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EFFECTS OF CHLORPROMAZINE AND TRIFLUOPERAZINE ON CHOLINE METABOLISM AND PHOSPHATIDYLCHOLINE BIOSYNTHESIS IN CULTURED CHICK HEART CELLS UNDER NORMOXIC AND ANOXIC CONDITIONS*

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Abstract—The effects of chlorpromazine and trifluoperazine on phosphatidylcholine biosynthesis in the heart were investigated in isolated cardiac cells under normoxic and anoxic conditions. The cells were obtained from 7-day-old chick embryos and were maintained in culture. After 96 hr, cells were maintained either in an incubator with oxygen at room air concentration (normoxia) or in an incubator containing 95% nitrogen and 5% CO_2 (anoxia). Pulse chase experiments with [*methyl*-^{3}H]choline were conducted using a 2-hr incubation with choline. Chlorpromazine and trifluoperazine at 10^{-5} M produced a significant ($P < 0.05$) increase in the incorporation of choline into both phosphocholine and phospholipid. High concentrations of chlorpromazine or trifluoperazine i.e. 10^{-4} M, damaged myocardial cells as reflected in a significant ($P < 0.05$) reduction in cellular protein and a further reduction in labelled choline in phosphocholine or phospholipid after adjusting for the lower protein concentrations. Anoxia altered choline metabolism but 6 hr of anoxia was the minimum time needed for the effect to be observable. Anoxia, for 24 hr, produced a significant ($P < 0.05$) reduction in labelled choline in phosphocholine without a significant change in incorporation of label in phospholipid or cellular protein. Both chlorpromazine and trifluoperazine at 10^{-5} M prevented anoxic-induced changes in phosphocholine metabolism. Thus, chloropromazine and trifluoperazine affect phospholipid biosynthesis in cardiac cells and prevent anoxia-induced changes in phosphatidylcholine biosynthesis.

The phenothiazines chlorpromazine and trifluoperazine have been found to limit the severity of myocardial cell damage from hypoxia and ischemia [1–4], but the mechanism underlying this effect is uncertain. One proposed mechanism is that chlorpromazine and trifluoperazine inhibit phospholipid degradation that is activated by hypoxia and ischemia [2, 3, 5, 6]. If these drugs affect phospholipid degradation in the heart, then they may affect phospholipid biosynthesis as well. The effects of anoxia and the phenothiazines on phospholipid biosynthesis, in the heart, have not been the focus of much attention. Phosphatidylcholine is a major component of the phospholipid composition of the heart and serves many important cellular functions [7]. The major pathway for phosphatidylcholine biosynthesis occurs via the CDP pathway in which choline is incorporated first into phosphocholine, then into CDP choline diacylglycerol and finally into phosphatidylcholine [8]. Zelinski *et al.* [9] demonstrated that, in hamster heart, this pathway accounts for 90% of total phosphatidylcholine biosynthesis. Thus, the purpose of this study was to examine the hypothesis that phosphatidylcholine biosynthesis in isolated cardiac cells is altered by the phenothiazine drugs, chlorpromazine and trifluoperazine, as well as by anoxia and to determine whether these drugs alter the potential impact of anoxia on myocardial phosphatidylcholine biosynthesis.

MATERIALS AND METHODS

Myocyte culture. Chick embryonic ventricular cells were cultured using previously described methods [10, 11]. Briefly, hearts from 7-day-old White Leghorn chick embryos were removed and the atria and blood dissected away. Isolated ventricles were minced and incubated at 37° in digestion medium containing 0.005% trypsin and 1×10^7 units of DNAase per ml of DMS8 solution. DMS8 consists of 116 mM NaCl, 5.4 mM KCl, 0.4 mM NaH_2PO_4, 0.8 mM $Na_2HPO_4 \cdot 7H_2O$ and 5.6 mM dextrose. After 5 min, the medium was removed and new digestion medium was added. This was repeated three times to complete the digestion of tissue. The disaggregate was then diluted 1:5 in fresh culture medium (20% M199, 73% DBSK, 7% fetal bovine serum) and spun at 800 *g* for 5 min. DBSK consists of 116 mM NaCl, 0.83 mM $MgSO_4 \cdot 7H_2O$, 1.0 $NaH_2PO_4 \cdot H_2O$, 5.6 mM dextrose, 1.8 mM $CaCl_2$ and 26 mM $NaHCO_3$. The cell pellet was resuspended in medium to a concentration of 1×10^6 cells/ml and plated into 35 × 10 mm dishes (2 ml/dish). A confluent culture was obtained after 96 hr. The cell population was estimated to be in excess of 80% myocytes as determined by the numbers of beating cells.

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Fibroblast culture. To differentiate the effect of anoxia on myocytes from that on fibroblasts, a pure culture of skin fibroblasts was studied. The cells (No. 3524, NIGMS Homon Genetic Mutant Cell Repository, Institute for Medical Research) were grown in Dulbecco's Modified Eagle Medium (GIBCO No. 430+600) with glucose (4.5 g/L). They were changed to DBSK and handled in the same manner as cardiac cells, starting 4 days before experiments.

Protocol. Chlorpromazine and trifluoperazine were added to the medium, and cells were exposed to drugs for 24 hr prior to commencing pulse chase experiments. Cells were maintained in an incubator with either an oxygen concentration equal to room air or under anoxic conditions. Anoxia was produced by placing cells, grown for 96 hr, in an incubator containing 95% nitrogen and 5% CO_2. For the pulse chase experiments, the cells were removed briefly from the incubator and then returned to the anoxic environment until the completion of the chase period. Control cells, cultured at the same time, were treated the same, during the pulse chase experiment, as the other cells except that they were in an incubator with a partial pressure of oxygen equal to that of room air.

Pulse chase studies. Cells were incubated in fresh medium containing [*methyl*-^{3}H]choline. After 2 hr (pulse period) the cells were either harvested or incubated for a further 3 hr in media that contained the same concentration of unlabelled choline (chase period). At predetermined times, the growth medium was removed, the cells were harvested, and the lipids were extracted as described by Pritchard and Vance [12]. The recovery of ^{3}H in choline, phosphocholine and CDP choline was determined following thin-layer chromatography using previously described methods [12, 13].

Protein determination. Cells that had been exposed to drug or anoxia or control conditions for 24 hr were rinsed with cold phosphate buffer (pH 7.0). Cells were disrupted by addition of 0.1 N NaOH, scraped into tubes, rinsed and pH-neutralized to 7.0 by HCl. Total protein was estimated with the Bio-Rad protein assay based on the method of Bradford [14].

Materials. Culture media and serum were obtained from GIBCO, Burlington, Ontario. [*Methyl*-^{3}H]Choline (78 Ci/mmol) was obtained from Amersham. Chlorpromazine was provided by Poulens Ltd., (Montreal, Canada), and trifluoperazine was purchased from Sigma Chemical Co., (St Louis, MO).

Data analysis. Hypothesis testing for between group comparisons used analysis of variance. Simple linear regression analysis and comparisons of straight lines were used for other analyses. For hypothesis testing, the null hypothesis was rejected when the probability of a Type I error was less than 5% ($P < 0.05$).

RESULTS

When cultured chick embryo cardiac myocytes were incubated in the presence of [^{3}H]choline for 2 hr, most of the label (about 85%) was recovered in phosphocholine and most of the remainder in phospholipid. Of the counts in phospholipid, over 95% were found, on TLC analysis, to represent phosphatidylcholine. During the chase incubation, label was transferred to phosphatidylcholine with relatively little accumulation of label in CDP choline. A small amount of label left the phosphocholine pool during the chase incubations and was recovered from the culture medium. TLC analysis of the chase media indicated that the label was released from the cells as choline.

Chlorpromazine and trifluoperazine. The effect of chlorpromazine on choline metabolism was dependent on its concentration (Fig. 1). At 10^{-6} M, chlorpromazine did not alter choline metabolism significantly. However, at 10^{-5} M there was a significantly ($P < 0.05$) greater amount of choline incorporated into phosphocholine and phospholipid. At 10^{-4} M, chlorpromazine markedly and significantly ($P < 0.05$) suppressed the amount of labelled choline in both phosphocholine and phospholipid. Most of the choline that entered the cells left the cell and was recovered in the chase media at this chlorpromazine concentration. The amount of choline in the chase was expressed per milligrams of cellular protein that was used to adjust for the amount of intracellular choline in phosphocholine or phospholipid. Chlorpromazine at 10^{-4} M, but not at the other concentrations, was associated with a marked reduction in an index of cell viability, namely the amount of cellular protein. The amounts of protein were 576.0 ± 11.7, 570.7 ± 15.4, 490.0 ± 21.4, 370.0 ± 56.9 and 168.0 ± 2.0 μg/dish (mean ± 1 SE) for chlorpromazine concentrations of 0, 10^{-7} M, 10^{-6} M, 10^{-5} M and 10^{-4} M, thus suggesting cell death at the highest concentration.

Trifluoperazine produced the same effects on choline metabolism as chlorpromazine with respect to the concentration–effect relationships (Fig. 2). At 10^{-5} M but not 10^{-6} M trifluoperazine, there were significant ($P < 0.05$) increases in labelled choline in both phosphocholine and phospholipid compared to controls. There were no differences in choline in the chase media for either 10^{-6} or 10^{-5} M trifluoperazine compared to control cells. At 10^{-4} M trifluoperazine, the amount of labelled choline in phosphocholine and phospholipid was markedly and significantly ($P < 0.05$) suppressed and a large amount of the choline left the cells. The amounts of protein were, respectively, 576.0 ± 11.7, 600.7 ± 5.5, 477.5 ± 74.8, 506.6 ± 58.6, and 141.5 ± 13.9 μg/dish for trifluoperazine concentrations of 0, 10^{-7} M, 10^{-6} M, 10^{-5} M and 10^{-4} M, again suggesting cell death at the highest concentration.

Anoxia. Prolonged durations of anoxia were necessary to show any changes in choline metabolism in cardiac cells (Fig. 3). Preliminary experiments with durations of anoxia of 6 hr or less produced little effect, while 7 hr of anoxia produced some reduction in incorporation of labelled choline into phosphocholine and 24 hr of anoxia produced a greater depression of labelled choline in phosphocholine. The rate of decline of labelled phosphocholine during the chase period was similar after 7 and 24 hr of anoxia. In contrast to phosphocholine, there was not a marked change in choline incorporation into

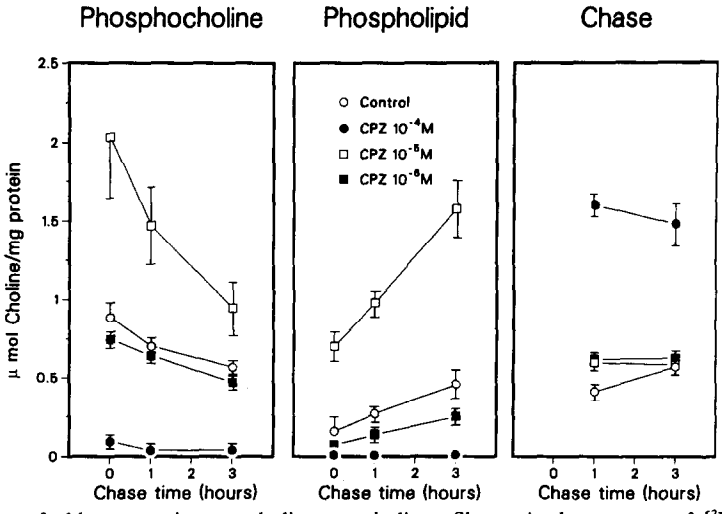

Fig. 1. Effect of chlorpromazine on choline metabolism. Shown is the amount of [^{3}H]choline in phosphocholine (left), phospholipid (middle), and chase medium (right) at the end of the pulse period and during the chase period for different concentrations of chlorpromazine (CPZ). The results are means ± 1 SE (N = 4 at each CPZ concentration).

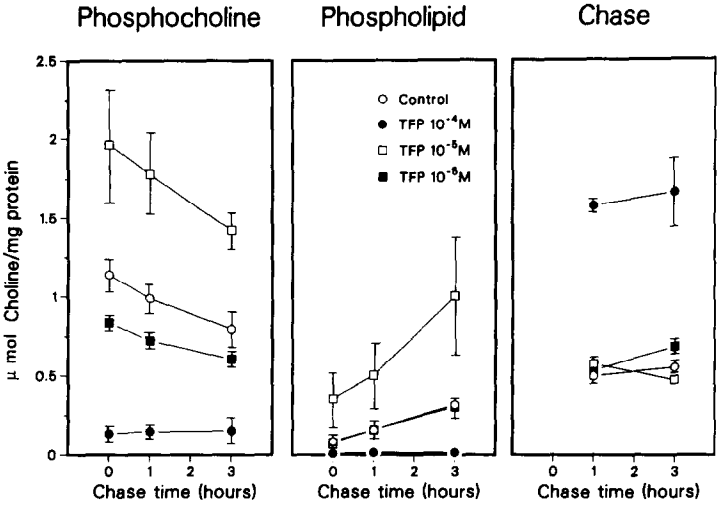

Fig. 2. Effect of trifluroperazine on choline metabolism. Shown is the amount of [^{3}H]choline in phosphocholine (left), phospholipid (middle), and chase medium (right) at the end of the pulse period and during the chase period for different concentrations of trifluorperazine (TFP). The results are means ± 1 SE (N = 4 at each TFP concentration).

phospholipid with anoxia. Increases in phospholipid over time were similar after 7 hr or 24 hr of anoxia, although perhaps slightly less after 24 hr of anoxia. TLC analysis did not show any alteration in CDP choline with anoxia.

A 24-hr anoxia period was chosen for more detailed evaluation of the effects of anoxia because the changes during this period were more readily distinguishable from the normoxic condition. There was no significant difference in the amount of protein between normoxic and anoxic cells (580.0 ± 55.1 vs 630 ± 39.4 μg protein/dish) respectively. The total amount of label in phosphocholine, at the end of the pulse period, was significantly ($P < 0.05$) less in anoxic than in normoxic cells (Fig. 4). Pulse chase experiments showed a significant ($P < 0.05$) reduction in phosphocholine in cells exposed to anoxia compared to normoxic controls. The rates of loss of label from phosphocholine were similar in the anoxic cells and the control cells, namely −0.120 ± 0.020 vs −0.099 ± 0.001 μmol choline/mg protein/hr (mean ± SE). During the chase period, anoxic cells had significantly ($P < 0.05$) less label in phosphocholine than normoxic cells. In contrast, the amounts of label in phospholipid were similar in anoxic and normoxic cells, although the rate of accumulation of labelled choline in phospholipid was slightly less in the anoxic than in the control cells, i.e. 0.080 ± 0.002 vs 0.119 ± 0.009 μmol choline/mg protein/hr. The smaller number of counts in

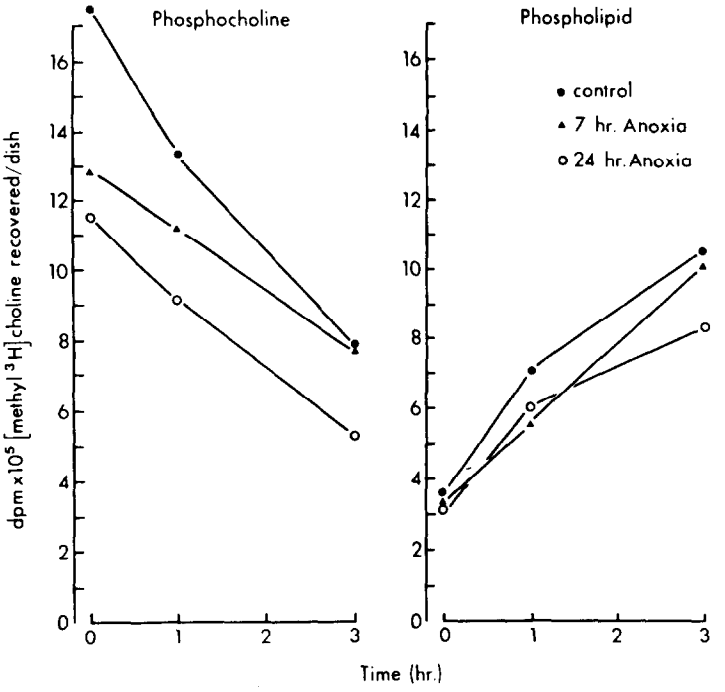

Fig. 3. Effect of anoxia on the metabolism of [*methyl*-^{3}H]choline. Cells were exposed to a total of 7 or 24 hr of anoxia. These cells were then compared to cells maintained under normoxic conditions. Results are expressed as the mean of duplicate incubations from a single preparation of heart cells exposed to each condition.

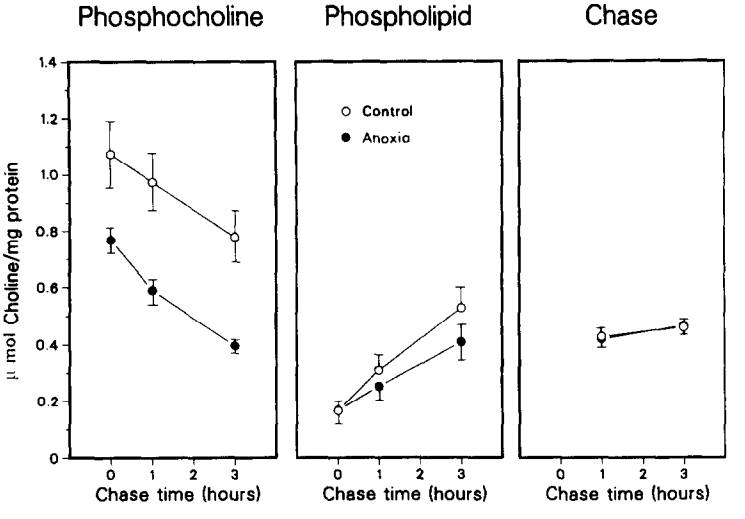

Fig. 4. Effect of anoxia on choline metabolism in cardiac cells. Cells were maintained for 24 hr in anoxic or normoxic conditions. The amount of [^{3}H]choline in phosphocholine (left), phospholipid (middle), and chase medium (right) at the end of the pulse period and during the chase period is shown for anoxic (N = 16) and normoxic cells (N = 16). The data are means ± 1 SE.

phosphocholine during the chase period was not due to an increase in choline leaving the cell as the amount of choline in the chase was the same for the control and the anoxic cells.

To determine the contribution of fibroblasts to these effects, pure cultures of fibroblasts were studied in the same manner. Culture dishes were maintained in an anoxic condition for 24 hr while others were maintained in a normoxic environment. Pulse chase experiments were conducted at the same time in cells under both conditions (Fig. 5). Fibroblasts showed a much smaller amount of choline uptake per milligram protein compared to myocytes. The amount of labelled choline in phosphocholine or

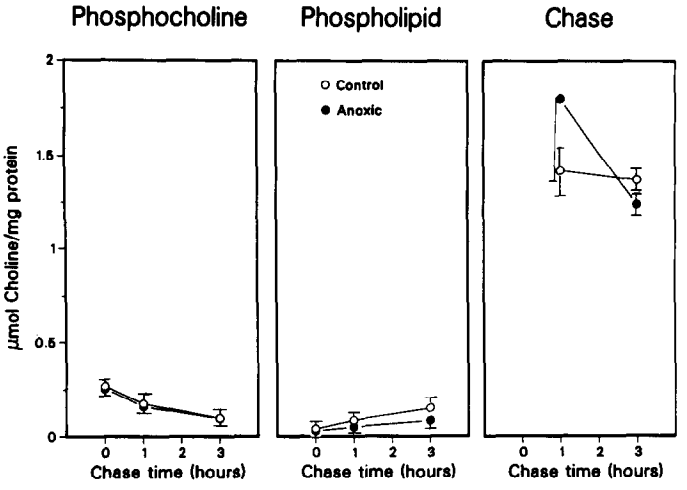

Fig. 5. Effect of anoxia on choline metabolism in fibroblasts exposed to normoxia (N = 3) or anoxia (N = 3) for 24 hr prior to and during the pulse chase experiment with [*methyl*-^{3}H]choline. The amounts of [^{3}H]choline in phosphocholine (left), phospholipid (middle) and chase medium (right) are shown at the end of the pulse and during the chase period. Results are means ± 1 SE.

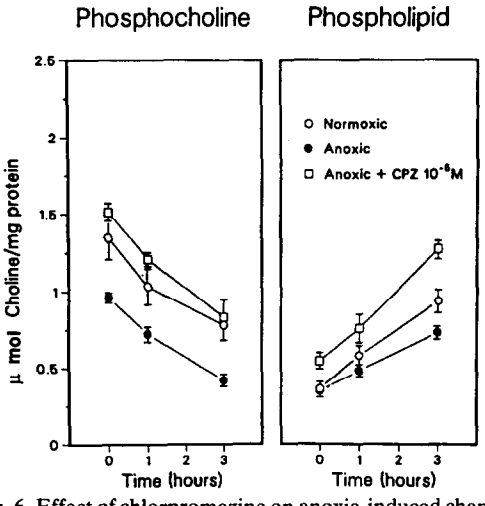

Fig. 6. Effect of chlorpromazine on anoxia-induced changes in choline metabolism. Shown is the amount of [^{3}H]choline in phosphocholine (left) and phospholipid (right) at the end of the pulse period and during the chase period for cells in normoxic conditions (N = 4), anoxia (N = 4) and anoxia with 10^{-5} M chlorpromazine in the medium (N = 4). The data are means ± 1 SE.

phospholipid was 25% or less than the amount in myocytes (Fig. 4 vs Fig. 5). The amount of choline in phosphocholine and phospholipid was reduced in cells exposed to anoxia but it was only significantly less for phospholipid ($P < 0.05$ vs normoxia). The magnitude of the reduction, especially for phosphocholine, was much less than in myocytes.

Interaction of chlorpromazine, trifluoperazine and anoxia. The effects of phenothiazines under anoxic conditions were assessed at 10^{-5} M because of the significant changes in phosphocholine found at this concentration. Chlorpromazine, at 10^{-5} M, prevented the anoxia-induced reduction in labelled choline incorporated into phosphocholine (Fig. 6). Anoxia significantly ($P < 0.05$) reduced the amount of label in phosphocholine compared to the control cells. In the presence of chlorpromazine, there was a significantly greater ($P < 0.05$) amount of choline in phosphocholine compared to cells exposed to anoxia without chlorpromazine. There were no significant differences in phosphocholine between the control and the anoxic cells pretreated with chlorpromazine. Although the effects of anoxia on phospholipid were less marked, the opposing effects of anoxia and chlorpromazine were also seen in the amount of label in the phospholipid pool. Anoxia significantly ($P < 0.05$) reduced the label in phospholipid only at 3 hr of the chase period. In contrast, there was significantly ($P < 0.05$) more label in phosphocholine in anoxic cells pretreated with chlorpromazine than in anoxic cells without chlorpromazine. The disappearance of label from phosphocholine was similar in control and anoxic cells (0.179 ± 0.0461 and 0.1773 ± 0.210 μmol choline/mg protein/hr, respectively) and was greater, although not significantly larger, in anoxic cells pretreated with chlorpromazine, i.e. 0.221 ± 0.029 μmol choline/mg protein/hr. The rate of accumulation of label in phospholipid was slightly lower in anoxic compared to control cells, i.e. 0.128 ± 0.001 vs 0.193 ± 0.009 μmol choline/mg protein/hr, and slightly, but not significantly, higher in anoxic cells pretreated with chlorpromazine (0.246 ± 0.010 μmol choline/mg protein/hr compared to controls).

The results were similar with trifluoperazine. Trifluoperazine prevented the significant ($P < 0.05$) reduction in label in phosphocholine produced by anoxia (Fig. 7). Anoxic cells pretreated with trifluoperazine did not have a significantly different amount of label in phosphocholine compared to control cells except at 3 hr of the chase period. The disappearance of the label from phosphocholine was reduced slightly in anoxic cells compared to controls and increased in anoxic cells pretreated with tri-

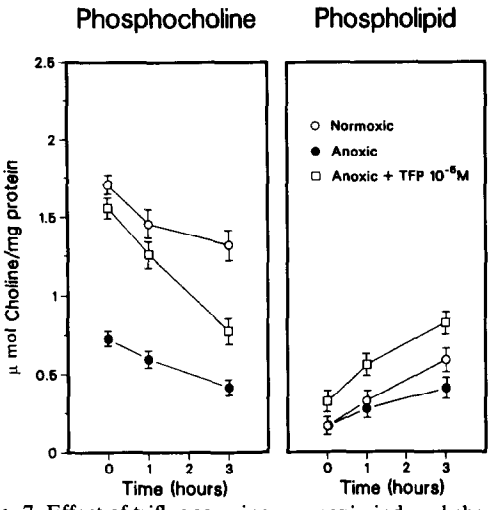

Fig. 7. Effect of trifluoperazine on anoxia-induced changes in choline metabolism. Shown is the amount of [^{3}H]choline in phosphocholine (left) and phospholipid (right) at the end of the pulse period and during the chase period for cells exposed to normoxia (N = 4), anoxia (N = 4) and anoxia with 10^{-5} M trifluoperazine in the medium (N = 4). The data are means ± 1 SE.

fluoperazine, although the differences were not statistically significant. The rate of accumulation of label in phospholipid, that was decreased in anoxic cells compared to controls, was prevented in anoxic cells pretreated with trifluoperazine.

DISCUSSION

In embryonic chick heart cells in culture, choline was found to be incorporated mainly into phosphocholine with subsequent metabolism into phospholipid. This is consistent with the observations of Zelinski *et al.* [9] in isolated perfused hamster heart. Oide [15] also found that the majority of choline is metabolised into phosphocholine in isolated chick embryo hearts not in culture. However, he reported significant amounts of label in betaine and choline, which neither we nor Zelinski *et al.* [9] found. Bosteels *et al.* [16] suggested that choline was not metabolised to a significant extent in isolated cat papillary and ventricular muscle but they used a less sensitive assay procedure, a different species, and another kind of preparation.

An important finding of the present study is that chlorpromazine and trifluoperazine increase the amount of choline incorporated into phosphocholine and phospholipid, in the heart, presumably by affecting the activity of choline kinase and CTP:phosphocholine cytidylyltransferase. Although these enzymes were not measured in this study, phenothiazines are known to affect a number of membrane and intracellular enzyme systems [17, 18] including enzymes involved in phosphatidylcholine synthesis in non-cardiac cell systems [19–21]. Pelech *et al.* [19] found that both chlorpromazine and trifluroperazine inhibit cytosolic but not microsomal cytidylyltransferase in the liver. HeLa cells are even more sensitive to these agents with more marked inhibition of phosphatidylcholine synthesis [20]. The mechanism of these effects is uncertain. Phenothiazines inhibit calcium influx in a wide variety of cellular membranes including the cardiac sarcolemma, sarcoplasmic reticulum microsomes, and mitochondria [18, 22]. It is unlikely that calcium would be the explanation as it has little effect on phospholipid biosynthesis in the heart [9, 23]. Chlorpromazine and especially trifluoperazine inhibit calmodulin, a calcium-dependent regulatory protein capable of activating a number of enzyme systems [24, 25]. However, effects on calmodulin are a possible but less likely explanation for the effects on phosphocholine and phospholipid because they were about equally affected by both drugs, yet trifluoperazine is a more potent inhibitor of calmodulin.

The findings of the present study may provide an explanation for the adverse effects of phenothiazines on the heart. High concentrations of both chlorpromazine and trifluoperazine were found to be toxic to cardiac myocytes despite showing protective effects on myocardial cells at low concentrations. This is similar to the report of Scott *et al.* [26]. Importantly, the remaining myocytes did not metabolize choline normally and most of the choline left the cell. Phenothiazines can induce a cardiomyopathy, the basis for which has been uncertain [27]. It is possible that a disturbance in phosphatidylcholine biosynthesis occurs at high doses of phenothiazines in some patients, altering the phospholipid composition of the myocardium and leading to the changes in myocardial function seen in phenothiazine-induced cardiomyopathy.

Phenothiazines have been found to improve myocardial viability when exposed to ischemic injury, but the mechanism of action for this effect has not been clearly defined. Scott *et al.* [26] suggested that phenothiazines preserve membrane integrity of cardiac myocytes, perhaps through inhibition of a calmodulin- or calcium-dependent process. Others have proposed that phenothiazines inhibit phospholipid degradation which can be induced by myocardial ischemia [1–4]. An important contribution of this work is that its findings suggest another possible mechanism whereby phenothiazines prevent myocardial damage, namely by inhibition of anoxic-induced changes in phosphotidylcholine biosynthesis.

The effect of anoxia on phosphatidylcholine biosynthesis is part of a general effect of anoxia on the heart. In isolated cardiac cells in culture, anoxia damages sarcolemmal integrity and decreases intracellular ATP, creatinine phosphate and protein synthesis [28–31]. Leakage of cellular enzymes during anoxia is inversely correlated with intracellular ATP concentration, suggesting that membrane integrity is dependent on oxidative metabolism in isolated cardiac cells [29, 31]. The effect of anoxia is dependent on the age of the heart (embryonic or adult), animals species, duration of anoxia, substrate availability, as well as a number of other factors relating to the model system studied. The present study used embryonic chick heart cells, studied only anoxia, and provided all other necessary substrates. Thus, prolonged durations of anoxia were necessary to show alterations in choline metabolism. Even longer durations of anoxia undoubtedly would have pro-

duced greater effects but would have damaged the myocardial cells, producing fewer viable cells. The duration of anoxia examined was not associated with a loss of viability as assessed by total cell protein, recognizing that protein is only one measure of viability. Our findings of duration of anoxia on myocyte viability are similar to the data of Acosta *et al.* [32]. The effect of anoxia on phosphatidylcholine biosynthesis has not been studied extensively in isolated cardiac cells in culture. The present study found, in viable cardiac myocytes, that deprivation of oxygen produces a reduction in label in phosphocholine but not in phospholipid. A likely explanation for this finding is an increased activity of phosphocholine cytidylyltransferase or phosphocholine transferase, presumably induced by a reduction in high energy triphosphate nucleotides, similar to the findings in the hearts of myopathic hamsters in which the enhanced activity of this enzyme was hypothesized to be a compensatory mechanism to maintain a minimal CDP choline level to prevent reduction in net phosphatidylcholine biosynthesis [33]. Thus, chlorpromazine and trifluoperazine can be effective in reversing anoxic-induced alterations in choline metabolism in cardiac cells. One concern was that the cardiac cells in culture were not a pure culture of myocytes. Thus, separate experiments were conducted with fibroblasts, the other cell type present in cardiac cell cultures. It is highly unlikely that the results of this study would be materially affected by choline metabolism in fibroblasts because their prevalence in the cell cultures was less than 20%, choline uptake in these cells was much less than in cardiac cells, and they were less affected by anoxia. An important caveat in the extrapolation of these findings is that we did not investigate whether the anoxic-induced changes in phospholipid metabolism were adaptive or maladaptive processes. However, effects on phospholipid biosynthesis maybe a potential explanation for some of the beneficial effects of phenothiazines on myocardial cell injury induced by ischemia or the calcium paradox [1–4, 34], in addition to their known effects on phospholipid degradation [2, 3, 6, 35, 36]. Another caveat is that embryonic chick heart cells were used. These cells are relatively resistant to hypoxia, as was shown herein, especially when compared to adult myocardial cells. It remains uncertain whether the adult heart will respond to anoxia and, indeed, ischemia in a manner similar to that found in the present study. Thus, extrapolation of these data to the intact adult heart made ischemic must be done with caution. Nevertheless, anoxia was found to affect phosphatidylcholine biosynthesis and it is uncertain whether the adult heart will respond in a different fashion. Importantly, the embryonic chick heart model has been studied intensively, and the data can be interpreted and are of value within the context of that literature.

In summary, choline metabolism to phosphatidylcholine occurred in cardiac cells. Anoxia, chlorpromazine and trifluoperazine altered phosphatidylcholine biosynthesis in a dose-dependent manner and, most importantly, both phenothiazines inhibited anoxic-induced changes in phosphocholine metabolism.

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