Compartmentalization of proline pools and apparent rates of collagen and non-collagen protein synthesis in arterial smooth muscle cells in culture

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Rates of collagen and non-collagen protein synthesis in rabbit arterial smooth muscle cells (SMC) were determined by using the specific (radio)activity of [^3H]proline in the extracellular, intracellular, and prolyl-tRNA pools. The intracellular free proline specific activity was only 25% of the extracellular value in cultures incubated for 12 h in 0.25 mM-proline. The specific activity of prolyl-tRNA was less than 10% of the extracellular specific activity. Increasing the extracellular proline concentration 10-fold (to 2.5 mM), while keeping the extracellular specific activity of proline constant, resulted in equilibration of the specific activities of intracellular and extracellular free proline, but the specific activity of prolyl-tRNA remained at less than 10% of the extracellular specific activity. Therefore, calculated rates of collagen and non-collagen protein synthesis were greatly underestimated using the intracellular or extracellular specific activity of proline. SMC were also incubated with 0.1 mM-[^{14}C]ornithine in 0.25 mM or 2.5 mM non-labelled proline to examine synthesis *de novo* of proline and prolyl-tRNA from ornithine. In SMC cultures containing 0.25 mM unlabelled proline, the specific activity of intracellular ornithine was approx. 45% of the extracellular specific activity, due to the production of unlabelled ornithine. The specific activity of ornithine-derived intracellular free proline in SMC incubated with 2.5 mM-proline was significantly lower than in SMC incubated in 0.25 mM-proline, due to the influx of unlabelled proline. However, a corresponding difference in the specific activity of [^{14}C]prolyl-tRNA between SMC in 0.25 mM- or 2.5 mM-proline was not observed. Ornithine-derived [^{14}C]proline was incorporated into proteins in a manner different from that of exogenously added radiolabelled proline. A much higher proportion of the proline synthesized *de novo* was channelled into collagen synthesis relative to total protein synthesis. Together, these results show that intracellular proline pools are highly compartmentalized in arterial SMC. They also suggest that proline synthesized from ornithine may enter a prolyl-tRNA pool separate from that of proline entering from the extracellular medium.

INTRODUCTION

We are interested in identifying factors which influence the rate of arterial smooth muscle cell (SMC) collagen synthesis and the subsequent accumulation of collagen in the developing atherosclerotic plaque. Many of the studies rely primarily on quantifying protein synthesis in SMC cultures. Radioactively labelled proline has been used extensively by many investigators to determine collagen synthesis, primarily because of its co-translational conversion to hydroxyproline which constitutes a relatively high proportion of the total amino acid composition of collagen. Apparent rates of collagen and non-collagen protein synthesis calculated from incorporation of labelled proline or any other amino acid depend on the accuracy in determining the specific radioactivity of the amino acid in the appropriate precursor pool. It cannot be assumed that the specific activity of prolyl-tRNA, the immediate precursor form of proline which is incorporated into newly synthesized protein(s), is identical with that of intracellular or extracellular free proline [1–5]. The specific activity in the

intracellular proline pool(s) could be decreased from that in the extracellular pool by dilution with unlabelled proline. Sources of unlabelled proline include synthesis *de novo* from ornithine [6–8] or glutamate [9,10] as well as from protein degradation [11,12]. Calculated rates of collagen and non-collagen protein synthesis employing the specific activity of extracellular proline may considerably underestimate the actual rates of synthesis [1–5]. Therefore, prior to utilizing SMC cultures for identification of factors which modulate collagen production, the present study was undertaken to compare the relative specific activities of proline in the extracellular, intracellular and prolyl-tRNA pools in cultures of rabbit arterial SMC incubated in the presence of labelled proline. The corresponding apparent rates of net collagen and non-collagen protein synthesis were calculated from the specific activities of proline in each of these three pools.

The influence of changes in extracellular proline concentration on the specific radioactivities of the intracellular free proline and prolyl-tRNA pools was also determined. In these experiments the extracellular

Abbreviations used: SMC, smooth muscle cells; PBS, phosphate-buffered saline.

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proline concentration was raised to 10-fold that in the plasma of fasted rabbits. A concomitant increase in labelled proline kept the specific activities of extracellular proline in these two incubation media identical.

Additional studies were undertaken to ascertain whether arterial SMC process endogenously synthesized proline differently from exogenous free proline. SMC were incubated with radiolabelled ornithine to determine if the proline synthesized from ornithine and aminoacylated onto tRNA enters a pool homogeneous with the pool containing prolyl-tRNA derived from labelled extracellular proline. Relative proportions of collagen and non-collagen proteins synthesized from the exogenously and endogenously labelled proline were also compared.

EXPERIMENTAL

Cell culture

Primary cultures of aortic SMC were derived from explants of the medial portion of thoracic aortas removed from New Zealand White rabbits weighing approx. 2.0 kg, as previously described [13]. Cells were grown in culture medium consisting of equal portions of Dulbecco-Vogt modified Eagle's medium with high glucose and Ham's F12 (DV/F12) supplemented with sodium pyruvate (1 mM), L-glutamine (4 mM), NaHCO₃ (0.24%) and 10% (v/v) fetal calf serum. Incubations were carried out at 37 °C in humidified air containing 5% CO₂. After the SMC reached confluency they were washed with a solution containing 0.15 M-NaCl and 0.01 M-phosphate, pH 7.5, (PBS) and freed from the substratum by incubation with 0.125% trypsin in PBS for 10 min at 37 °C. Cells were reseeded at 10⁴ cells/cm² in 75 cm² culture flasks. Growth medium was changed twice weekly, and cells for experimentation were from the second subculture. Media and supplemental components were obtained from Gibco (Grand Island, NY, U.S.A.) and tissue culture plates were from Costar (Cambridge, MA, U.S.A.).

Subcultured SMC were seeded at 10⁴ cells/cm² in 8 cm² wells. Confluent layers of cells were washed with PBS and then preincubated for 3 h in DV/F12 culture medium containing 10% (v/v) fetal calf serum, 50 µg of ascorbate/ml, 0.1 mM-ornithine and the same concentration of proline subsequently used in the incubation. Cells were then incubated for 12 h with either 0.25 mM-proline containing 25 µCi of L-[5-³H]proline/ml (13.6 Ci/mmol; New England Nuclear) or 2.5 mM-proline with 250 µCi of L-[5-³H]proline/ml. In addition, SMC were also incubated for 12 h in the above medium containing 0.1 mM-ornithine with 2.0 µCi of L-[1-¹⁴C]ornithine/ml (55.9 mCi/mmol; New England Nuclear) and either 0.25 mM- or 2.5 mM-proline with no added [³H]proline.

Collagen and non-collagen protein synthesis rates were determined by using the specific activities of extracellular and intracellular free proline and prolyl-tRNA as precursor pools. Differences between experimental groups were determined by Student's *t* test.

Specific activities of extracellular and intracellular free proline and ornithine

Extracellular and intracellular proline or ornithine specific activities were determined from the incubation

medium and washed cell layer of each SMC culture. The specific activity of proline in the non-conditioned medium was also measured. The extracellular media were removed and a solution of cold trichloroacetic acid was added to a final concentration of 5% (w/v). After 1 h at 4 °C, the suspension was centrifuged at 5000 *g* for 10 min to collect the precipitated extracellular proteins. The supernatant was removed and passed through a column containing a mixed cation and anion exchange resin (AG 11A8; Bio-Rad Laboratories) to desalt and remove trichloroacetic acid [14]. An aliquot of this effluent was taken for subsequent determination of the specific activity of either proline or ornithine in the extracellular media by reverse-phase h.p.l.c.

The cell layer was washed five times with PBS to remove residual extracellular free proline and then scraped off the plastic substratum in the presence of PBS. Trichloroacetic acid was added to a final concentration of 5% to precipitate the cell layer proteins from the suspension. After centrifugation at 5000 *g* for 10 min, the supernatant was removed and passed over an AG 11A8 column. As with the media supernatants, the subsequent quantification of intracellular free proline or ornithine was performed on aliquots of the effluent by h.p.l.c. analysis.

Protein precipitates of the extracellular media and cell layers were washed seven times with 5% (w/v) trichloroacetic acid followed by a final wash with cold (4 °C) 95% ethanol to remove the trichloroacetic acid. The protein precipitates were hydrolysed in 6 M-HCl in tightly sealed screw-capped tubes under N₂ for 24 h at 120 °C in preparation for amino acid analysis. Samples were analysed by h.p.l.c. as described below or applied to cation-exchange columns (AG 50W-X8) for the separation of labelled proline and hydroxyproline as previously described [15].

Specific activity of proline in the aminoacyl-tRNA fraction

Proline bound to tRNA was isolated by a method modified from Airhart *et al.* [1]. After removing the incubation medium the cells were rinsed five times with ice-cold 0.165 M-sodium acetate buffer, pH 5.0, to remove residual free proline. Washed cells were lysed with 1 ml of 1% (w/v) SDS in 0.05 M-Mes at pH 6.0. The lysates were transferred to separate tubes and an equal volume of 88% phenol in the 1% SDS buffer solution was added. After shaking for 1 h and centrifugation at 10000 *g* for 15 min at 4 °C, the upper aqueous phase was carefully aspirated. The separation was repeated three times with additions of the 1% SDS buffer solution to the phenol phase. The aqueous phases were pooled and sodium acetate buffer, pH 6.5, was added to a final concentration of 0.1 M along with the subsequent addition of 2.5 vol. of 95% ethanol at -20 °C. After 24 h at -20 °C, the precipitated nucleic acids were collected by centrifugation at 10000 *g* for 15 min, washed three times with 95% ethanol and suspended in 150 µl of water. The suspension was adjusted to pH 9.0 by addition of NaOH and the deacylation of the amino acids from tRNA was allowed to continue for 1 h at 37 °C. The pH was then adjusted with 0.5 M-HCl to 2.0 and the solution was centrifuged at 10000 *g* for 15 min to remove the precipitated nucleic acids. The supernatant was recovered for amino acid analysis by h.p.l.c. and eventual determination of proline specific activity in prolyl-tRNA.

Amino acid analyses by h.p.l.c.

Amino acid analyses were performed on protein hydrolysates and free amino acids from the extracellular and intracellular pools. The h.p.l.c. PICO-TAG system [16] developed by Henrikson & Meredith [17] was used for the quantification of proline and hydroxyproline mass. The amino acids were first derivatized with phenylisothiocyanate as described by Edman [18] in a solution containing ethanol/triethylamine/water/phenylisothiocyanate (7:1:1:1, by vol.). The phenylthiocarbamoyl derivatives were dried under vacuum and suspended in a sample diluent containing 5 mM-sodium phosphate buffer, pH 7.4, in 5% (v/v) acetonitrile. The samples were applied to an octadecyl (C_{18}) PICO-TAG reverse-phase column (4 μ m; 3.9 mm $\times$ 150 mm). The column temperature was maintained at 38 °C by a Waters column heater. The starting buffer (buffer A) consisted of 0.05 M-sodium acetate, pH 6.4, in water containing 0.05% (v/v) triethylamine and 6% (v/v) acetonitrile. The mobile phase (buffer B) consisted of water/acetonitrile (2:3, v/v). A convex gradient was run from 0 to 46% buffer B at a flow rate of 1 ml/min with a wash of 100% buffer B and an equilibration phase of buffer A. The gradient eluents were maintained by two Waters 510 pumps controlled by an M72 gradient controller. Phenylthiocarbamoyl amino acids were monitored at 254 nm by an M440 fixed-wave-length u.v. detector. Quantifications of retention time, area, area/height, and percentage area were performed with a Hewlett Packard 3390A integrator. The retention times of glutamate, hydroxyproline, proline and ornithine were approx. 2.1 min, 3.1 min, 6.9 min and 11.8 min, respectively. Fractions were initially collected at 20 s intervals and thereafter at longer intervals for 15 min after radioactive amino acids were shown to be sufficiently separated.

Calculation of collagen and non-collagen protein synthesis rates

Net collagen and non-collagen protein synthesis rates were determined from the radioactive hydroxyproline and proline in collected fractions as previously described [13,15,19]. The amount (nmol) of hydroxyproline in newly synthesized collagen was calculated by dividing the measured hydroxyproline radioactivity (c.p.m.) by the specific activity of proline. The mass of collagen synthesized was determined by multiplying the amount (mol) of hydroxyproline by its molecular mass (131.1) divided by the fraction of collagen mass accounted for by hydroxyproline (0.14). Non-collagen protein synthesis was calculated from the labelled proline incorporated into non-collagen protein. The radioactivity of proline contributed by collagen-bound proline must first be subtracted from the proline radioactivity in the total protein hydrolysate. The radioactivity in collagen-bound proline is equivalent to the radioactivity of hydroxyproline in collagen since approx. 50% of all proline in the mixed types of SMC collagen is hydroxylated. To determine the mass of non-collagen protein the calculated amount (nmol) of proline was then multiplied by the molecular mass of proline (115.1) and also by 20, based on the assumption that cell proteins contain an average of 5% proline by weight. Determinations of net synthesis rates were then normalized for the number of SMC incubated. Collagen synthesis as a percentage of total

protein synthesis was also determined by dividing net collagen synthesis ($\times 100$) by total protein synthesis. This percentage is independent of the specific activity of the precursor proline since the same value is used in the calculation of net collagen and non-collagen protein synthesis rates.

RESULTS

In SMC cultures incubated for 12 h in 0.25 mM- or 2.5 mM-proline, the extracellular specific activity of proline decreased by less than 10%. Proline concentration in the media also was not significantly altered. Therefore, it was assumed that the specific activity of the proline pools was at steady state at 12 h, as shown with fibroblasts incubated at these proline concentrations [2]. At 0.25 mM-proline, the intracellular specific activity was only 25% of the extracellular specific activity (Fig. 1). The specific activity of intracellular free proline in cells incubated with [3 H]proline at 2.5 mM-proline was significantly higher than in cells incubated with 0.25 mM-proline, even though the specific activity of proline in the extracellular medium was not different at these concentrations. In addition, the specific activity of intracellular free proline nearly reached equilibration with the extracellular pool in cells incubated at 2.5 mM-proline. This increase in the intracellular specific activity (c.p.m./nmol) from 22900 ± 1570 in SMC incubated with 0.25 mM-proline to 84400 ± 4700 at the higher concentration was due to an increased influx of extracellular proline, as seen by comparing the molar ratios of proline to phenylalanine. The proline/phenylalanine molar ratio in SMC incubated with labelled proline was 3.35 ± 0.6 with a medium concentration of

Fig. 1. Specific activity of proline in the extracellular incubation medium (EC) and in the intracellular free proline pools (IC) from rabbit arterial SMC

SMC were incubated for 12 h in medium containing either 0.25 mM- or 2.5 mM-proline with 25 or 250 μ Ci of [3 H]proline/ml. Specific activities ($n = 3$) are expressed as a percentage of that in the extracellular medium. The specific activities of hydroxyproline in proteins precipitated by trichloroacetic acid from the EC medium (Protein Hyp) are also presented ($n = 6$). *Denotes values which are significantly different from each other (0.25 versus 2.5 mM) at $P < 0.001$ (Student's t test).

Fig. 2. Specific activity of ornithine and ornithine-derived prolyl-tRNA in arterial SMC

Cells were incubated for 12 h in medium containing 0.1 mM-ornithine with 2.0 μ Ci of [14 C]ornithine/ml at either 0.25 mM- or 2.5 mM proline concentrations. The specific activities of intracellular ornithine (IC) and prolyl-tRNA synthesized from ornithine are expressed as a percentage of the specific activity of extracellular ornithine (EC). *Denotes differences ($P < 0.05$).

0.25 mM-proline and increased to 23.5 ± 2.5 with 2.5 mM-proline. In SMC labelled with ornithine, intracellular free proline concentration relative to phenylalanine also increased at higher proline concentrations from 4.0 ± 0.6 to 28.5 ± 4.7 . Proline concentrations were compared with phenylalanine since the concentration of phenylalanine, an essential amino acid, would not be influenced by changes in extracellular or intracellular proline.

The prolyl-tRNA specific activity in cells labelled with [3 H]proline clearly did not equilibrate with the intracellular pool and was less than 10% of the specific activity of proline in the extracellular medium (Fig. 1). Although

the mean specific activity of proline bound to tRNA appeared to be increased by approx. 50% in SMC incubated in 2.5 mM-proline, the difference did not reach statistical significance. However, the specific activity of hydroxyproline in proteins precipitated from the incubation medium was doubled when incubations were carried out in 2.5 mM- versus 0.25 mM-proline. The specific activity of hydroxyproline in these newly synthesized collagen-containing protein precipitates would equal that of proline, since hydroxylation occurs only after proline is incorporated into the nascent polypeptide chains, and therefore should be identical with the specific activity of proline in the corresponding precursor prolyl-tRNA pool. The data show (Fig. 1) that the specific activities of prolyl-tRNA and protein-bound hydroxyproline are both quite significantly lower than those of either intracellular or extracellular free proline but they are not equal in either the 0.25 mM- or 2.5 mM-proline groups. The accuracy of the hydroxyproline specific activity determination cannot be verified by direct comparison with the specific activity of proline in proteins secreted into the incubation medium, as just described, since the medium contained a large quantity of unlabelled proline from proteins contributed by the fetal calf serum supplement. The much smaller mass of hydroxyproline present in these serum proteins was subtracted from the total hydroxyproline in the incubation medium prior to calculating hydroxyproline specific activity, as described in Table 1.

The specific activity of extracellular ornithine decreased by $35.8 \pm 3.9\%$ and $42.9 \pm 8.1\%$ in cells incubated for 12 h in 0.25 mM- and 2.5 mM-proline with 0.1 mM-ornithine. The specific activities of intracellular ornithine and ornithine-derived proline bound to tRNA are shown in Fig. 2. Comparisons were made with extracellular values determined after 12 h. The specific activity of intracellular ornithine was reduced to less than 50% of that in the extracellular medium due to the intracellular production of free ornithine. SMC incubated with 2.5 mM-proline showed an intracellular specific activity of ornithine which was 25% lower than that of cells in

Table 1. Calculated rates of collagen, non-collagen and total protein synthesis in SMC incubated with labelled proline

Net collagen and non-collagen protein synthesis rates were determined by using the calculated specific activity of each of the proline pools presented in Fig. 1. Synthesis rates are expressed in ng/h per 10^5 cells. Values are means $\pm$ S.D.; *significantly different ($P < 0.001$) from corresponding 0.25 mM samples. Each value represents the combined precipitable proteins from the cell layer and extracellular medium ($n = 6$). 'Protein hydroxyproline' represents the specific activity of hydroxyproline in newly synthesized procollagen(s) and collagens precipitated from the extracellular medium by 5% (w/v) trichloroacetic acid. This value should be equivalent to that of proline in the 'precursor pool'. The mass of precipitable hydroxyproline contributed by the 10% fetal bovine serum supplement, presumably from Clq, was determined by h.p.l.c. and subtracted from total hydroxyproline prior to calculating 'protein hydroxyproline' specific activity.

Precursor proline pool	[Proline] in medium (mM)	Protein synthesis (ng/h per 10^5 cells)		
		Collagen	Non-collagen	Total
Free extracellular	0.25	23 ± 3	255 ± 14	278 ± 10
	2.5	$35 \pm 1^*$	$455 \pm 11^*$	$490 \pm 12^*$
Free intracellular	0.25	85 ± 10	941 ± 50	1026 ± 36
	2.5	$38 \pm 1^*$	$495 \pm 12^*$	$533 \pm 13^*$
Prolyl-tRNA	0.25	833 ± 102	9270 ± 495	10103 ± 356
	2.5	786 ± 29	10300 ± 253	11086 ± 356
Protein hydroxyproline	0.25	215 ± 21	2406 ± 116	2655 ± 135
	2.5	173 ± 43	2263 ± 205	2436 ± 206

0.25 mM-proline. The influx of unlabelled proline into these cells incubated with 2.0 μCi of [^{14}C]ornithine/ml in 2.5 mM unlabelled proline also decreased the specific activity (c.p.m./nmol) of the ornithine-derived intracellular free proline from 561 ± 13.6 in SMC in 0.25 mM-proline to 98 ± 17.4 with 2.5 mM-proline. Although the mean specific activity of [^{14}C]prolyl-tRNA in the 2.5 mM-proline group also appeared to be lower than that in the 0.25 mM-proline group (Fig. 2), this difference was not statistically significant nor was it at all comparable with the 6-fold difference in specific activities of intracellular free proline.

Calculations of collagen and non-collagen protein synthesis rates based on the specific activity of proline from different pools are shown in Table 1. Values presented are net synthesis rates since a portion of the procollagen synthesized is degraded intracellularly [20]. The linearity of protein synthesis with time had been previously established in several experiments, including one in which confluent monolayers of SMC were incubated from 4 to 24 h in wells containing radiolabelled proline. Relative rates of collagen synthesis (d.p.m. of hydroxyproline/ μg of cell layer protein) were 77 ± 12 , 321 ± 97 and 467 ± 49 for 4, 16, and 24 h incubations, respectively. Rates of non-collagen protein synthesis (d.p.m. of proline/ μg of cell layer protein) were 500 ± 66 , 1708 ± 151 , and 2475 ± 222 for the same three incubation periods. Therefore, it was assumed that protein synthesis rates in SMC would be linear over the 12 h incubation period. Synthesis rates based on the specific activity of extracellular proline grossly underestimated both collagen and non-collagen protein synthesis compared with rates calculated from tRNA-bound proline specific activity. Rates of total protein synthesis were approx. 75% higher when calculated from the specific activity of proline in the extracellular medium containing excess proline compared with 0.25 mM-proline. This increase must be attributed to the higher degree of labelling of the prolyl-tRNA pool, since the extracellular proline specific activities did not differ (Fig. 1). Apparent synthesis rates using the intracellular free proline specific activities were also significantly different even though the actual rates would not be expected to vary between cells incubated in 0.25 mM- and 2.5 mM-proline. In fact, synthesis rates based on the corresponding specific activities of

prolyl-tRNA were not different between cultures incubated with 0.25 mM- or 2.5 mM-proline. Synthesis rates calculated from the specific activity of hydroxyproline in newly synthesized proteins precipitated from the incubation medium were likewise similar at both proline concentrations, but were significantly lower than those based on prolyl-tRNA. Thus, the actual synthesis rates of collagen and other proteins are independent of the proline concentration of the incubation medium. Furthermore, the differences in protein synthesis rates using prolyl-tRNA versus hydroxyproline specific activities for the calculations are due directly to the difference in the two specific activities (Fig. 1).

Net collagen and non-collagen protein synthesis rates were determined in SMC cultures incubated with labelled ornithine ($n = 6$). Synthesis rates were determined by using the ornithine-derived prolyl-tRNA specific activity determined at the end of the 12 h incubation. Synthesis rates, expressed as ng/h per 10^5 cells, represent radiolabelled proteins from the cell layer and incubation medium combined. In arterial SMC incubated in 0.25 mM-proline, collagen and non-collagen protein synthesis rates (means $\pm$ s.d.) were 48.2 ± 1.6 and 148.3 ± 8.9 ng/h per 10^5 cells whereas the corresponding values in cells incubated with 2.5 mM-proline were 64.5 ± 8.1 and 38.6 ± 3.9 . Therefore, increasing the extracellular proline concentration of the medium 10-fold while keeping the ornithine concentration similar produced an apparent decrease in non-collagen protein synthesis but collagen synthesis was increased. These results differ substantially from those calculated from the specific activities of prolyl-tRNA in SMC incubated with labelled proline.

Collagen synthesis relative to non-collagen protein synthesis was determined in arterial SMC incubated with labelled proline or ornithine (Table 2). In SMC incubated with [^3H]proline in 0.25 mM- or 2.5 mM-proline there was no difference in collagen synthesis expressed as a percentage of total proteins synthesized. Separate analyses of proteins precipitated from the extracellular medium and cell layer showed a small but significant lowering of the percentage in the cell layer of SMC in 2.5 mM-proline; however, collagen represents only a small portion of total proteins in this fraction. In contrast with collagen synthesis in SMC, which averaged

Table 2. Collagen synthesis as a percentage of total protein synthesis

Collagen synthesis expressed as a percentage of the total quantity of protein synthesized was determined in arterial SMC incubated with labelled proline or labelled ornithine. Cells were incubated at proline concentrations of 0.25 mM or 2.5 mM. Values calculated from the radioactivity in labelled hydroxyproline and proline residues in precipitable proteins were independent of precursor specific activity (see the Experimental section). Each group ($n = 6$) represents the mean $\pm$ s.d.; *significantly different at $P < 0.001$ (Student's t test).

Labelled amino acid	[Proline] in medium (mM)	Collagen synthesis/total protein synthesis (%)		
		Extracellular medium	Cell layer	Extracellular medium + cell layer
[^3H]Proline	0.25	22.3 ± 0.9	2.3 ± 0.01	8.2 ± 0.7
	2.5	20.8 ± 1.9	$1.8 \pm 0.09^*$	7.1 ± 0.3
[^{14}C]Ornithine	0.25	57.1 ± 3.5	14.2 ± 2.3	24.5 ± 1.3
	2.5	100.0^*	$58.2 \pm 3.9^*$	$69.3 \pm 3.0^*$

7.6% of the total protein synthesis in cells incubated with [^3H]proline, collagen synthesized from ornithine-derived proline accounted for 24% of total protein synthesis in SMC incubated in 0.25 mM-proline and 69% of total proteins synthesized by cells in 2.5 mM-proline.

DISCUSSION

Equilibration of extracellular specific activities of amino acids with the intracellular and aminoacyl-tRNA pools can be attained with certain essential amino acids if the extracellular concentration of the amino acid is sufficiently elevated [3,21–25]. The results with proline, a non-essential amino acid, clearly showed that, even after 12 h incubations, specific activities of proline in the various precursor pools did not equilibrate even when SMC were exposed to excess extracellular proline *in vitro*. Intracellular dilution of the specific activity of proline may arise from synthesis *de novo* of unlabelled proline [6–10] or proline from protein degradation [11,12]. The significantly lower specific activity of proline in prolyl-tRNA compared with intracellular free proline apparently arises from endogenous unlabelled proline being incorporated directly into prolyl-tRNA without appreciable mixing with the intracellular pool containing exogenously-derived labelled proline. Therefore, it appears that intracellular proline pools in SMC are compartmentalized so that certain pools may be preferentially aminoacylated to tRNA. A model in which a large intracellular free proline pool containing a major portion of the extracellular proline taken up via a receptor site on or near the plasma membrane that does not feed into the prolyl-tRNA synthetase pathway has been proposed for human lung fibroblasts by Hildebrand *et al.* [2].

Further evidence that prolyl-tRNA is not derived solely or even primarily from the free intracellular pool was demonstrated by increasing the proline concentration in the medium from 0.25 mM to 2.5 mM with a corresponding increase in [^3H]proline. The specific activity of intracellular proline rose nearly 4-fold to a value almost equal to extracellular proline but without a proportionate increase in the prolyl-tRNA specific activity (Fig. 1). This equilibration of extra- and intra-cellular specific radioactivity was due to an influx of extracellular proline, as demonstrated by the 7-fold increase in the proline/phenylalanine ratio. Although the 50% increase in prolyl-tRNA specific activity in cells incubated in the 10-fold higher concentration of proline was not statistically significant ($n = 3$), the increase in the specific activity of hydroxyproline in the precipitable proteins isolated from the incubation medium was significant ($n = 6$). These data suggest that the compartmentalized intracellular free proline pool does interact to some degree with the prolyl-tRNA precursor pool in SMC *in vitro*. That changes in extracellular proline concentrations can influence the specific activity of the prolyl-tRNA precursor pool(s) of collagen was shown *in vivo* by Nissen *et al.* [26], who reported a decrease in recycling of labelled proline from the breakdown of previously labelled protein in aorta, skin and tendon tissue of rats by repeated injections of large amounts of unlabelled proline, and by Laurent [27], who demonstrated that the specific activity of hydroxyproline in newly synthesized procollagen from skin was similar to plasma and tissue free proline specific activity in rabbits

receiving a bolus of radiolabelled proline along with large amounts of nonlabelled proline.

In SMC incubated with 0.1 mM-[^{14}C]ornithine for 12 h, the decrease in specific activity of extracellular ornithine and of intracellular free ornithine compared with that in the extracellular medium was due to dilution by endogenous ornithine synthesized presumably from arginine by arginase. Many types of cells in which collagen synthesis accounts for a relatively high percentage of total protein synthesis have high arginase activities, even though they lack a complete urea cycle [6]. Labelled ornithine was readily converted to [^{14}C]proline, as expected for SMC since levels of proline metabolic enzymes frequently correlate with proline requirements in several connective tissue cells. The proline catabolic enzymes, proline oxidase and pyrroline-5-carboxylate dehydrogenase, are nearly undetectable, whereas there are relatively high activities of the anabolic enzymes pyrroline-5-carboxylate reductase and pyrroline-5-carboxylate synthetase in bone, cartilage and skin [7,8,10,28]. Results with arterial SMC are consistent with those observations since no radioactivity was found in glutamate in cells labelled with proline or ornithine, yet proline derived from ornithine was detectable in intracellular free proline, aminoacylated-tRNA and in proteins.

The specific activity of ornithine-derived free proline was significantly decreased in cells incubated in 2.5 mM-proline versus 0.25 mM-proline, due to the 7-fold increase in net incorporation of unlabelled proline into the intracellular proline pool. The 25% further reduction in intracellular free ornithine specific activity in the 2.5 mM-proline group compared with SMC in 0.25 mM-proline, however, is not as easily explained (Fig. 2). Proline is known to inhibit pyrroline-5-carboxylate reductase, which catalyses the conversion of pyrroline-5-carboxylate to proline [28]. Inhibition of this enzyme by the higher intracellular free proline may cause the accumulation of ornithine synthesized *de novo* in the cell, resulting in the subsequent decrease in intracellular ornithine specific activity seen in SMC (Fig. 2). A decrease was also observed in the extracellular medium of cells incubated with 2.5 mM-proline. However, this small decrease (7.1%) could not entirely account for differences in the intracellular specific activities of ornithine or proline. In contrast with these results, Smith & Phang [7] have shown that the ornithine pool size in xyphoid cartilage cells was not affected by higher concentrations of proline in the medium, since the rate of incorporation of ornithine-derived proline into protein was not altered. The specific activity of [^{14}C]prolyl-tRNA derived from [^{14}C]ornithine was lower than that of intracellular free ornithine regardless of proline concentration in the medium (Fig. 2). The apparent decrease in the mean specific activity of ornithine-derived prolyl-tRNA shows that, although increasing intracellular proline concentration by incubation in 2.5 mM-proline had a dramatic effect on free proline specific activity, it had a much lesser effect on the specific activity of the tRNA aminoacylated pool. It thus appears that intracellular proline synthesized *de novo* from labelled ornithine in SMC does not equilibrate completely with the precursor proline pool prior to prolyl-tRNA synthesis. This observation is similar to that just described in which exogenously-derived labelled proline was also found to be compartmentalized in SMC rather

than establishing isotopic equilibrium with the intracellular free proline pool prior to aminoacylation with tRNA.

It is obvious from the calculations of net collagen and non-collagen protein synthesis rates employing the different specific activities observed in the various precursor proline pools that use of either the extracellular or intracellular free proline values severely underestimates synthesis in SMC incubated with [^3H]proline (Table 1). Rates calculated from the specific activities of prolyl-tRNA were 10–40 times higher than those calculated from intra- and extra-cellular specific activities. Although synthesis rates estimated from the specific activity of hydroxyproline in collagen secreted into culture media were about 75% lower than those calculated from prolyl-tRNA, since the specific activity was three times that of prolyl-tRNA they were still in much closer agreement than those derived from extra- and intra-cellular free proline. It is important to note that altering the proline concentration of the incubation medium did not change the synthesis rates of collagen and non-collagen protein from [^3H]proline regardless of whether the calculations were based on labelled prolyl-tRNA or [^3H]hydroxyproline in newly synthesized collagen (Table 1).

Collagen synthesis as a percentage of total protein synthesis was also independent of proline concentration in the medium when cells were incubated with labelled proline (Table 2). Similar results have been demonstrated in our previous experiments with arterial SMC in culture [13]. However, when labelled ornithine served as the precursor, a much greater proportion of the endogenously labelled proline which was incorporated into trichloroacetic acid-precipitable proteins was recovered as hydroxyproline. These results indicate that a higher percentage of ornithine-derived proline was channelled into collagen synthesis than was seen with exogenously-added [^3H]proline (Table 2). Collagen and non-collagen protein synthesis from labelled ornithine were both affected by the 10-fold increase in unlabelled proline in the medium, in contrast with incubations with [^3H]proline. Therefore the observed ratio of collagen synthesis to total protein synthesis may vary when labelled proline is derived from different intracellular compartmentalized pools with different specific activities.

Hildebran *et al.* [2] have shown in fibroblasts that the specific activities of proline and hydroxyproline in proteins labelled with proline are similar to that of steady state prolyl-tRNA. Although there was evidence of compartmentalization of amino acid pools in the intracellular space, their results suggest that the prolyl-tRNA pool may be considered homogeneous. Similar results have been demonstrated in myocardial cells [20,21], lung tissue [22], and skeletal muscle cells [5,20]. However, our results with [^3H]proline and [^{14}C]ornithine suggest that intracellular proline in SMC can enter separate compartmentalized tRNA pools which may be involved in the synthesis of specific proteins. Similarly, Tanaka & Ichihara [29] have shown that endogenous amino acids derived from protein breakdown can be used to synthesize proteins which differ from those synthesized from amino acids in the extracellular medium. Fern & Garlick [30] have also demonstrated differences in the specific activity of glycine and serine among isolated proteins synthesized by the liver. In addition, Ilan & Singer [31] have shown differences in the specific activity

of leucine in nascent peptide chains isolated from membrane-bound and free ribosomes. Consistent with these studies are the observations of multiple forms of amino acyl-tRNA synthetases which are also compartmentalized in the cell [32–34]. It has been suggested that they are involved in the protein synthetic machinery and use amino acids from separate intracellular pools [32,35,36]. Together, these results suggest that aminoacyl-tRNA complexes do not exist as a homogeneous pool in SMC and the compartmentalization of these intracellular components may result in errors in the calculated rates of protein synthesis if the specific activity of the amino acid pool used in the estimation of protein synthesis is not accurately determined. Therefore, the specific activity of proline or hydroxyproline in newly synthesized collagen or procollagen measured under steady state conditions should be used as the appropriate precursor value for the quantification of collagen synthesis. In addition, determination of prolyl-tRNA values will yield a far closer approximation of the true rates of total protein synthesis in SMC than calculations employing the specific activity of intracellular or extracellular free proline.

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