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PROTEIN KINASE C MODULATION OF QUEUINE UPTAKE IN CULTURED HUMAN FIBROBLASTS

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Protein kinase C modulates the activity of a highly specific uptake mechanism for queuine in cultured human fibroblasts. Activators of protein kinase C induce an increased uptake rate for the radiolabeled analog of queuine, rQT_3. The protein kinase C inhibitors, H-7 ,staurosporine and sphingosine all induced a dramatic decrease in the uptake rate of rQT_3. This suggests that protein kinase C is tied to efficient cellular uptake of queuine. Uptake is prerequisite to the modification of transfer RNA with queuine. Perturbation of queuine-modified transfer RNA levels has been associated with neoplastic transformation, differentiation and growth control.

The queuosine modification of mammalian transfer RNA (tRNA) occurs post-transcriptionally as an exchange of queuine for guanine in the first position of the anticodon in tRNA isoacceptors accommodating aspartic acid, asparagine, histidine and tyrosine (1-4). This reaction is irreversibly catalyzed by tRNA-guanine ribosyltransferase (3,4). Mammals are incapable of synthesizing their own queuine, and therefore must obtain it from their diet or gut flora (5,6). Mammalian cells grown in culture obtain queuine from animal sera used to supplement the growth media (3,7).

Transfer RNA isolated from neoplastic tissues is hypomodified to various degrees with respect to queuosine (2,8,9). Furthermore, the degree of hypomodification has been associated with the severity of disease in leukemias and lymphomas (10). The biochemical lesions leading to queuine-hypomodification of tRNA may occur at the level of dietary availability of queuine, cellular transport rates, enzymatic insertion of queuine into tRNA, tRNA turnover, or activity of a specific queuine salvage pathway (8).

Chronic exposure of phorbol ester tumor promoters to cultured human fibroblasts, demonstrates a transient inhibition of queuosine modification in tRNA (11). This accompanies morphological effects, where a decrease in queuosine content of tRNA always precedes an

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increase in cell population density in culture (11). The addition of exogenous purified queuine concurrently with phorbol ester exposure, blocked the tumor promoter induced increase in population density and maintained the tRNA in a queuosine modified form.

Phorbol-12,13-didecanoate (PDD) was unable to directly inhibit a partially purified preparation of tRNA-guanine ribosyltransferase in vitro (12). Since phorbol esters demonstrate non-specific interaction with membranes and specific interaction with protein kinase C (13), studies were initiated to analyze PDD effects on queuine uptake into cultured human fibroblasts. Uptake studies were performed with a radiolabeled analog of queuine, rQT_3, which demonstrated the same affinity for the uptake mechanism as queuine (12). Uptake of rQT_3 exhibits biphasic kinetics with low and high affinity components, demonstrating Kms of 350 nM and 30 nM respectively. It was shown that PDD, and a structurally unrelated stimulator of protein kinase C, teleocidin, were both capable of inducing modest inhibition of the high affinity component of rQT_3 uptake. However, inhibition was observed in relatively few early passage human fibroblast cultures, and this inhibition was tied to the endogenous expression of a protein factor, believed to be interferon (14, 15). In general, most of the early passage human fibroblast cultures demonstrated an increase in rQT_3 uptake rates in the presence of PDD. Here we discuss the effects of positive and negative modulators of protein kinase C activity on queuine (rQT_3) uptake in cultured human fibroblasts.

METHODS AND MATERIALS

Materials: Cell cultures were established and maintained in neonatal calf serum supplemented minimum essential media (GIBCO, Grand Island NY). Media was further supplemented with 25 mM HEPES, sodium pyruvate, non-essential amino acids, vitamins, penicillin-streptomycin, sodium bicarbonate, and the additional amino acids; asparagine, aspartic acid, histidine, tyrosine and phenylalanine as previously described (12,14). The radiolabeled reduced analog of queuine, tritiated dihydroqueuine (rQT_3), was a gift from Dr. Jon R. Katze (University of Tennessee, Memphis). The compound was prepared from queuine by catalytic reduction and exchange performed by Amersham (Arlington Heights, IL). Lyophilized rQT_3 was dissolved in water to yield a stock concentration of 1 mM with a specific activity of 0.19 uCi/ug. The queuine analog was stored as a 200 x stock solution at -20'C. Phorbol-12,13-didecanoate (PDD) was obtained from Sigma Chemical Company (St.Louis, MO) and dissolved to a 1 mM stock solution in acetone. All solutions containing PDD were wrapped in foil to protect them from light and then stored at 4'C. The protein kinase C modulators, diolein, phosphatidylserine, 1,2-dicapryloyl-rac-glycerol, and staurosporine were purchased from Sigma Chemical Company. Sphingosine was purchased from Biomol Research Laboratories of Plymouth Meeting PA. The calcium

ionophore, A23187 was a generous gift from Dr. Michael Beatrice (Old Dominion University). The protein kinase C antagonist, H-7 (1-[5-isoquinoline sulfonyl]-2-methyl piperazine dihydrochloride) was obtained from Seikagaku America, Inc. (St. Petersburg, FL). All culture work was performed in sterile polystyrene plasticware from Corning.

Methods: Human skin cell cultures were established from neonatal foreskins (Sentara Norfolk General Hospital) using the method of Riegner et al. (12,14,16),which results in a predominately fibroblast culture. Cultures were maintained in 25 cm^2 flasks with 5 ml of media supplemented with 10% calf serum. Confluent cell cultures were trypsinized and passaged at a 1 to 4 ratio into new flasks.

For assay of rQT_3 uptake, fibroblasts were subcultured into 35 mm dishes at a density of $4x10^4$ cells/ml in a final volume of 2ml of media with 10% serum. When the cells reached confluency, the media was decanted and 1 ml of media supplemented with 10% calf serum and 100 nM rQT_3 was added to the cultures. Uptake of rQT_3 into untreated control cultures was compared to cultures treated with protein kinase C modulating agents at various concentrations. Incubations were terminated by rinsing the cell sheet four times with ice-cold phosphate buffered saline, and then the cells were lysed with 1.0 ml of 95% ethanol for five minutes. The lysate was aspirated and radioactivity determined by liquid scintillation.

RESULTS

In the course of these studies twelve separate primary cultures were established from individual foreskin samples. These cultures were serially subcultured through four passages. Uptake of rQT_3 was assessed at passages two, three and four for many of these cultures. Passage dependent variation in uptake levels was not observed in any of these cultures. Culture to culture variation in rQT_3 uptake levels in untreated cells was noted but considered to be minimal.

Treatment of cell cultures with 10 nM PDD induced an increase in rQT_3 uptake in all cultures analyzed. There was a culture to culture variation in sensitivity to PDD, ranging from increases of less than 10% to more than 40% above control rQT_3 uptake levels. Variability of sensitivity was attributed to genetic variation between individual primary cultures. Since it had been reported that protein kinase C is the specific receptor of phorbol ester tumor promoters (13), we attempted to mimic PDD behavior with other stimulators of protein kinase C activity. Treatment of early passage fibroblasts cultures with 0.2 mg/ml diolein, 0.06 mg/ml dicapryloyl glycerol,0.05 mg/ml phosphatidylserine and 0.5 uM of the calcium ionophore A23187, increased rQT_3 uptake to a greater extent than PDD. Again some culture to culture variation was observed, with a range of stimulation between 30% to 80% above untreated control values. Figure 1 is representative of a typical

experiment involving stimulation of rQT_3 uptake in cultured fibroblasts using both 10 nM PDD and the protein kinase C activators; diolein, diacylglycerol, phosphatidylserine and calcium (through A23187).

Various growth factors are believed to affect cell function by mobilization of a series of second messengers through interaction with a specific cell surface receptor. Growth factor specific activation of phospholipases induces the release of diacylglycerol and phosphatidylinositol (which mobilizes calcium). These agents activate protein kinase C; therefore, growth factors should induce an increase in rQT_3 uptake in cultured fibroblasts. Figure 2 demonstrates that 10 ng/ml of the growth factors; platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF), each induce an increase in rQT_3 uptake. Furthermore the level of increase is consistent

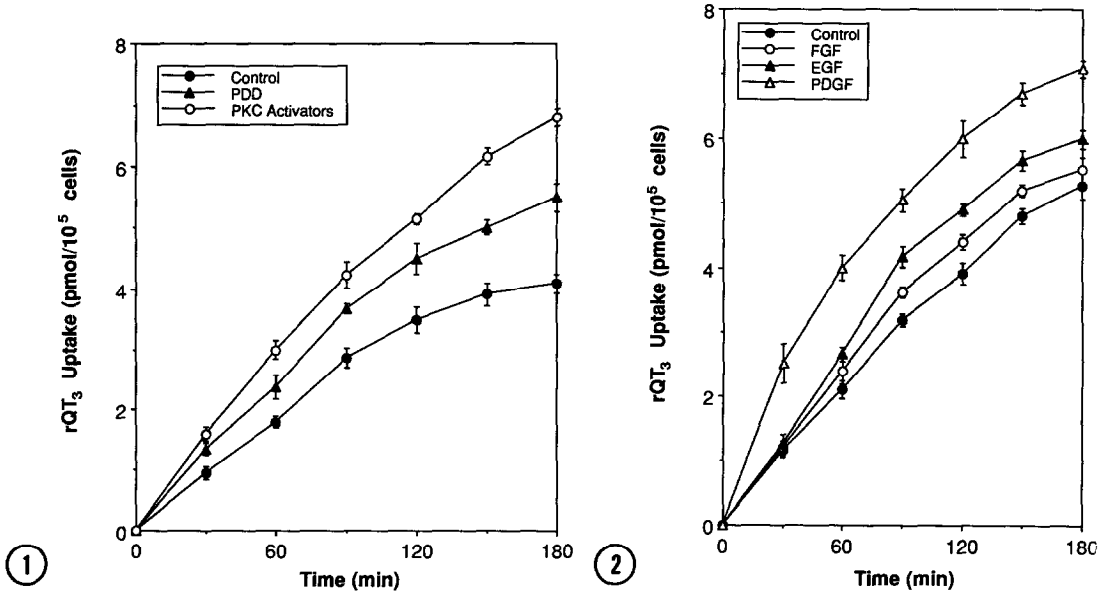
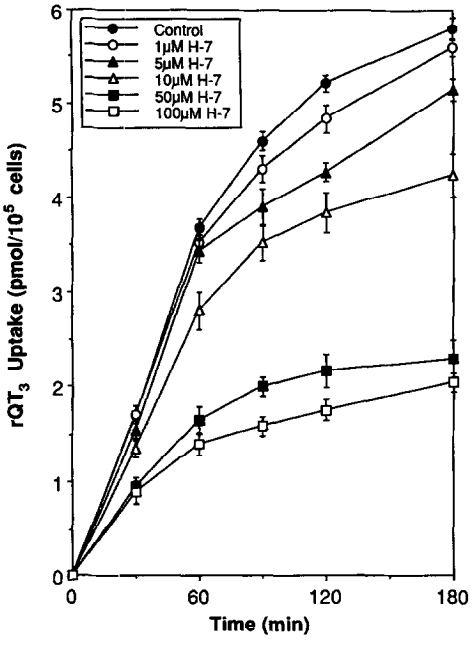


Figure 1. Effect of protein kinase C stimulation on rQT_3 uptake. Confluent 35mm dishes of human fibroblast cell cultures were treated with 1 ml of serum supplemented media containing 100 nM rQT_3 (●), and further supplemented with 10 nM PDD (▲), or 0.2 mg/ml diolein, 0.06 mg/ml dicapryloyl-glycerol, 0.05 mg/ml phosphatidylserine and 0.5 uM calcium ionophore A23187 (○). All points represent the mean of triplicate assays from duplicate experiments.

Figure 2. The effects of PDGF, EGF, and FGF on rQT_3 uptake. Confluent 35mm dishes of human fibroblast cell cultures were treated with 1ml of serum supplemented media containing 100 nM rQT_3 (●), and further supplemented with 10 ng/ml PDGF (△), 10 ng/ml EGF (▲), or 10 ng/ml FGF (○). All points represent the mean of triplicate assays from duplicate experiments.

with the level of uptake observed with PDD or diacylglycerol and calcium ionophore treatment.

The protein kinase C inhibitor, H-7, was added to fibroblast cultures and induced significant concentration dependent inhibition of rQT_3 uptake (Figure 3). Maximal inhibition of uptake was observed at 100 uM H-7. The protein kinase C inhibitor, sphingosine, also demonstrated concentration dependent inhibition of rQT_3 uptake (Figure 4), with maximal inhibition observed with 100 uM sphingosine. Staurosporine also induced concentration dependent inhibition of rQT_3 uptake into treated cell cultures with 100 nM demonstrating maximal inhibition (Figure 5). Staurosporine required overnight exposure to the cell culture prior to the uptake experiment to generate optimal inhibition. H-7 and sphingosine required no pre-incubation to illicit inhibitory effects on rQT_3



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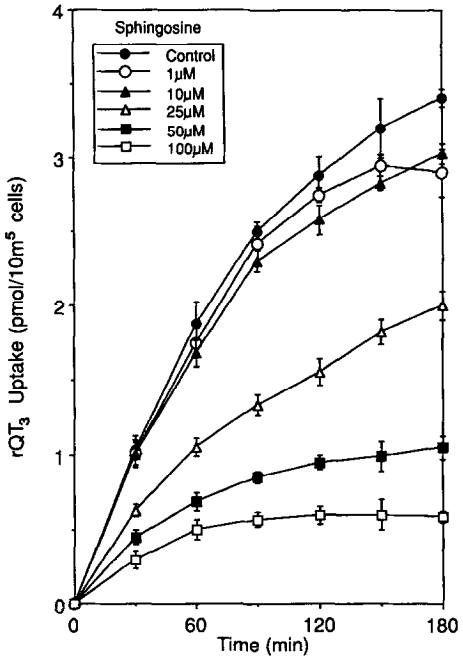

(4)

Figure 3. Effect of H-7 concentration on induction of rQT_3 uptake inhibition. Confluent 35mm dishes of human fibroblast cell cultures were treated with 1 ml of serum supplemented media containing 100 nM rQT_3 (●), and further supplemented with 1 uM (○), 5 uM (▲), 10 uM (△), 50 uM (■) or 100 uM (□) H-7. The mean of triplicate assays from duplicate experiments is represented at each point.

Figure 4. Effect of sphingosine concentration on induction of rQT_3 uptake inhibition. Confluent 35mm dishes of human fibroblast cell cultures were treated with 1 ml of serum supplemented media containing 100 nM rQT_3 (●), and further supplemented with 1 uM (○), 10 uM (▲), 25 uM (△), 50 uM (■),or 100 uM (□) sphingosine. The mean of triplicate assays from duplicate experiments is represented at each point.

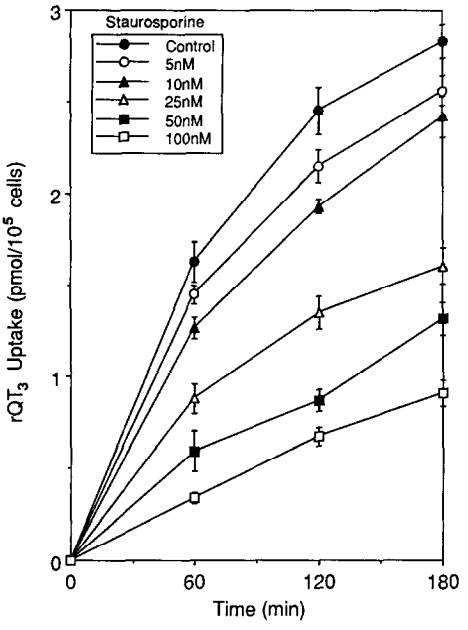

Figure 5. Effect of staurosporine concentration on induction of rQT_3 uptake inhibition. Confluent 35mm dishes of human fibroblast cell cultures were treated with 1 ml of serum supplemented media containing 100 nM rQT_3 (●), and further supplemented with 5 nM (○), 10 nM (▲), 25 nM (△), 50 nM (■), or 100 nM (□) staurosporine. Cells were pre-incubated with staurosporine containing media overnight (except control cultures) prior to the addition of fresh media containing rQT_3 and fresh staurosporine. The mean of triplicate assays is represented at each point.

uptake. Simultaneous treatment of the cultures with 10 nM PDD did not effect the level of inhibition exerted by H-7, staurosporine, or sphingosine.

DISCUSSION

Activation of protein kinase C in cultured human fibroblasts was achieved by addition of diolein, dicapryloyl-glycerol, phosphatidylserine and the ionophore A23187. Treatment with these protein kinase C agonists induced stimulation of rQT_3 uptake to 155% (+/- 25% , n=10) of control uptake levels. Treatment of fibroblast cultures with 10 nM PDD enhanced uptake to 120% (+/- 10%, n=10) of untreated control levels. Our data also suggests that growth factors (PDGF, EGF, and FGF) all tend to stimulate rQT_3 uptake to various extents. It has been shown that a number of growth factors, by interacting with their respective receptors, can mobilize intracellular calcium and release transiently elevated levels of diacylglycerol (17). Therefore, the growth factor results may also be representative of a protein kinase C modulated

stimulatory event exerted on the queuine uptake mechanism. Analogous stimulation of a glucose transport system by protein kinase C has been documented previously (18,19).

Inhibition of protein kinase C was examined with H-7 , an agent that inactivates protein kinase C by interacting directly with the active site of the enzyme (20,21). H-7 (50 uM) depressed uptake of rQT_3 to 40% (+/- 5% , n=3) of untreated control levels. Sphingosine (50 uM), a protein kinase C specific inhibitor (22,23,24), inhibited rQT_3 uptake to an equivalent level, 35% (+/- 5%, n=3) of untreated control cultures. An additional inhibitor of protein kinase C , staurosporine (24,25), was also efficient at inhibiting rQT_3 uptake. 100 nM staurosporine inhibited rQT_3 to 30% (+/- 7%) of control levels. Our data suggest that there is a low basal rate of queuine uptake across a cell membrane, and that a major portion of the queuine uptake efficiency is regulated by the activity of protein kinase C.

Chronic exposure of PDD to human fibroblasts in culture has been shown to induce transient hypomodification of tRNA with respect to queuosine (11). Studies have also shown that chronic exposure of cells to phorbol esters results in the down regulation of protein kinase C activity (26). We suggest that a portion of the hypomodification of tRNA observed after chronic exposure to PDD, could have been due to a reduced efficiency of the queuine uptake mechanism by down regulating the positive influence of protein kinase C. This would result in a lower cytosolic concentration of queuine and less efficient modification of tRNA.

It is now evident that modulation of protein kinase C activity is closely tied to the efficiency of queuine uptake in cultured human fibroblasts. Events leading to the down regulation of protein kinase C may have a profound effect on the overall queuine modification of tRNA. Queuine-hypomodified tRNA has been closely associated with the neoplasia, and is proposed to be a component of the events involved with tumor promotion. By associating protein kinase C to queuine modification of tRNA, we may be a step closer to explaining tumor promotion at the molecular level of nucleic acids.

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