**Old Dominion University**
**ODU Digital Commons**

Chemistry & Biochemistry Faculty Publications | Chemistry & Biochemistry

1996

# Modulation of Queuine Uptake and Incorporation into tRNA by Protein Kinase C and Protein Phosphatase

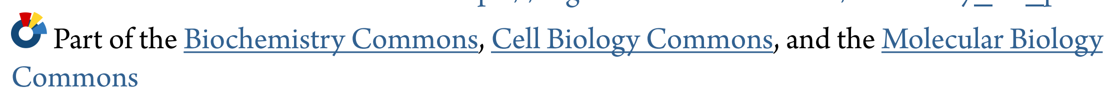
Rana C. Morris
*Old Dominion University*

Bonnie J. Brooks
*Old Dominion University*

K. Lenore Hart

Mark S. Elliot

Follow this and additional works at: https://digitalcommons.odu.edu/chemistry_fac_pubs


Part of the Biochemistry Commons, Cell Biology Commons, and the Molecular Biology Commons

## Repository Citation

Morris, Rana C.; Brooks, Bonnie J.; Hart, K. Lenore; and Elliot, Mark S., "Modulation of Queuine Uptake and Incorporation into tRNA by Protein Kinase C and Protein Phosphatase" (1996). *Chemistry & Biochemistry Faculty Publications*. 162.
https://digitalcommons.odu.edu/chemistry_fac_pubs/162

## Original Publication Citation

Morris, R. C., Brooks, B. J., Hart, K. L., & Elliott, M. S. (1996). Modulation of queuine uptake and incorporation into trna by protein kinase c and protein phosphatase. *Biochimica et Biophysica Acta, 1311*(2), 124-132. doi:10.1016/0167-4889(95)00184-0

This Article is brought to you for free and open access by the Chemistry & Biochemistry at ODU Digital Commons. It has been accepted for inclusion in Chemistry & Biochemistry Faculty Publications by an authorized administrator of ODU Digital Commons. For more information, please contact digitalcommons@odu.edu.

ELSEVIER

Biochimica et Biophysica Acta 1311 (1996) 124–132

BBA Biochimica et Biophysica Acta

# Modulation of queuine uptake and incorporation into tRNA by protein kinase C and protein phosphatase

Rana C. Morris, Bonnie J. Brooks [1], K. Lenore Hart [2], Mark S. Elliott *

*Department of Chemistry and Biochemistry, Old Dominion University, Norfolk, VA 23529, USA*

Received 1 September 1995; revised 31 October 1995; accepted 15 November 1995

**Abstract**

It has been suggested that the rate of queuine uptake into cultured human fibroblasts is controlled by phosphorylation levels within the cell. We show that the uptake of queuine is stimulated by activators of protein kinase C (PKC) and inhibitors of protein phosphatase; while inhibitors of PKC, and down-regulation of PKC by chronic exposure to phorbol esters inhibit the uptake of queuine into cultured human fibroblasts. Activators of cAMP- and cGMP-dependent kinases exert no effect on the uptake of queuine into fibroblast cell cultures. These studies suggest that PKC directly supports the activity of the queuine uptake mechanism, and that protein phosphatase activity in the cell acts to reverse this. Regardless of the modulation of uptake rate, the level of intracellular queuine base saturates in 6 h. However, there is still an effect on the incorporation rate of queuine into tRNA of fibroblast cultures even after 24 h. We now show that the incorporation of queuine into tRNA in cultured human fibroblasts by tRNA-guanine ribosyltransferase (TGRase) is also stimulated by activators of PKC and inhibitors of protein phosphatase; while inhibitors of PKC decrease the activity of this enzyme. These studies suggest that PKC supports both the cellular transport of queuine and the activity of TGRase in cultured human fibroblasts, and that protein phosphatase activity in fibroblasts acts to reverse this phenomenon. A kinase-phosphatase control system, that is common to controlling both intracellular signal transduction and many enzyme systems, appears to be controlling the availability of the queuine substrate and the mechanism for its incorporation into tRNA. Since hypomodification of transfer RNA with queuine is commonly observed in undifferentiated, rapidly growing and neoplastically transformed cells, phosphorylation of the queuine modification system may be a critical regulatory mechanism for the modification of tRNA and subsequent control of cell growth and differentiation.

*Keywords:* Queuine; Protein kinase C; Protein phosphatase

## 1. Introduction

There are a large number of modified nucleosides found in tRNA molecules. Various individual modifications have been suggested to play important roles in molecular biology. One example is the queuosine modification of tRNA. The pre-formed base, queuine, is incorporated into tRNA as an irreversible post-transcriptional exchange for guanine in the first position of the anticodon of four tRNA isoacceptors (Asp, Asn, His and Tyr) to generate the nucleoside queuosine. The unique base exchange reaction is catalyzed by the enzyme tRNA-guanine ribosyltransferase (TGRase) (EC 2.4.2.29) [1–4].

Research concerning the function of queuosine-modified tRNA indicates it may be a controlling agent in the differentiation, development, and stress management of several cell types. Alterations in the levels of queuosine-modified tRNA have been observed during differentiation and development for *D. discoideum* [5], plants [6], *Drosophila* [7–9], as well as development and aging in the rat [10]. Queuosine-modified tRNA in *Escherichia coli* protects the organism from stress induced by suboptimal growth conditions [11]. Queuosine-modified tRNA also is involved with lactate dehydrogenase and cytochrome *b*-559 expression in mammalian systems, and therefore may be involved with management of oxidative stress in eukaryotic cells [12–14]. The presence of the queuine base has been proposed to have effects on controlling protein synthesis [15], as well as aspects of protein phosphorylation

* Corresponding author. Fax: +1 (804) 6834628.

[1] Present address: Department of Internal Medicine, Spokane Internal Medicine, Spokane, WA 99220, USA.

[2] Present address: Reagents Division, American Medical Laboratories, Fairfax, VA 22032, USA.

0167-4889/96/$15.00 © 1996 Elsevier Science B.V. All rights reserved
*SSDI* 0167-4889(95)00184-0

[15], and regulation of cell signaling by receptor tyrosine kinases [16]. Therefore, the queuine base and queuosine-modified tRNA appear to exert important controlling effects on cellular physiology.

Transfer RNA isolated from neoplastic tissues and transformed cell lines is hypomodified with queuosine to various degrees [2,17,18]. The degree of hypomodification is related to the severity or malignancy of disease in human lymphomas, leukemias [19], lung cancers [20], and ovarian carcinomas [21]. Murine erythroleukemia cells are also typically queuosine-deficient. These cells can be forced to differentiate and have been shown to demonstrate a significant concurrent increase in tRNA queuosine levels [22,23]. The differentiation of these cells and the queuosine-level increase was blocked effectively by chronic treatment with the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [22]. These observations suggest that hypomodification of tRNA with queuosine is related to growth enhancing or tumor promoting events.

Chronic exposure of cultured normal human fibroblasts to phorbol-12,13-didecanoate (PDD) induces a large inhibition of the queuosine modification of tRNA [24]. The decrease in queuosine content of tRNA precedes an increase in the population density of the fibroblast cultures. Concurrent addition of purified queuine effectively blocks the population density increase in fibroblast cultures chronically exposed to PDD, and maintains the queuosine level in those cells at near normal levels. These data imply that the free queuine base or the queuosine modification of tRNA is related to a controlled growth rate in normal human fibroblasts.

Mammals are incapable of synthesizing their own queuine, and therefore must obtain it from their diet or gut flora [25,26]. Mammalian cells grown in culture obtain queuine from animal serum used to supplement their growth media [3,26]. This suggests that efficient cellular uptake of dietary queuine across the cell membrane is a prerequisite to the insertion of the base into tRNA by TGRase. It was demonstrated that cellular uptake of queuine may be sensitive to phorbol esters [27,28], and it was suggested that queuine uptake may be modulated by protein kinase C (PKC) [29]. Chronic exposure of cultured cells to phorbol esters has been shown to induce a proteolytic down-regulation of PKC and its activity [30]. Since we observed a decrease in the level of queuosine-modified tRNA with chronic exposure of human fibroblasts to PDD [28], it is proposed that the down-regulation of PKC activity levels in the cell cultures may be decreasing the activity of the mechanism for the formation of queuosine-modified tRNA.

Evidence presented in this manuscript demonstrates that a PKC-protein phosphatase system is important for maintenance of queuine uptake rates. The phosphorylation level of the queuine uptake mechanism in cultured human fibroblasts is proposed to modulate the baseline activity for queuine uptake into cultured normal human fibroblasts. However, the profound level of queuosine-hypomodification observed in tRNA after chronic exposure to PDD argues against substrate limitation as a sole mediating influence, since queuine still gets into the cell to a saturable level by 6 h, albeit at a modestly reduced rate. Our results suggest that a PKC-protein phosphatase system is also important for maintenance of the TGRase activity level in cultured cells. The level of phosphorylation of both the queuine uptake mechanism and the incorporation enzyme (TGRase) in cultured human fibroblasts modulates the baseline activity for the formation of the queuosine modification in the anticodon of four tRNAs.

## 2. Materials and methods

### 2.1. Cell culture

Cell cultures were established and maintained in neonatal calf serum supplemented minimum essential media (GIBCO, Grand Island NY) as previously described [24]. Queuine-deficient fibroblast cultures for the queuine incorporation studies were generated by growing the cells in media supplemented with 10% charcoal stripped calf serum [24], for at least three passages before the studies were initiated.

The addition of a radiolabelled analog of queuine, tri-tritiated dihydroqueuine ($rQT_3$), to cell cultures was employed to monitor cellular uptake of this base and its incorporation into tRNA. This agent was obtained as a gift from Dr. Ronald W. Trewyn (Kansas State University). The $rQT_3$ substrate was prepared by dissolving lyophilized $rQT_3$ in water to yield a stock concentration of 1 mM with a specific activity of 0.19 $\mu$Ci/$\mu$g and stored at $-20$°C.

cGMP- and cAMP-dependent kinase, and PKC activators were obtained from Sigma Chemicals (St. Louis, MO). The cGMP and cAMP analogues (dibutyryl and 8-bromo) were applied to cell cultures at a concentration of 10 $\mu$M. 12-tetradecanoyl phorbol-13-acetate (TPA) and phorbol-12,13-didecanoate (PDD) were used at concentrations of 20 nM. The PKC inhibitors sphingosine (used at 20 $\mu$M), staurosporine (used at 100 nM), and calphostin C (used at 100 nM) were purchased from Biomol Research Laboratories of Plymouth Meeting, PA. The PKC inhibitor H-7 (1-(5-isoquinoline sulfonyl)-2-methyl piperazine dihydrochloride) (utilized at 10 $\mu$M) was obtained from Seikagaku America (St. Petersburg, FL). The protein phosphatase inhibitors okadaic acid and calyculin A (utilized at varying nM concentrations) were purchased from Biomol Research Laboratories. All culture work was performed in sterile polystyrene plasticware from Corning.

### 2.2. Assay for $rQT_3$ uptake into cells

Fibroblasts were subcultured into 35-mm dishes at a density of $4 \times 10^4$ cells/ml in a final volume of 2 ml of media containing 10% serum. When the cells reached

confluence, the media was decanted and 1 ml of media supplemented with 10% calf serum and 100 nM $rQT_3$ (0.10 $\mu$Ci) was added to the cultures. Uptake of $rQT_3$ into untreated control cultures was compared to cultures treated with kinase or protein phosphatase modulating agents at concentrations as stated above. For short-term exposure studies, PKC and protein phosphatase inhibition agents were added to the culture at the same time as the $rQT_3$ substrate. For chronic exposure studies, the agents were introduced to the cell growth media and exposed to the cells for seven days prior to the introduction of $rQT_3$. Incubations for $rQT_3$ uptake analysis were conducted at 37°C for up to 4 h and terminated by rinsing the cell monolayer four times with 5 ml of ice-cold phosphate-buffered saline, followed by cell lysis with 1.0 ml of 95% ethanol for 5 min. The lysate was aspirated and radioactivity determined by liquid scintillation. The level of radioactivity in the cell lysate is reflective of $rQT_3$ uptake into the cultured cells.

### 2.3. Assay for $rQT_3$ incorporation into tRNA

Fibroblasts were subcultured into 35-mm dishes at a density of $4 \times 10^4$ cells/ml in a final volume of 2 ml of media containing 10% charcoal stripped calf serum. When the cells reached confluence, the media was decanted and 1 ml of media supplemented with 10% charcoal stripped calf serum and 100 nM $rQT_3$ (0.10 $\mu$Ci) was added to the cultures. Incorporation of $rQT_3$ into the acid precipitable fraction (tRNA) of control cultures was compared to cultures treated with kinase activators or inhibitors and protein phosphatase inhibitors. PKC and protein phosphatase inhibitors were added to the cultures at the same time as $rQT_3$ for short-term studies, or 7 days prior to the addition of $rQT_3$ for chronic exposure studies. Incubations for $rQT_3$ incorporation analysis were conducted at 37°C for timed intervals of up to 12 h. Incubations were terminated by rinsing the cell monolayer four times with 5 ml of ice-cold phosphate-buffered saline, followed by disruption of the cells with 0.5 ml of lysis buffer (10 mM Tris (pH 7.5), 0.01% SDS, 0.01% Triton X-100) for 10 min at room temperature. The lysate was transferred to a small test tube and treated with 0.25 ml of ice-cold 30% trichloroacetic acid (TCA). The lysate was placed on ice for 10 min and the resulting precipitate was collected by vacuum aspiration through GFA 2.4 cm glass fiber filter disks. Each disk was thoroughly rinsed with 40 ml of ice-cold 5% TCA and a final rinse of 5 ml ice-cold 95% ethanol. The filters were analyzed for bound radioactivity by liquid scintillation. The level of radioactivity on the filter disks was reflective of $rQT_3$ incorporation into the acid precipitable fraction (tRNA) from cultured fibroblast cells.

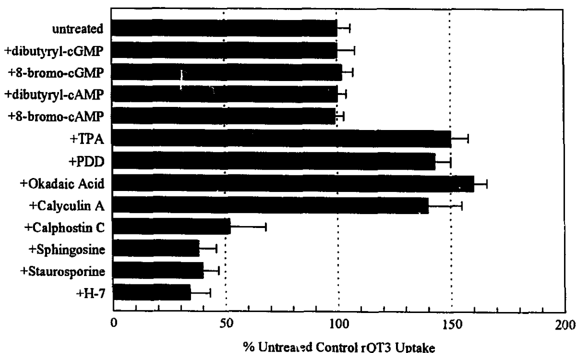



Fig. 1. The effect of protein kinase and protein phosphatase modulators on queuine uptake in human fibroblasts. The 'untreated' bar represents $rQT_3$ uptake in untreated control cultures, with experimental samples representing $rQT_3$ uptake in cultures exposed to various kinase and phosphatase modulators for 3 h. The cultures were treated with modulators at the following concentrations: 10 $\mu$M dibutyryl-cGMP, 8-bromo-cGMP, dibutyryl-cAMP and 8-bromo-CAMP, 20 nM TPA and PDD, and 10 nM okadaic acid, 1 nM calyculin A, 100 nM calphostin C, 20 $\mu$M sphingosine, 100 nM staurosporine and 10 $\mu$M H-7, respectively. The uptake of the radiolabelled queuine in all cultures ($4 \times 10^5$ cells) was measured after a 3-h incubation with 100 nM $rQT_3$ and modulators at 37°C. These values were normalized to number of pmol $rQT_3$ taken up by $10^5$ cells, and converted to percentage uptake with the control culture value ($5.5 \pm 0.3$ pmol/$10^5$ cells) serving as 100%. Deviation bars indicated represent the standard deviation with $n = 6$.

## 3. Results

### 3.1. Regulation of the queuine uptake mechanism

Due to the reported sensitivity of queuine uptake to phorbol esters [28,29], it was suggested that this system might be activated by PKC-catalyzed phosphorylation. In this study, several kinase and phosphatase modulators were used to identify the specific kinase and phosphatase families involved in the regulation of the queuosine modification system. Activators for cAMP-dependent protein kinase (butyryl-cAMP and 8-bromo-cAMP), cGMP-dependant protein kinase (butyryl-cGMP and 8-bromo-cGMP), and PKC (TPA and PDD) were employed to identify the participating kinase family. PKC activators (TPA and PDD) and inhibitors (calphostin C, sphingosine, staaurosporine, and H-7), as well as phosphatase inhibitors (okadaic acid and calyculin A) were studied to establish and verify phosphorylation as the control mechanism for queuine uptake.

Cultured human fibroblasts (passages 3–5) were exposed to these agents to discern influences on $rQT_3$ uptake after a 3-h incubation (Fig. 1). The agents that are reported to stimulate the cAMP- or cGMP-dependent kinase systems had no effect on the rate of $rQT_3$ uptake. However, stimulators of PKC increased the rate of $rQT_3$ uptake by 50% when compared to untreated controls. By contrast, cellular exposure to inhibitors of PKC all decreased the rate of $rQT_3$ uptake. Additional fibroblast cultures were exposed to the protein phosphatase inhibitors in order to maintain higher protein phosphorylation levels within the cell. These cultures showed a similar increase in queuine

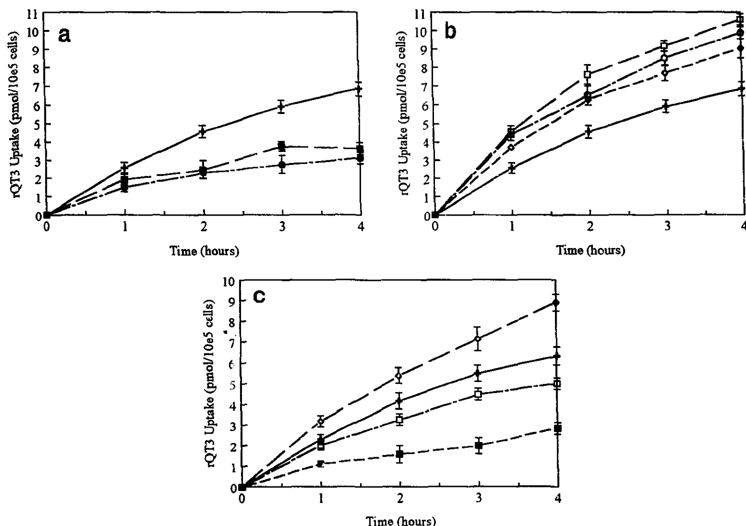



Fig. 2. The effect of protein kinase C and protein phosphatase modulators on queuine uptake in short-term and chronic exposure studies in human fibroblasts. Graph 2a displays the uptake of $rQT_3$ during short-term exposure of cultures to protein kinase C inhibitors. The top line (+) represents $rQT_3$ uptake in untreated control cultures, with the lower two lines representing cultures treated with 100 nM staurosporine (■), and 100 nM calphostin C (●), respectively. Graph 2b illustrates the uptake of $rQT_3$ during short-term exposure of cultures to protein kinase C activators and protein phosphatase inhibitors. The bottom line (+) represents an $rQT_3$ uptake in untreated control cultures, with the upper lines representing cultures treated with 10 nM okadaic acid (◇), 1 nM calyculin A (○), and 20 nM TPA (□), respectively. In these short-term exposure studies, modulators were added to cultures at time 0. Graph 2c shows the uptake of $rQT_3$ during chronic exposure of cultures to the modulators. The traces represent $rQT_3$ uptake in untreated control cultures (+), and those pre-exposed for 7 days to 20 nMTPA (□), 1 nM okadaic acid (◇), and 10 nM staurosporine (■). The uptake of the radiolabelled queuine in all cultures was measured after a 37°C incubation with 100 nM $rQT_3$ at 0, 1, 2, 3 and 4 h. These values were then normalized to number of pmol $rQT_3$ taken up by $10^5$ cells. Deviation bars indicated represent the standard deviation with $n = 4$.

uptake when compared to those treated with PKC activators, further suggesting that phosphorylation is involved in the regulation of queuine transport.

Parallel time course studies of $rQT_3$ uptake with short-term treatment to staurosporine, calphostin C, TPA, calyculin A, and okadaic acid were performed and compared to the $rQT_3$ uptake rate in control cells at hourly intervals over 4 h. Exposure to the PKC inhibitors resulted in a decrease in the uptake rate for $rQT_3$ as compared to untreated control fibroblast cultures (Fig. 2a). The PKC activator (TPA) nearly doubled the rate of $rQT_3$ uptake, while the protein phosphatase inhibitors also stimulated the $rQT_3$ uptake rate (Fig. 2b). This further indicates that queuine uptake efficiency is linked to protein phosphorylation levels.

Fibroblast cultures were pre-treated for 7 days with TPA, staurosporine, and okadaic acid in order to measure effects of long-term exposure to these modulators on queuine uptake (Fig. 2c). This is particularly revealing since chronic exposure to TPA results in proteolytic down-regulation of PKC activity [30]. As expected, exposures to the PKC inhibitor significantly decreased the uptake of queuine at 3 h of exposure to $rQT_3$, while treatment with low levels of the phosphatase inhibitor induced a two-fold increase in $rQT_3$ uptake. Furthermore, chronic exposure to TPA resulted in an inhibition of $rQT_3$ uptake rate when compared with untreated control cultures. This is a reversal of the effect observed in Fig. 2b with short-term exposure to TPA, and supports the PKC modulation scheme for queuine uptake based on the idiosyncrasy of differential effects on PKC with short-term vs. chronic exposure to phorbol esters.

The relatively high concentrations of the protein phosphatase inhibitors used in the short-term exposure study was cause for concern given their reported toxicity levels [31–33]. A range of concentrations of calyculin A and okadaic acid were studied to analyze their effects on the 3-h time point for $rQT_3$ uptake relative to untreated control fibroblast cultures (Fig. 3). These studies were performed for both short-term (simultaneous exposure to inhibitor and $rQT_3$) and for chronic exposure to the inhibitors (pre-treatment: 7 days prior to addition of $rQT_3$).

Concentrations of okadaic acid in excess of 100 nM proved to be cytotoxic for the 3-h short-term exposure studies. However, increasing concentrations of okadaic acid from 5 to 100 nM demonstrated progressively increas-

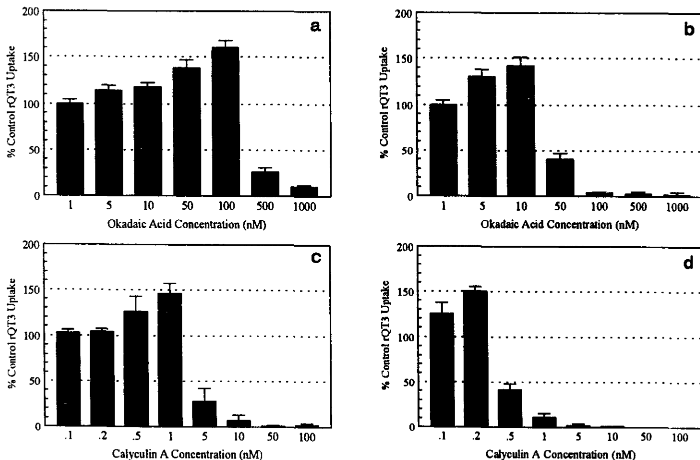



Fig. 3. The effect on queuine uptake of short-term and chronic exposure to protein phosphatase inhibitors in human fibroblasts. These bar graphs represent the effects of exposure of varying concentrations of okadaic acid (graphs a and b) and calyculin A (graphs c and d) on $rQT_3$ uptake in cultures. Short-term exposure studies (graphs a and c) were conducted with exposure of cells to the modulator at time 0. Chronic exposure studies (graphs b and d) were conducted with pre-treatment of cultures for 7 days prior to time 0. The uptake of the radiolabelled queuine in all cultures ($4 \times 10^5$ cells) was measured after a 3-h incubation with 100 nM $rQT_3$ at 37°C. These values were normalized to number of pmol $rQT_3$ taken up by $10^5$ cells, and converted to percentage uptake with the control culture value ($5.5 \pm 0.3$ pmol/$10^5$ cells) serving as 100%. Deviation bars indicated represent the standard deviation with $n = 6$.

ing $rQT_3$ uptake (Fig. 3a). Similar trends were observed in studies of chronic exposure to this phosphatase inhibitor (Fig. 3b). Increasing concentrations of okadaic acid from 1 to 10 nM demonstrated progressively increasing $rQT_3$ uptake rates, while concentrations over 10 nM were cytotoxic to the fibroblast cultures. Comparable results were seen with studies using the protein phosphatase inhibitor calyculin A. However, the fibroblast cultures were much more sensitive to this compound. Exposure for 3 h to 1 nM calyculin A effectively increased the rate of $rQT_3$ uptake (Fig. 3c). For chronic exposure (7 day pre-treatment), 0.2 nM calyculin A demonstrated equivalent maximal induction of $rQT_3$ uptake rate (Fig. 3d). Concentrations above these levels were cytotoxic in both short-term and chronic exposure studies, respectively.

It has been reported that calyculin A is a more effective inhibitor of protein phosphatase I than okadaic acid. In one study, $IC_{50}$ values of approx. 50 nM for okadaic acid and 0.3 nM for calyculin A were reported for muscle phosphatase I [31]. Based on the reported sensitivity of protein phosphatases type I and II to okadaic acid and calyculin A with pre-treatment exposure [31–33], our data suggest that protein phosphatases of the type I group are participating in the modulation of $rQT_3$ uptake in human fibroblasts.

### 3.2. Regulation of the queuine incorporation mechanism

Although the rate of uptake can be modulated, it was observed that maximal uptake of the queuine base to a saturating level occurs in 6 h (unpublished data). After this point, uptake and efflux are balanced [27]. Thus, the modulation of the uptake mechanism might not be solely responsible for the dramatic queuosine-modification deficiency seen in neoplastic transformation and progression of cancer cells to metastasis. Thus, the queuine incorporation mechanism (TGRase) was studied to determine if PKC acts in a dual role in modulating both components of the queuine modification system.

First, cultured human fibroblasts (passages 3–5) were exposed to dibutyryl and 8-bromo analogs of cAMP and cGMP, as well as TPA, PDD, calphostin C, sphingosine, staurosporine, H-7, okadaic acid or calyculin A for up to 12 h in order to determine effects on the incorporation of $rQT_3$ into tRNA by TGRase (Fig. 4). The queuine incorpo-

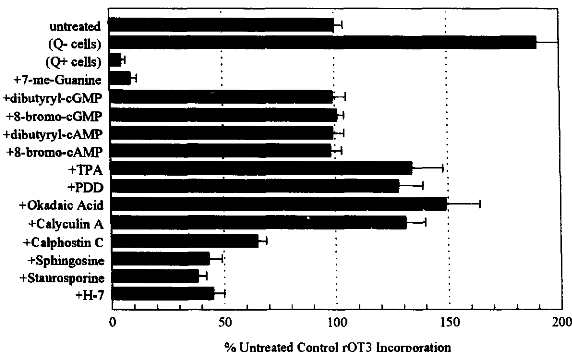



Fig. 4. The effect of protein kinase and protein phosphatase modulators on queuine incorporation into tRNA in human fibroblasts. The top four bars represent $rQT_3$ incorporation into the acid-precipitable fraction (tRNA) of control cultures. The 'untreated' bar represents $rQT_3$ incorporation in untreated 'normal' cultures and is, therefore, used as a point of reference for 100% $rQT_3$ incorporation. The 'Q -cells' bar represents $rQT_3$ incorporation in queuine-deficient cells that were preincubated with charcoal-stripped media for three passages. Therefore, this represents the maximal limit for the incorporation of $rQT_3$. The 'Q + cells' bar represents $rQT_3$ incorporation in queuine-saturated cells that were preincubated with normal media plus an additional 0.10 $A_{260}$ queuine for three passages. Thus, this sets the minimal limit for incorporation. The last control bar represents $rQT_3$ incorporation in cells treated with 10 $\mu$M 7-methylguanine, a competitive inhibitor for the incorporation enzyme TGRase. This also helps to verify the minimal level of incorporation activity in this study. Remaining bars represent $rQT_3$ incorporation into the tRNA of cultures exposed to various kinase and phosphatase modulators for 8 h at concentrations of 10 $\mu$M for dibutyryl-cGMP, 8-bromo-cGMP, 10 $\mu$M dibutyryl-cAMP and 8-bromo-cAMP, 20 nM for TPA and PDD, and 10 nM okadaic acid, 0.2 nM calyculin A, 100 nM calphostin C, 20 $\mu$M sphingosine, 100 nM staurosporine and 10 $\mu$M H-7, respectively. The incorporation of radiolabelled queuine into tRNA in all cultures ($4 \times 10^5$ cells) was measured after a 37°C incubation with 100 nM $rQT_3$ and modulators at 8 h. These values were normalized to number of pmol $rQT_3$ incorporated into the acid-precipitable fraction of $10^5$ cells. The 'untreated' bar represents $rQT_3$ incorporation in untreated 'normal' cultures (measured at $3.0 \pm 0.2$ pmol/$10^5$ cells), and was used as the basis for the conversion of experimental cultures' values into percentage. Deviation bars indicated represent the standard deviation with $n = 6$.

ration rates of cells exposed to these modulators were compared to an untreated control culture and three additional control (positive and negative) cultures. These additional control fibroblast cultures were utilized to establish both the maximum and minimum incorporation limits for comparison to the experimental sample.

Fibroblasts deficient in queuosine-modified tRNA were established in queuine-deficient media supplemented with charcoal-stripped serum. Cells grown under these conditions for three passages are completely deficient in queuosine-modified tRNA (unpublished data). Queuosine-deficient tRNA cultures were used as substrate targets for demonstrating the maximal incorporation rate of $rQT_3$ into tRNA by TGRase (positive control). These cells demonstrated a nearly two-fold increase in $rQT_3$ incorporation rate when compared to normal serum-supplemented controls. The slower rate of incorporation in serum-supplemented control cells is likely due to partial queuosine-modification of the existing tRNA population in the cell. Two negative control cultures were grown in 10% serum-supplemented media. One culture was treated with additional 100 nM queuine and the other was treated with 10 μM 7-methylguanine. Addition of excess queuine completely and irreversibly saturates the cell's tRNA with queuosine and eliminates the cell's tRNA as a substrate for $rQT_3$ incorporation. 7-Methylguanine is a competitive inhibitor of TGRase and blocks the ability of TGRase to incorporate $rQT_3$ into tRNA at concentrations used here [34]. These two cultures both represent the background levels of non-specific $rQT_3$ association to the acid precipitable fraction of solubilized cell cultures, which is expected with the absence of TGRase activity.

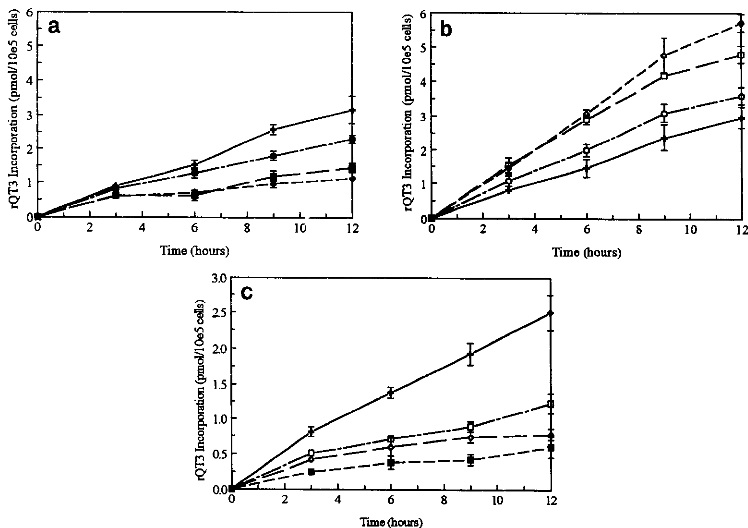



Fig. 5. The effect of protein kinase C and protein phosphatase modulators on queuine incorporation into tRNA in human fibroblasts. Graph 5a) displays the incorporation of $rQT_3$ into tRNA during short-term exposure of cultures to protein kinase C inhibitors. The top line (+) represents incorporation of $rQT_3$ into acid-precipitable fractions of untreated control cultures; the lower lines represent values for cultures treated with 100 nM calphostin C (●), 100 nM staurosporine (■), and 20 μM sphingosine (♦), respectively. Graph 5b illustrates the incorporation of $rQT_3$ into tRNA during short-term exposure of cultures to protein kinase C activators and protein phosphatase inhibitors. The bottom line (+) represents incorporation of $rQT_3$ into tRNA of untreated control cultures; the upper lines represent cultures treated with 10 nM okadaic acid (◇), 20 nM TPA (□), and 0.2 nM calyculin A (○), respectively. In these short-term exposure studies, modulators were added to cultures at time 0. Graph 5c shows the incorporation of $rQT_3$ into tRNA during chronic exposure of cultures to the modulators. The traces represent the rate of $rQT_3$ incorporation into the tRNA of untreated control cultures (+) and those pre-treated for 7 days to 20 nM TPA (□), 10 nM okadaic acid (◇), and 100 nM staurosporine (■). The incorporation of radiolabelled queuine into tRNA in all cultures ($4 \times 10^5$ cells) was measured after a 37°C incubation with 100 nM $rQT_3$ at 0, 3, 6, 9 and 12 h. These values were normalized to number of pmol $rQT_3$ incorporated into the acid-precipitable fraction of $10^5$ cells. Deviation bars indicated represent the standard deviation with $n = 4$.

Cell cultures treated with agents that increase the activity of cAMP- and cGMP-dependent kinases appear to have no effect on the incorporation rate of $rQT_3$ (Fig. 4). However, PKC activators and phosphatase inhibitors increase the incorporation of $rQT_3$ toward that observed in totally queuosine-deficient cells. While cells treated with inhibitors of PKC reduce the incorporation of $rQT_3$ to a baseline level just above that of non-specific binding. This further demonstrates that PKC-catalyzed phosphorylation is important in regulation of TGRase activity, thus the formation of the queuosine modification of tRNA.

Next, a 12-h time course for $rQT_3$ incorporation into tRNA of queuosine-deficient tRNA fibroblast cultures was performed in cells grown for three passages in 10% charcoal-stripped serum (queuine-free) supplemented media (Fig. 5a,b). In short-term exposure studies, $rQT_3$ and various modulating agents (staurosporine, sphingosine, calphostin C, TPA, okadaic acid and calyculin A) were simultaneously added to parallel cultures. Then, these were analyzed for $rQT_3$ incorporation into tRNA at 4-h intervals for 12 h. Inhibitors of PKC decreased the rate of $rQT_3$ incorporation into tRNA to a baseline level as compared to untreated controls (Fig. 5a). The protein kinase activator and phosphatase inhibitor, when added to cell cultures at the same time as $rQT_3$, both increased the $rQT_3$ incorporation rates when compared to untreated control cells (Fig. 5b). Queuosine-deficient cells pre-treated with various agents for 7 days were studied for $rQT_3$ incorporation rates into tRNA over a 12-h time course (Fig. 5c). Chronic exposure to TPA, which induces a proteolytic down-regulation of PKC [30], resulted in a decrease in $rQT_3$ incorporation into tRNA. However, treatment with staurosporine was more effective at inhibiting the rate of $rQT_3$ incorporation in cell cultures. It is both possible and likely that residual PKC activity may still be present in the cells chronically exposed to TPA.

Chronic pre-treatment of these cells for 7 days with okadaic acid showed $rQT_3$ incorporation levels lower than that of the control. Since this treatment maintains the phosphorylation level in the cell, the activity of TGRase should be enhanced during this preparative step. TGRase would effectively recycle queuine from tRNA turnover and incorporate it into new transcripts of tRNA. At the time of the $rQT_3$ addition, the cognate tRNA would be saturated with the queuosine modification and would no longer be used as a substrate for incoming $rQT_3$ due to the irreversible nature of the queuine incorporation reaction. Therefore, chronic exposure to okadaic acid should actually decrease the rate of $rQT_3$ incorporation into tRNA as compared to control cultures, and this is indeed the case (Fig. 5c).

## 4. Discussion

The uptake of queuine into human fibroblasts appears to be modulated by phosphorylation. Activators of cAMP and cGMP dependent kinases have no effect on $rQT_3$ uptake. However, increasing PKC activity with phorbol ester treatment elevates the rate of $rQT_3$ uptake. Conversely, chronic exposure of TPA to fibroblast cultures, which is known to down-regulate PKC activity, decreases $rQT_3$ uptake. A spectrum of protein kinase inhibitors, with various specificities to PKC, all inhibit $rQT_3$ uptake into fibroblast cultures in concentration ranges reported to be most specific for PKC. These results make a strong case for the role of PKC in enhancing the uptake rate of queuine into fibroblast cultures.

The inhibition of protein phosphatases by both okadaic acid and calyculin A also induces an increase in the uptake of $rQT_3$. These results indicate that maintenance of protein phosphorylation is important to maximize the rate of queuine uptake. Due to the concentrations of the two phosphatase inhibitors involved, it is likely that protein phosphatases of type I are down-regulating queuine uptake activity by removing a phosphate group from the queuine-specific membrane transporter. This analysis is based on reported sensitivity of protein phosphatases type I and II to okadaic acid and calyculin A [31–33].

TGRase activity in cultured human fibroblasts also appears to be modulated by PKC and protein phosphatase activity. Activators of cAMP- and cGMP-dependent kinases have no effect on $rQT_3$ incorporation. However, activators of PKC and inhibitors of protein phosphatase both increase TGRase incorporation rates above a baseline incorporation level, while inhibitors of PKC decrease incorporation rates of $rQT_3$ to the baseline level. Chronic exposure to TPA, which is known to induce a proteolytic down-regulation of PKC, also decreases $rQT_3$ incorporation into fibroblast tRNA when compared to untreated controls. These results make a strong case for the role of PKC in enhancing the incorporation rate of queuine into tRNA in fibroblast cultures. Furthermore, supporting evidence recently published by this laboratory demonstrates direct phosphorylation and activation of purified rat liver TGRase by PKC in vitro [35]. This indicates that control of TGRase activity by phosphorylation may be a universal mammalian phenomenon.

We propose that PKC and protein phosphatase are important modulators of both queuine uptake and incorporation into tRNA in cultured human fibroblasts. These are likely to be independently regulated sites for PKC due to (1) the ability of the uptake mechanism to fully saturate the cell with queuine in 6 h despite down-regulation by dephosphorylation, (2) the observation of the modulation of TGRase activity up to 24 h, and (3) the in vitro evidence that shows direct phosphorylation and regulation of rat liver TGRase activity by PKC [35].

PKC activity stimulates queuine uptake and incorporation rates, while competing phosphatase activity reduces these to a baseline level. Kinase-phosphatase control systems are important components of the cell's signal transduction system, and are known to be critical components

involved with differentiation, control of cell growth and neoplastic transformation. This work ties an important signal transduction network to mechanisms responsible for tRNA modification with queuine. Alterations in the signal transduction system can effect both the availability of queuine base and the activity of TGRase which can potentially lead to the queuosine-hypomodification of tRNA commonly observed in neoplastically transformed and undifferentiated cells.

## Acknowledgements

The authors thank the National Institutes of Health, National Cancer Institute for financial support toward the research presented in this manuscript (NIH/NCI 1 R29 CA45213); Dr. Ronald W. Trewyn for the generous supply of radiolabelled queuine analog, $rQT_3$, required to analyze queuine modification in cultured cells; Dr. James Yuan and Dr. Werner Langgut for their insightful critical analysis and suggestions regarding this manuscript; our husbands and wives for tolerating our ridiculous hours in performing this research; and Speedy's Tacos for moral and caloric support.

## References

[1] Harada, F. and Nishimura, S. (1972) Biochemistry 11, 301–308.
[2] Nishimura, S. (1983) Prog. Nucleic Acids Res. Mol. Biol. 28, 49–80.
[3] Katze, J.R. and Farkas, W.R. (1979) Proc. Natl. Acad. Sci. USA 76, 3217–3275.
[4] Shindo-Okada, N., Okada, N., Ohgi, T., Goto, T. and Nishimura, S. (1980) Biochemistry 19, 345–400.
[5] Schachner, E. and Kersten, H. (1984) J. Gen. Microbiol. 130, 135–144.
[6] Beier, H., Zech, U., Zubrod, E. and Kersten, H. (1987) Plant Mol. Biol. 8, 345–353.
[7] White, B.N., Tener, G.M., Holden, J. and Suzuki, D.T. (1973) J. Mol. Biol. 74, 635–651.
[8] Owenby, R.K., Stulberg, M.P. and Jacobsen, K.B. (1979) Mech. Aging and Develop. 11, 91–103.
[9] Farkas, W.R. and Jacobsen, K.B. (1980) Insect Biochem. 10, 183–188.
[10] Singhal, R.P., Kopper, R.A., Nishimura, S. and Shindo-Okada, N. (1981) Biochem. Biophys. Res. Commun. 99, 120–126.
[11] Noguchi, S., Nishimura, Y., Hirota, Y. and Nishimura, S. (1982) J. Biol. Chem. 257, 6544–6550.
[12] Reisser, T., Eicher, A. and Langgut, W. (1993) Biochem. Biophys. Res. Commun. 197, 1319–1325.
[13] Reisser, T., Langgut, W. and Kersten, H. (1994) Eur. J. Biochem. 221, 979–986.
[14] Schachner, E., Aschhoff, H.-J. and Kersten, H. (1984) Eur. J. Biochem. 139, 481–487.
[15] Mahr, U., Bohm, P. and Kersten, H. (1990) BioFactors 2, 185–192.
[16] Langgut, W. (1995) Biochem. Biophys. Res. Commun. 207, 306–311.
[17] Katze, J.R., Beck, W.T., Cheng, C.S. and McCloskey, J.A. (1983) Recent Results in Cancer Res. 84, 140–159.
[18] Okada, N., Shindo-Okada, N., Sato, S., Itoh, Y.H., Oda, K.I. and Nishimura, S. (1978) Proc. Natl. Acad. Sci. USA 75, 4247–4251.
[19] Emmerich, B., Zubrod, E., Weber, H., Maubach, P.A., Kersten, H. and Kersten, W. (1985) Cancer Res. 45, 4308–4314.
[20] Huang, B.-S., Wu, R.-T. and Chien, K.-Y. (1992) Cancer Res. 52, 4696–4700.
[21] Baranowski, W., Dirheimer, G., Jakowicki, J.A. and Keith, G. (1994) Cancer Res. 54, 4468–4471.
[22] Shindo-Okada, N., Terada, M. and Nishimura, S. (1981) Eur. J. Biochem. 115, 423–428.
[23] Chen, Y.-L. and Wu, R.-T. (1994) Cancer Res. 54, 2192–2198.
[24] Elliott, M.S., Katze, J.R. and Trewyn, R.W. (1984) Cancer Res. 44, 3215–3219.
[25] Farkas, W.R. (1979) J. Biol. Chem. 255, 6832–6835.
[26] Reyniers, J.P., Pleasants, J.R., Wostman, B.S., Katze, J.R. and Farkas, W.R. (1981) J. Biol. Chem. 256, 11591–11594.
[27] Elliott, M.S., Trewyn, R.W. and Katze, J.R. (1985) Cancer Res. 45, 1079–1085.
[28] Elliott, M.S. and Katze, J.R. (1986) J. Biol. Chem. 261, 13019–13025.
[29] Elliott, M.S. and Crane, D.L. (1990) Biochem. Biophys. Res. Commun. 171, 393–400.
[30] Parker, P.J. and Millrich, A. (1987) J. Cell Phys. 5, 53–56.
[31] Ishihara, H., Martin, B.L., Brautigan, D.L., Karaki H., Ozaki, H., Kato, Y., Fusetani, N., Watabe, S., Hashimoto, D., Ueura, D. and Hartshorne, D.J. (1989) Biochem. Biophys. Res. Comm. 159, 871–877.
[32] Hardie, D.G., Haystead, I.A.J. and Sim, A.T.R. (1991) Methods in Enzymol. 201, 469–476.
[33] Cohen, P. (1991) Methods in Enzymol. 201, 389–398.
[34] Elliott, M.S. and Trewyn, R.W. (1982) Biochem. Biophys. Res. Commun. 104, 326–332.
[35] Morris, R.C., Brooks, B.J., Eriotou, P., Sagar, S., Kelly, D.F., Hart, K.L. and Elliott, M.S. (1995) Nucleic Acids Res. 23, 2492–2498.