipid mixtures. These results differ from published observations. A study described neutral pH as optimal for CCT activity, the activity at pHs 6.5 and 7.5 being similar and only a bit lower than at pH 7 [39]. Another study found that the optimum pH for CCT was 6 [40]. In the present study, when CCT activity was measured in membrane and cytosolic fractions from cells that had been pre-incubated at different pHs, it was observed that CCT activity could not be further increased by lipid mixtures in membrane fractions from cells pre-incubated at a low pH. This finding is compatible with the existence of a putative pool of CCT that is more active under acidosis than at pH 7.4. We therefore suggest that the activation of CCT by acidosis is either an indirect or whole-cell-dependent effect. Other authors after monitoring an enhancement of the pool size of CDP-Cho have suggested that acidosis, secondary to the action of apoptotic agents, inhibits the last step in PtdCho synthesis catalysed by CPT [41]. Interestingly, it is remarkable that this would also be the predicted result after the activation of CCT.

The observation that LDH release correlated with CCT activity in whole cells suggested that more so than the rate of the incorporation of [³H]Cho in to PtdCho through the Kennedy pathway, which did not correlate with LDH release, what seems to influence the likelihood of necrotic cell death during hypoxia or ischaemia is the rate of the CCT-catalysed step. Moreover, no general correlation between ATP and CCT activity was found, although a significant correlation was observed when the analysis was limited to data obtained from hypoxic cells. The correlation between CCT activity and LDH release thus cannot be solely explained by ATP levels.

Our interpretation that CCT activity plays a role in cell death elicited by simulated ischaemia is in accordance with previous work [17,18,20–25] demonstrating the involvement of CCT activity in cell death. Whether the inhibition of CCT takes part in intracellular signalling pathways that lead to cell death, or induces a net decrease of the PtdCho pool, which presumably renders the membrane fragile and facilitates its rupture, is still unknown. Both hypotheses are compatible with ours and others' previously published experimental data. On one hand, it has been shown that there is no net decrease of the PtdCho pool after up to 7 h of hypoxia [1], which supports the signalling hypothesis, but on the other hand, it has also been demonstrated that exogenous PtdCho or lyso-PtdCho [16,20,24,25,42,43] rescues cells with inhibited PtdCho synthesis from death, which supports the idea that the loss of PtdCho is the determining factor. Nonetheless, the protective effect of lyso-PtdCho has to be interpreted cautiously. It is known that inhibitors of phospholipase A₂, which are expected to decrease the levels of lyso-PtdCho, protect isolated rat hearts against global ischaemia [29], and it has also been described that exogenously added lyso-PtdCho induces cell injury in perfused rat hearts [44,45].

In summary, our results show that the inhibition of [³H]Cho incorporation in to PtdCho induced by hypoxia and glucose deprivation precedes the onset of LDH release and that the modulation of the CCT-catalysed reaction may have a role in sarcolemmal disruption during ischaemia.

PI030135, A-2003 and FIS-RECAVA. E.S. is the recipient of a postdoctoral fellowship from Institut de Recerca Hospital Universitari Vall d'Hebron. A.A. is the recipient of a doctoral fellowship from the Spanish Ministry of Science.

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We thank Angeles Rojas for technical assistance with cell cultures. This study was supported by Fondo de Investigación Sanitaria of Spanish Ministry of Health, grants FIS

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Received 20 May 2005/12 October 2005; accepted 20 October 2005

Published as BJ Immediate Publication 20 October 2005, doi:10.1042/BJ20050834