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CHOLINE RESISTANCE AND PHOSPHATIDYLCHOLINE BIOSYNTHESIS IN TOBACCO CELL SUSPENSIONS OR CALLI

MYRIAM GAWER, NOÉMI GUERN, DAISY CHERVIN and PAUL MAZLIAK*

Laboratoire de Physiologie Cellulaire et Moléculaire, CNRS (UA 1180) Université P. et M. Curie, 4 place Jussieu 75252, Paris Cedex 05, France

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Key Word Index—*Nicotiana tabacum*; Solanaceae; cell and callus culture; choline-resistance; ATP:choline phosphotransferase; CTP:phosphocholine cytidylyltransferase; CDP-choline:DAG cholinephosphotransferase.

Abstract—Addition of choline to the culture medium of tobacco cells or calli of a wild cell line promoted an increase in the level of phosphatidylcholine (PC) as well as a rise in the phosphatidylcholine:phosphatidylethanolamine (PC/PE) molar ratio. Both of these events are inconsistent with cell or tissue survival. The *in vitro* activities of the enzymes involved in the synthesis of PC by the choline diphosphate (CDP) pathway, were examined in tobacco cells sensitive to (ChS line) or resistant to choline (ChR line), grown in suspension in a liquid medium or as calli on a solid medium. During the whole growth cycle, ChR cells or calli showed no changes in the enzymatic activities related to the CDP pathway, in contrast to ChS cells or calli which exhibited large changes. Alterations in the levels of these enzymatic activities mostly occurred at the onset of the subculture of ChS calli or cells transferred on an enriched choline medium. During the lag phase of ChS calli development, an abrupt drop in the *in vitro* activity of phosphocholine cytidylyltransferase (CT) occurs, which may be linked to a high level of the microsomal PC. Moreover during this phase an increase in the phosphotransferase activity (CPT) occurred in ChS calli, by contrast with a transient decrease of this activity found in suspension-cultured ChS cells. In choline-treated ChS cultures, an extended elevation of cholinekinase (CK) activity promoted a large entry of exogenous choline into ChS cells. By comparison with the behaviour of choline-sensitive cells, the choline-resistance might be correlated with low and stable levels of CK and CT activities associated with a high level of CPT activity both in tobacco cells or calli.

INTRODUCTION

It has been shown [1] that the changes in lipid content which accompanied the growth of suspensions of wild type tobacco cells in culture, could be altered by the addition of choline to the culture medium. Choline supplementation resulted in a transient increase in phosphatidylcholine (PC) content preceding cell death. However, addition of high concentrations of sucrose or phosphate may mask the deleterious effects of choline [2]. We have also observed that culture on a solid medium leads to a slow growth and reduced changes in the lipid content of calli as compared with suspension-cultured cells. Using the wild tobacco cell line, choline-sensitive (ChS), and a choline-resistant (ChR) cell line, isolated in our laboratory [1], we have tried to further characterize the activities of the enzymes involved in PC biosynthesis both in suspension-cultured cells and calli.

Three enzymes are involved in the synthesis of PC by the CDP-choline pathway (see Ref. [3] for a review): (1) ATP:choline kinase (CK, EC 2.7.1.32) which catalyses the production of choline phosphate (ATP + choline → choline phosphate + ADP); (2) CTP:phosphocholine cytidylyltransferase (cytidylyltransferase, CT, EC 2.7.7.15) which catalyses the production of CDP-choline (choline phosphate + CTP → CDP-choline + PPi); (3) CDP-choline:diacylglycerol cholinephosphotransferase (cholinephosphotransferase, CPT, EC 2.7.8.2) which allows the transfer of choline phosphate groups to diacylglycerols (CDP-choline + diacylglycerol → PC + CMP). These enzymes play a central role in sustaining cellular PC level. It has been stated that the rate of PC synthesis was modulated by changes in the amount of membrane-associated CT [3–5]. This binding of CT might depend on the lipid composition of membranes [6–10] and particularly on the intracellular PC:PE molar ratio. We have determined previously the *in vitro* activities of CK and CT from tobacco cell growing in suspension, the phospholipid composition of which had been altered by growth in the presence of high concentrations of choline [11, 12]. In the present study we have analysed changes in the *in vitro* CPT activity from ChS and ChR cells cultivated in a liquid medium in the presence or absence of choline, as well as the modifications of the *in vitro*

*Author to whom correspondence should be addressed.

activities of the three enzymes involved in PC biosynthesis induced by the culture on a solid medium. As a consequence of these studies, we expected to establish some correlation between choline-resistance and the activities of the enzymes involved in PC biosynthesis.

RESULTS

CK, CTP:phosphocholine CT and CDPcholine:DAG CPT in vitro activities in tobacco ChS and ChR suspension-cultured cells

The changes in the activities of the three enzymes involved in PC synthesis are depicted in Fig. 1. These data are expressed as ratios of the specific activity in subcellular fractions of each enzyme at the different stages of cell growth relative to the specific activity of the same enzyme extracted from the inoculum. The results concerning CK and CT activities have been discussed in great detail in earlier reports [11, 12] and are indicated here only for comparison. We have observed no change in the activity of CK (Fig. 1A) from the soluble fractions of ChS or ChR cells grown in suspension on their standard mediums. Addition of choline to the culture medium of ChS cells resulted in a large increase of CK activity during all phases of the culture period (Fig. 1A). The CT activity (Fig. 1B) from microsomal fractions of ChR cells remained stable during the whole growth cycle, whereas the CT activity of ChS cells cultivated in the absence or presence of choline abruptly peaked during the first 2–3 days of the subculture and later declined. In non-treated ChS cells, the decline of CT activity was significant enough to achieve a level lower than that observed in ChR cells. Nevertheless the CT activity remained high in choline-fed ChS cells.

The CPT activity from the microsomes of suspension-cultured ChS cells (Fig. 1C) changed during the growth cycle. After a decrease, weak but always detected by day 1, the CPT specific activity increased to 940 ± 150 pmol min^{-1} mg^{-1} protein (*ca* twice the value of the inoculum) at days 5–6, and gradually decreased during the stationary growth phase. Thus, the maximum stimulation of CPT activity occurred prior to the last doubling of the cell population, unlike that of CT activity which occurred much earlier. This increase in CPT activity was correlated with a high PC content in ChS cells (Fig. 2F). The CPT relative activity of microsomal fractions from choline-fed ChS cells exhibited a similar pattern as control ChS cells (Fig. 1C). However, the peak of CPT specific activity during the exponential growth phase reached 1440 ± 250 pmol min^{-1} mg^{-1} protein, (*ca* three-fold the value of inoculum) (Fig. 1C) and the decline of CPT in the stationary phase was less abrupt than in control ChS cells. In ChR cells, the microsomal CPT activity remained relatively unchanged throughout the growth cycle, with some fluctuations occurring by day 5 of the subculture (725 ± 110 pmol min^{-1} mg^{-1} protein in ChR culture versus 580 ± 90 in the inoculum). During the late stationary phase, the CPT specific activity did not significantly differ in ChR and control ChS cells, both reaching respectively *ca* 550 ± 80 and 545 ± 60 pmol min^{-1} mg^{-1} protein at day 14. Therefore, the presence of choline in the culture medium greatly enhanced the *in vitro* microsomal CPT activity of ChS suspension-cultured cells and did not influence that of ChR ones.

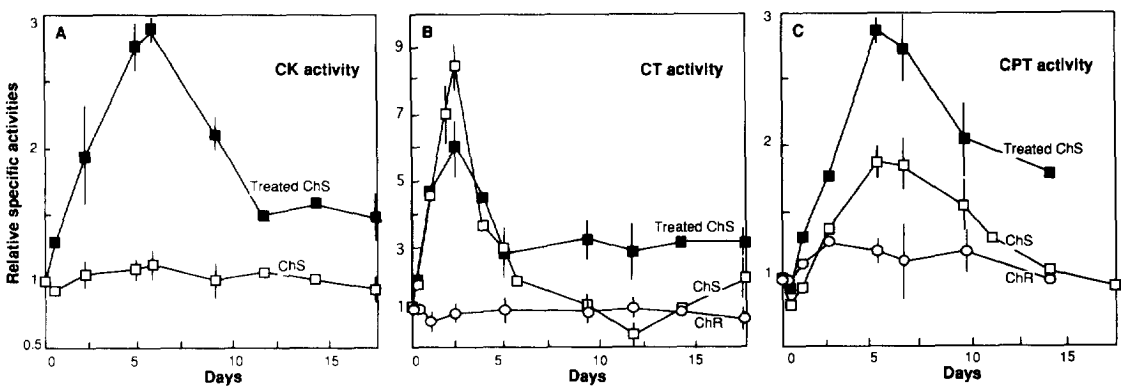

Fig. 1. Changes in the specific activities of CK (A), CT (B) and CPT (C) of extracts from tobacco cell suspensions cultivated in the absence or presence of choline. ChS cells were cultivated on their standard choline-free medium. Treated ChS or control ChR cells were cultivated on a 10 mM choline medium. The activities were assayed with soluble (CK) or microsomal (CT and CPT) fractions from cellular extracts as described in the Experimental section and expressed as the ratio of the specific activities estimated at various times of the culture to the specific activities of the inoculum at day 0. The absolute specific activities at day 0 for the three enzymes are the followings: 1.75 and 1.25 nmol P-choline formed min^{-1} mg^{-1} protein for CK in ChS and ChR cells, respectively; 6.3 and 31.8 nmol CDP-choline formed min^{-1} mg^{-1} protein for CT in ChS and ChR cells respectively; 0.495 and 0.580 nmol PC formed min^{-1} mg^{-1} protein for CPT in ChS and ChR cells, respectively. The relative CK activity profile corresponding to ChR cell suspension is not indicated because it closely follows that of the ChS control. Results are the means of three to five experiments run in duplicate or in triplicate. Some standard errors are indicated as vertical bars.

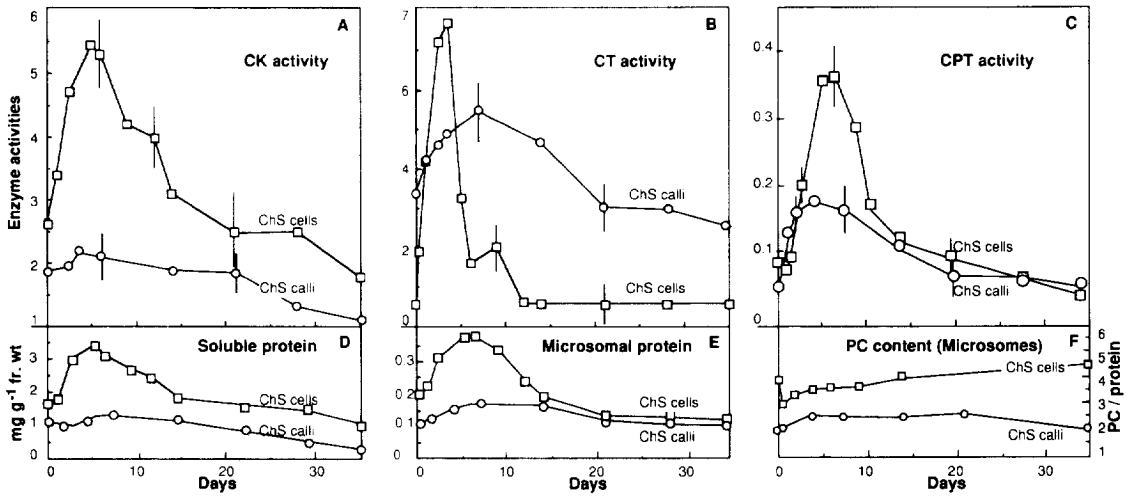

Fig. 2. Changes in the activities of CK (A), CT (B), CPT (C) and in the protein (D, E) and PC (F) contents of ChS suspension-cultured cells (ChS cells) as compared with those of ChS calli over a culture period. Culture on liquid or solid medium and assays conditions are described in the Experimental section and Fig. 1. The CK, CT or CPT activities are indicated as nmol of P-choline, CDP-choline or PC formed, $min^{-1} g^{-1}$ fr. wt, respectively. In (F), PC content is indicated as the ratio of PC content ($mg g^{-1}$ fr. wt) relative to microsomal protein content ($mg g^{-1}$ fr. wt). These reported ratios seem too high, probably because of a systematic under-estimation of the protein content. Results are the means of three experiments run in duplicate or in triplicate. Some S.E. are indicated as bars.

Comparison of the in vitro *activities of the enzymes involved in PC biosynthesis in tobacco wild (ChS) cell suspension and calli*

Figure 2(A–C) presents the overall profiles of the activities of CK, CT and CPT of tobacco suspension-cultured cells or calli during a growth cycle. These parameters are expressed as total activity per g of fresh mass in order to take account of the changes in the protein contents of soluble or microsomal cell fractions (Fig. 2D and E).

During a given growth cycle, the changes in CK activity, recovered in soluble fractions of suspension-cultured ChS cells or ChS calli (Fig. 2A), paralleled the changes in their respective soluble protein content (Fig. 2D). This fact reflected the stable value of the specific CK activities of soluble fractions in both types of culture. Moreover, the increase in soluble protein content in ChS suspension-cultured cells during the exponential growth phase resulted in a large increase in total enzymatic activity of cells, which was not observed in callus culture.

The acute peak of the *in vitro* microsomal CT activity which is partially cycloheximide independent [12] occurred by days 1–2.5 of the subculture of ChS cell suspensions and was not observed in ChS calli (Fig. 2B). However, with the exception of this early stimulation, suspension-cultured ChS cells exhibited a total CT activity lower than that of calli. To explain such discrepancies in the behaviour of both types of culture, we hypothesized that CT activity might be correlated with the level of PC content relative to microsomal protein: the lower the PC content, the higher would be the CT activity. In ChS cells, we observed the highest CT activity during the lag phase and during the subsequent first cell divisions (Fig. 2B). During this period, we observed a decreased PC content when expressed on a microsomal protein basis (Fig. 2F). The microsomal CT activity of calli extracts changed little as did the PC content. The CT activity in calli during the late exponential growth phase was greater than that of suspension-cultured cells during the same phase, while the PC content in calli was lower. The lack of an early increase in CT activity during growth of calli might be due to a high initial value of this activity in the inoculum, which was correlated with a low PC content relative to microsomal protein levels.

This hypothesis was confirmed by the following experiment with ChS suspension-cultured cells. We have observed that the presence of choline in the culture medium of ChS cells led to a relatively high CT activity level in the late growth phase (Fig. 1B). A 2.5 mM choline concentration did not alter the pattern of the first growth cycle of a given cell population (data not shown). An inoculum of 2.5 mM choline-fed ChS cell suspensions exhibited a greater CT activity than that of control ChS cell suspensions (Fig. 3, day 0). When this inoculum was transferred to choline-free medium, the growing cells did not exhibit the early CT stimulation, whereas the control culture did, and CT activity remained high in these pretreated cells throughout the growth cycle. This stable and high CT activity was concomitant with a lower PC content in the inoculum of choline-pretreated cells (2.35 $mg mg^{-1}$ microsomal protein versus 4.1 in the control). This value, 2.35, was close to that of the inoculum of ChS calli cultivated in the choline-free medium (Fig. 4). Given the above data, we assumed that changes in PC content might regulate the activity of the membrane-

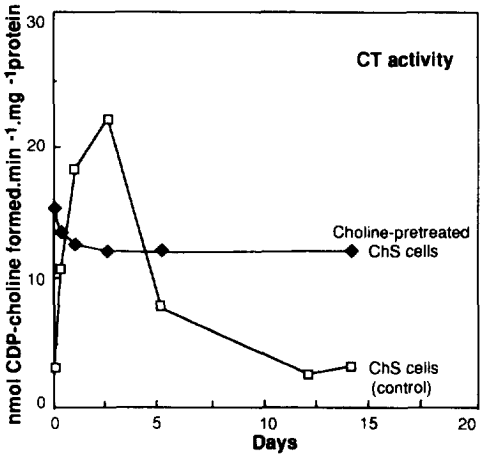

Fig. 3. Effect of a choline pretreatment on the change of CT activity from microsomal fractions of ChS suspension-cultured cells subcultured on a choline-free medium. The choline-pretreated suspensions had been grown during 14 days in a 2.5 mM choline-enriched medium and were then cultivated on a choline-free medium as control ChS cells. Results are the means of two experiments run in duplicate.

bound CT in ChS suspension-cultured cells or calli of ChS tobacco lines: a low and stable PC content in calli microsomes would correspond to a high and stable specific CT activity. The changes in total CT activity of ChS calli in Fig. 2B would thus be due to the changes in the microsomal protein content (Fig. 2E).

During the stationary growth phase of suspensions or during the late growth phase of calli, the total *in vitro* activities of CPT in both types of cultures followed similar patterns (Fig. 2C). By the first days of subculture corresponding to the exponential growth phase in suspension-cultured cells and to the lag phase and the start of the cell divisions in calli, an increase in total CPT activity occurred, more marked in suspension-cultured cells than in calli. Both increases paralleled the high levels of microsomal protein in tissue extracts. Thus, whereas the specific CPT activity in the microsomes of ChS calli was greater than that measured in the microsomes of suspension-cultured cells, the total CPT activity remained lower in calli because of the low level of microsomal protein. The specific activities expressed as pmol of PC formed min^{-1} mg^{-1} microsomal protein in callus or suspension-cultured cell extracts were, respectively, 1210 ± 190 versus 940 ± 110 for maximal values and 630 ± 90 versus 380 ± 80 for minimal values.

In conclusion, the changes in the *in vitro* activities of the enzymes of PC synthesis appeared different throughout the growth of calli or cell suspensions. The culture on a solid medium markedly reduced these changes. The most striking difference between the culture on a liquid or solid medium concerned the large peak in CT microsomal activity of suspension-cultured cells early in the exponential growth phase, a peak which was not detected in calli.

Effects of choline supplementation on the in vitro *activities of the enzymes of PC biosynthesis in tobacco ChS or ChR calli*

The PC:PE molar ratio is a good marker of the steady state of cellular membranes. Supply of choline to the solid culture medium led to an increase in PC content and in PC:PE molar ratio of ChS calli whereas in ChR calli, PC:PE molar ratio was not significantly distinct from that of ChS control [2]. On the other hand, the PC content (expressed on a microsomal protein basis) was high in choline-fed ChS calli over a growth period whereas it was low in late growth phase of ChR calli (Fig. 4).

Cell-free soluble extracts of ChR calli, routinely maintained on a choline-supplemented solid medium, displayed a specific CK activity stable over a growth cycle, as did ChS calli maintained on a standard choline-free medium (Fig. 5A). This CK activity was higher in ChS than in ChR calli. Moreover, addition of choline to the culture medium of ChS calli greatly stimulated this activity. Such a stimulation, previously observed in ChS suspension-cultured cells fed with choline, has been correlated with the high capability of ChS cells to accumulate choline [13].

The specific CT activity from the microsomal fractions of ChR calli was stable over a growth cycle, except for a small decline in the lag phase, and was lower than that of control ChS calli (Fig. 5B). In choline-fed ChS calli, the specific CT activity of microsomes strongly declined during the lag phase, and then recovered a mean value lower than that of the control calli cultivated in free-choline medium. This decrease corresponded to a high PC content relative to microsomal protein (Fig. 4).

Except for a 35% increase in the lag phase of the growth, the CPT activity of microsomal fractions from ChR calli was stable over a culture period (Fig. 5C). In control ChS calli, this activity was marked by an early

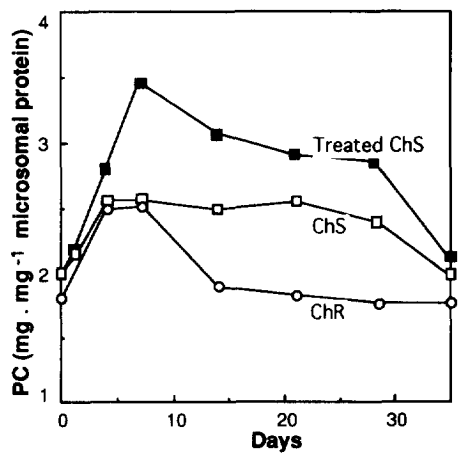

Fig. 4. Comparison of changes in PC contents of ChR or ChS tobacco calli cultivated in the presence or absence of choline. Data were expressed as the ratio of PC content ($mg\,g^{-1}$ fr. wt) relative to microsomal protein content ($mg\,g^{-1}$ fr. wt). See legend of Fig. 2 (F).

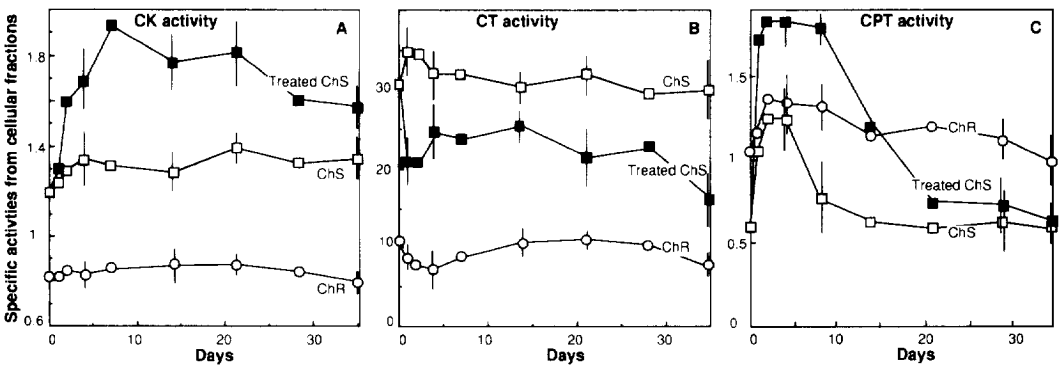

Fig. 5. Comparison of the specific activities of CK from soluble fractions (A) and CT (B) or CPT (C) from microsomal fractions of ChS or ChR tobacco calli. The calli were cultivated in the absence (ChS) or in the presence of 10 mM choline (Treated ChS and ChR). For fractionation and enzyme assays, see the Experimental section. The CK, CT or CPT activities are indicated as nmol of choline phosphate formed $min^{-1} mg^{-1}$ soluble protein, CDP-choline or PC formed $min^{-1} mg^{-1}$ microsomal protein, respectively. Results are the means of three to five experiments run in duplicate. Some S.E. are indicated as bars.

two-fold increase. Addition of choline to the culture medium of ChS calli emphasized and extended this increase. In late growth phase, the CPT activity levelled at a high value in ChR calli but recovered a low value both in control and choline-fed ChS calli.

In brief, addition of choline to the culture medium of tobacco calli of both lines had different effects on the *in vitro* activities of the enzymes of PC biosynthesis. It promoted a stimulation of choline kinase activity and a reduction of CT activity in ChS calli, whereas it maintained both enzymes at a low level in ChR calli. Moreover, the same choline supply kept the CPT activity at a high level in ChR calli and only promoted a transient increase in ChS calli. Thus, choline-resistant callus extracts might be characterized by a low CK and CT activities and high CPT activity compared with the corresponding activities in ChS calli.

DISCUSSION

In the presence of choline in the culture medium, suspension-cultured cells or calli from the ChS tobacco cell lines accumulated choline or its soluble derivatives which enhanced the osmolarity of cell fluids [13]. Moreover, the cells or callus changed the membrane PC:PE molar ratio [1, 2]. Because of these alterations, ChS cells did not survive for long in a choline-enriched medium. During a growth cycle, the profiles of PC contents expressed relatively to fresh mass, microsomal protein or total lipids were highly affected by the culture conditions [2]. All of these changes mainly originated from the changes in the *in vivo* activities of the enzymes involved in PC biosynthesis. Such *in vivo* enzymatic activities were difficult to consider for technical reasons. So, we rather attempted to correlate the *in vitro* enzyme activities with the *in vivo* changes in PC contents of suspension-cultured cells or calli. On the basis of the specific activities, our results indicated that CK and CT, the first two enzymes of the synthesis of PC via the CDP-choline pathway, were more active in ChS than in ChR cells or calli; the specific CPT activity was not significantly different in ChR and ChS isolated cells but was high in ChR calli during the log phase of growth. Addition of choline to the culture medium of ChS suspension-cultured cells or calli altered PC synthesis and disturbed the activities of the three enzymes by inducing a continuous stimulation of CK, a reduction of CT and a transient increase in CPT during the log phase of growth. The special behaviour of CT awarded to this enzyme a key role in the regulation of PC biosynthesis, as already suggested in animal cells [7, 14] and in plant cells [15, 16].

CK promoted the entry of choline in plant cells [13, 17–19]. In the presence of high choline concentrations, the reduced CK activity in ChR tobacco cells or calli contributed to limit the uptake of choline. In marked contrast, ChS cells or calli, cultivated in the presence of 10 mM choline, because of their stimulated CK activities, could not limit the entry of choline and were not able to regulate PC biosynthesis. A correlation between choline uptake and the regulation of PC synthesis has also been found in CPT deficient *Saccharomyces cerevisiae* [20, 21]. A feedback loop has been suggested to coordinate choline uptake with ongoing PC biosynthesis. On the other hand, PC is also a potent feedback inhibitor of its own biosynthesis by modulating the CT [7, 17]. It has been demonstrated that translocation of CT from the cytosol to the endoplasmic reticulum was required for the activation of the enzyme [22–24]. The binding of CT to the membrane is sensitive to the lipid environment [25–29]. Jamil and co-workers [10] have shown that the binding of CT is also sensitive to the ratio of bilayer to non-bilayer-forming lipids in membrane. PC is the major bilayer-forming lipid in the membranes of eucaryotes while unsaturated PE tends to favour an hexagonal H II phase [30, 31]. It has been suggested that a balanced presence of both bilayer and non-bilayer lipids is essential for membrane structure and function [32]. Thus, the

shifts observed in CT activity might reflect membrane lipid composition; the proportion of PC or the level of PC: PE molar ratio might modulate CT activity and PC synthesis as well [33]. Both of these parameters were changing in tobacco cultures. In ChS calli cultivated in standard conditions, PC concentration in the membrane and PC: PE ratio [2] were low, associated with a high CT activity. In marked contrast, in the presence of choline, ChS or ChR calli displayed an increase in PC content and in PC: PE ratio associated with a drop in CT activity during the lag phase. Likewise, in ChS cell suspensions cultivated in the absence of choline, the minimal CT activity occurred at days 9–11 when PC concentration was maximal. However, in ChR calli or suspension-cultured cells the low and stable CT activity was not apparently correlated with high PC contents or PC: PE ratio. Such a discrepancy might suggest that CT activity was submitted to additional regulatory mechanisms in ChR tobacco cell line.

Some studies have shown that diacylglycerols (DAG) might also regulate the biosynthesis of PC by two mechanisms: as a substrate for CPT reaction and as a mediator in the CT activation following its translocation [7, 34, 35]. Moreover, CPT activity was dependent on the culture conditions of plants [36, 37]. The specific CPT activity of tobacco cells and calli changed with culture conditions and age. These changes might result from changing amounts of protein or DAG available in the microsomal membranes. At the beginning of the subculture of calli from both lines, we observed an early increase in CPT specific activity which might result from the liberation of DAG following phospholipid degradation. As a matter of fact, several degradative processes occur during the lag phase of callus development, because of the cutting of inoculum explants. In suspension culture cells, the transfer of the stock-cell suspension to a new medium is not so injurious and no stimulation of CPT activity was observed during the lag growth phase. The maximal CPT activity occurred in the late exponential growth phase and might result from a greater amount of enzymatic protein in the microsomes of dividing cells.

In summary, let us consider the respective activities of the three enzymes involved in PC synthesis: firstly, in ChR suspension-cultured cells or calli; and secondly, in choline-treated ChS cultures. We suggest that a low CK activity promoted a limited uptake of choline in ChR cells, and the low level of CT activity—considered as the limiting factor of PC biosynthesis—would not allow the formation of a large amount of PC, in spite of a high *in vitro* CPT activity. In marked contrast, the high CK activity, induced in ChS tissues by the presence of choline in the culture medium, promoted the uptake of choline, an accumulation of phosphocholine inside the cells and a subsequent elevation of PC synthesis; this excess in PC content resulted from the greater activity of CT compared with that of ChR cells. However, we also noticed that the high PC content of choline-treated cells or calli might be correlated to a CPT activity continuously higher in ChS cells or transiently higher in ChS calli than in the corresponding ChR cultures. From these facts, we concluded that one key to the choline resistance of ChR cells probably lies in the limitation of the activities of the enzymes involved in PC biosynthesis via the CDP pathway, particularly in CK activity, leading to a steady level of PC content compatible with cell survival and similar to that of the wild cell type cultivated in the absence of choline.

EXPERIMENTAL

Culture conditions. Tobacco suspension-cultured cells and calli of both ChS or ChR lines were grown as described elsewhere [9, 12]. The routinely used choline concn was 10 mM except as indicated in the text. The ChR lines were routinely maintained on 10 mM choline medium without any injury. The cell suspensions and calli were, respectively, subcultured every 14 and 28 days and the culture was prolonged for some experiments. The lag phase was, respectively, $\leqslant$ 24 hr in cell suspensions and $\leqslant$ 4 days in calli. The exponential growth phase lasted, respectively, 7–9 days (cell suspensions) and 4–5 weeks (calli).

Tissue homogenization and enzyme assays. The activities of CK, CT and CPT have been studied in cell-free extracts. The cell suspensions or calli were harvested during the growth-cycle traverse, frozen in liquid N_2 and stored at $-70°$. Freezing and storage of the cell material up to 2 months did not significantly modify the measured initial rate of the enzyme activities.

After thawing of the material, the CK activity was studied in the 100 000 *g* supernatant of cell or calli homogenates as previously described [11] according to a modified method of Ref. [38]. The activity of CT was measured only in the microsomal fraction (100 000 *g* pellet) of the homogenates [12] according to the procedure outlined in Ref. [39].

Some precautions had to be taken for the study of the CPT from thawed material; this enzyme was labile and its activity reduced to 50% after 0.5 hr of the storage of the microsomal fractions in ice. So the initial rates were estimated on short term assays. The reaction proceeded linearly for 12 min and the activities increased proportionally to the amount of the microsomes up to 55 μg protein. The CPT activity was measured according to Ref. [37] using a reaction mixt. containing 50 mM Tris–HCl (pH 8), 10 mM $MgCl_2$, 1.25 mM dithiotreitol, 17.2 μM [^{14}C]CDP-choline (5.8 mCi mmol^{-1}) in a final vol. of 100 μl. The reactions, at 26°, were started with the addition of 20–35 μg protein (microsomal fraction) and terminated after appropriate time points by boiling for 2 min. The lipid fractions were extracted by the method of Ref. [40] in a solvent mixt. $CHCl_3$–MeOH–H_2O (1:1:1, v/v/v). The $CHCl_3$ phase (0.6 ml) containing the formed labelled PC was transferred to a scintillation vial, evaporated and quantitated by scintillation counting in Permafluor (Packard). The upper phase (1.2 ml), containing the unchanged [^{14}C]CDP-choline, was counted, after evaporation, in liquid scintillation Ready Solv. (Beckman). The transformation ratio allowed estimation of the enzyme activity.

Depending on the experiments, the data concerning enzyme activities were expressed either as relative activity (ratio of enzyme activity at different stages of the culture to the activity at time 0 of the subculture), or as sp. act. of soluble or microsomal fractions of extracts (nmol formed min^{-1} $protein^{-1}$) or as total activity of the tissues (nmol formed min^{-1} gfw^{-1}).

Protein and lipid determination. Protein was determined by the method of Ref. [41] with BSA as a standard. PC analysis was carried out as described in Ref. [11] and PC content was expressed on microsomal protein content.

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