Selective Inhibition of Collagen Synthesis by the Ca^{2+} Ionophore A23187 in Cultured Human Fibroblasts*

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The question of whether the Ca^{2+} ionophore A23187 affects collagen production relative to total protein synthesis or has possible effects on collagen degradation was investigated. Cultured normal human fibroblasts were incubated with radioactive proline, and the radioactivity of collagenase-sensitive and -resistant proteins was used to calculate the rates of protein production. The net production of collagen relative to total proteins was inhibited by A23187 in a dose-related manner, and 50% inhibition of basal collagen production was achieved with 0.6 μM A23187. There was a 70% decrease in the absolute rate of collagen production in the presence of 0.8 μM A23187 which represented a 4-fold greater inhibition of collagen production than of noncollagen protein production. The major mechanism for the decreased net production of collagen was decreased synthesis, rather than increased degradation. Ca^{2+} mobilization induced by cholecystokinin octapeptide was also associated with selective inhibition of collagen production in normal human fibroblasts. These studies establish that the Ca^{2+} ionophore A23187 induces a selective decrease in collagen polypeptide synthesis by normal human fibroblasts and suggest a modulatory role of Ca^{2+} on collagen metabolism.

Collagen, the most abundant of mammalian proteins, is a major constituent of the extracellular matrix (1). Regulation of net collagen production involves complex interaction between the processes of collagen synthesis, post-translational modification, secretion, extracellular organization, and degradation (1). Abnormal regulation of fibrogenesis is responsible for the excess accumulation of connective tissue in many diseases, including those of the skin (2), lungs (3), and liver (4). The control of collagen production is therefore a critical step of tissue homeostasis. Although most cells have the potential to produce collagen in vitro, fibroblasts and related cells have been implicated in the pathogenesis of tissue fibrosis (5–8). It has been shown that collagen production in cultured fibroblasts can be modulated by different stimuli, including cAMP (9–12). Increased intracellular levels of cAMP are associated with a selective decrease in collagen production by normal human fibroblasts (12), apparently by enhancement of intracellular procollagen degradation (13).

Both cyclic nucleotides (14) and Ca^{2+} (15) have been proposed as "second messengers," molecules whose varying intracellular concentrations couple extracellular stimuli to alterations in cellular physiology, yet little is known about the role of intracellular Ca^{2+} on collagen metabolism. Treatment of isolated rat skeletal muscle with the Ca^{2+} ionophore A23187, which induces mobilization of cellular Ca^{2+}, appears to increase overall protein degradation without significant changes in protein synthesis (16). However, the question of whether the Ca^{2+} ionophore affects collagen production relative to total protein synthesis or has possible effects on collagen degradation remains to be established. Therefore, we evaluated the effects of the Ca^{2+} ionophore A23187 on collagen production by normal human fibroblasts.

**EXPERIMENTAL PROCEDURES**

Materials—[5-3H]Proline (19 mCi/μmol), [4-3H]proline (16 mCi/μmol), [14C]proline (290 mCi/ml), DL-hydroxy-[2-14C]proline (19 mCi/μmol), 4Ca (10-40 mCi/mg of Ca), and ACS scintillation fluid were obtained from Amersham Corp.; [3H]toluene and [14C]toluene standards were purchased from New England Nuclear. Chromatographically purified bacterial collagenase form III was from Advance Biofactures Company (Lynbrook, NY). Sources of other chemicals were: AG-50W-X8 resin (100–200 mesh) from Bio-Rad Laboratories; absolute ethanol from Aaper Alcohol and Chemical Co. (Louisville, KY); L-ascorbic acid, calcium ionophore A23187, α,α'-dipyridyl (2,2'-bipyridine), L-proline, hydroxy-L-proline, calcium chloride, sodium hydroxide, hydrochloric acid, Trizma buffer, N-ethylmaleimide, soybean trypsin inhibitor type I, acetylsalicylic acid, indomethacin, and trichloroacetic acid from Sigma. Phorbol dibutyrate was the gift of Michael Boyle (Salk Institute, La Jolla, CA). Synthetic COOH-terminal octapeptide of cholecystokinin (CCK') was a gift from Miguel Ondetti (Squibb Institute for Medical Research, Princeton, NJ). Leupeptin and pepstatin were the gift of Karl Hostetter (University of California, San Diego, CA). Human fetal AF2 fibroblasts and media were obtained from the Core Cell Culture Facility (University of California, San Diego, CA). Fibroblast Cultures—Cells were cultured under an atmosphere of 5% CO2, 95% air in tissue culture dishes using Dulbecco's minimal essential medium; DMEM-0, DMEM without serum; and media were obtained from the Core Cell Culture Facility (University of California, San Diego, CA).

Fibroblast Cultures—Cells were cultured under an atmosphere of 5% CO2, 95% air in tissue culture dishes using Dulbecco's minimal essential medium (DMEM) containing 1.5 mM CaCl2 and 10% fetal calf serum (17, 18). Human fetal AF2 fibroblasts were used at subcultivation 5 to 21 as previously described (17, 18). Cells were plated at a density of 9 × 10^4/P-100 dish and radiolabeling studies were performed after 5 days as described below. The number of cells at 5 days was approximately 2.7 × 10^6/plate.

Radiolabeling of Cells—Confluent cell cultures were incubated in 2.5 ml of DMEM without serum (DMEM-0) but containing 1.5 mM CaCl2 and 0.1 mM L-proline, at 37 °C in 5% CO2, 95% air. After a 15-min preincubation period in the presence of 0.2 mM ascorbic acid, a cofactor for prolyl and lysyl hydroxylases, 10 μCi of L-[5-3H]proline...

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were added and the incubation was continued for up to 4 h (17). Labeling of cells was terminated by cooling the plates to 0 °C. When determining the degree of collagen degradation, an isotopic mixture containing 33.8 μCi of L-[4-3H]proline, 5.4 μCi of L-[14C]proline was added to each plate as previously described (19). Before use, the isotope were purified on a 1 × 35 cm Bio-Gel P-100W-X8 column with 1 HCl followed by elution of proline from 6 N HCl (20). The effects of the Ca2+ ionophore on collagen metabolism were examined by the addition of A23187 (0–12 μM) to plates at the time of preincubation. The Ca2+ ionophore A23187 was dissolved in ethanol and stored at –20 °C. In order to avoid interexperimental variability, the concentration of the A23187 solutions was determined by absorbance at 325 nm using standards. When A23187 was added to the incubation medium, the final concentration of ethanol did not exceed 17 mM; the control media were incubated in medium containing the same concentration of ethanol. However, the final concentration of ethanol did not affect basal rates of collagen production (17).

The effects of CCK and tumor-promoting phorbol esters on collagen production by confluent AF2 cells were also analyzed. After a 1-h preincubation with or without phorbol dibutyrate (1 or 20 μM), or 30-min preincubation with CCK (0.1 nM) in the presence of 0.2 mM calcium ions, the proline was added to the incubation and continued for 3 h. Also, the influence of indomethacin (20 μM), aspirin (0.2 mM), and leupeptin (50 and 100 μM) or without peptatin (100 μM), on the A23187-treated cells was assessed by the addition of a compound to the incubation 30 min prior to addition of the Ca2+ ionophore (0.6 μM).

Measurement of Collagen Production—After the incubation of cells was completed, the media were removed and the cell layers were rinsed with cold 0.1 M NaCl, 0.05 M Tris, pH 7.4. Media and washes were pooled and stored at –20 °C. The cell layers were scraped off the dishes with 0.11 M NaCl, 0.05 M Tris, pH 7.4, and sonicated at 0 °C for 15 s at 20% maximum voltage in a Biosonik sonicator (needle probe) to rupture the cells (19). Trichloroacetic acid was added to yield a final concentration of 10%. Samples were centrifuged at 1000 × g for 10 min. Precipitates were resuspended and recentrifuged in 5% trichloroacetic acid. The combined trichloroacetic soluble fractions were stored at –20 °C to determine the specific activity of proline in the free cellular pool. The pooled media and washes were added to the precipitate from the cell layers, and the combined suspension was precipitated with 10% trichloroacetic acid. Proteins were separated by centrifugation at 1000 × g for 10 min and washed four times with 5% trichloroacetic acid. Combined cell layer and media precipitates were dissolved in 0.2 N NaOH. The amount of radioactive collagen was determined by the collagenase digestion method (21), as previously described (22). The collagenase-resistant material was hydrolyzed in 6 N HCl at 120 °C for 3 h (22). The hydrolysates were semicarbazide-treated and the semicarbazide-resistant proteins were used to calculate the relative rate of collagen production (23). The absolute rate of collagen production was calculated from the 14C radioactivity incorporated into collagenase-sensitive proteins and the specific radioactivity of the proline free pool as follows: radioactivity in collagen (disintegrations/minute)/proline specific activity (disintegrations/minute/picomole). The value is expressed as picomoles of proline incorporated per 106 cells/h (24).

Specific Radioactivity of the Free Proline Pool—In some experiments, the specific activities of the free [14C]proline pools were determined in AF2 fibroblasts incubated in the presence of A23187. Separate portions of the trichloroacetic acid-soluble fraction of cell sonicates were used for quantitation of proline by ninhydrin reaction on a Beckman 119 Amino Acid Analyzer (W-3H ion-exchange resin) and for measurement of radioactivity (24). The specific radioactivity was then calculated as disintegrations/minute/picomole.

Specific Activity of Newly Synthesized Proteins—Newly secreted proteins were purified by dialysis (50 kDa cutoff) for 4 h at 4 °C against distilled water containing 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, pH 7.0. Samples were evaporated in siliconized glass tubes, hydrolyzed in 6 N HCl, and evaporated to dryness. The proline was then quantitated as previously described (25), (26). The sample was dissolved in 10 μl of 0.2 M NaOH, 1 M trichloroacetic acid transport, the rate of collagen production was expressed as the percentage of [14C]hydroxyproline in the low molecular weight fraction compared to the total protein [14C]hydroxyproline. The amount of [14C]hydroxyproline in the high molecular weight fraction was calculated by multiplying the total [14C]collagen radioactivity, determined by the procedure of Cline et al. (24), by the specific activity of proline in the free cellular pool as follows: radioactivity in collagen (disintegrations/minute)/proline specific activity (disintegrations/minute/picomole). The value is expressed as picomoles of proline incorporated per 106 cells/h (24).

Determination of 45Ca Fluxes—AF2 fibroblasts, cultured as described above, were incubated for 2 h with DMEM-0 (1.5 mM CaCl2) in the presence of 5 μCi of 45Ca. Media were removed and cells washed four times with 5 ml of DMEM-0, and incubation begun in the presence or absence of 0.6 μM A23187 or 0.1 nM CCK. 50 μl of media were sampled at 0, 1, 2, 5, 10, 20, and 30 min. Radiochemical and cell layers were removed and sonicated, and total radioactivity determined. 45Ca efflux was calculated as the percentage of total radioactivity eluting in the hydroxyproline region. The recovery of internal [14C]hydroxyproline standards, added to unhydroxylated samples, processed and analyzed as described above, was 82%, and data were corrected accordingly.

Degradation of newly synthesized collagen was calculated as the percentage of [14C]hydroxyproline in the low molecular weight fraction compared to the total protein [14C]hydroxyproline. The amount of [14C]hydroxyproline in the high molecular weight fraction was calculated by multiplying the total [14C]collagen radioactivity, determined by the procedure of Cline et al. (24), by the specific activity of proline in the free cellular pool as follows: radioactivity in collagen (disintegrations/minute)/proline specific activity (disintegrations/minute/picomole). The value is expressed as picomoles of proline incorporated per 106 cells/h (24).

Proline Transport—In order to correct for differences in amino acid transport, the rate of collagen production was expressed as the percentage of the total radioactive proline taken up into the cells (trichloroacetic acid-soluble plus trichloroacetic acid-precipitable) that had been incorporated into collagen-sensitive protein (29).
counting solution, and counted by standard procedures. Intra-sample variation in cell counts was less than 1%.

A final assay of viability involved the evaluation of cellular growth potential after experimental treatment. Following a 4-h incubation, cells were washed, and fresh DMEM media with 10% fetal calf serum was added. After 48 h, aliquots with known numbers of cells were transferred from the original cultures to new plates along with fresh media and the growth of these cells was evaluated for up to 14 days by cell counting as described.

Statistical Analysis—All the results are expressed as mean ± S.E. unless stated otherwise. The Student's t test was used to evaluate the differences of the means between groups, accepting p < 0.05 as significant (38).

RESULTS

Effect of A23187 on Cellular Ca²⁺ Fluxes—As expected, A23187 increased ⁴⁰Ca efflux in human fetal fibroblasts to an extent similar to that previously reported for other biological systems (15, 34, 35). The ⁴⁰Ca efflux was increased by the Ca²⁺ ionophore at 1 min and remained at least 2-fold greater than control values throughout a 30-min period (Fig. 1). CCK, a hormone found in plasma, small intestine, and brain (36-37) stimulated Ca²⁺ mobilization in AF² fibroblasts to an extent similar to A23187 (data not shown), as previously described in other biological systems (38). A very good correlation has been reported between atomic absorption and ⁴⁰Ca fluxes in evaluating cellular Ca²⁺ concentrations (34). Also, changes in free cytosolic Ca²⁺ measured by the quin-2 method parallel ⁴⁰Ca fluxes under different experimental conditions (35).

Collagen and Noncollagen Protein Production—The net production of labeled collagen relative to total labeled proteins in AF₂ human fibroblasts was markedly inhibited by A23187 (Fig. 2). To correct for possible differences in proline transport, the production of collagen was calculated as the percentage of total [¹⁴C]proline taken up into the cells which had been incorporated into collagenase-sensitive protein. This value was also significantly decreased by A23187 (Fig. 2). This selective inhibitory effect of A23187 on collagen production in fibroblasts was exponentially related to the concentration of the Ca²⁺ ionophore (Fig. 3) and was observed in the presence or absence of Ca²⁺ in the media (2.1 ± 0.1 versus 2.2 ± 0.2 dpm/10⁶ cells/h; not significant). Since a ~50% inhibition of basal collagen production was consistently achieved with 0.6 μM A23187, we examined the effects of 0.6 μM A23187 on collagen metabolism in greater detail. In the presence of the Ca²⁺ ionophore, collagen production was linear over the course of 4-h incubation, but only 40% (2 h) to 31% (4 h) of control values. However, procollagen secretion was not affected by A23187 (data not shown).

The expression of collagen production as a relative rate eliminates consideration of changes in amino acid transport or other factors that would modify the specific activity of the precursor proline pool. A decrease in the relative rate of collagen production expressed as a percentage, however, may result from either decreased collagen production or increased noncollagen protein production. To determine which of these possibilities caused the observed change, the rates of proline incorporation into proteins were assessed. We found that the inhibition of collagen production by A23187 was selective. The incorporation of [¹⁴C]proline into collagen was decreased by 65% in the presence of 0.6 μM A23187, whereas incorporation of [¹⁴C]proline into noncollagen protein was decreased only by 19%. Thus, total [¹⁴C]protein was only decreased by 24% (12.8 ± 0.5 versus 16.9 ± 0.5 dpm/10⁶ cells/h; p < 0.01).

Absolute Rates of Collagen Synthesis and Degradation—The
absolute rates of collagen production were determined from
the specific activity of [14C]proline in the free amino acid pool
of fibroblasts from the experiment described above. There
were only small differences between the specific activities of
free [14C]proline in control and A23187-treated cells (9.4 ±
0.7 versus 11.5 ± 0.5 dpm/pmol; p < 0.05), suggesting that the
Ca2+ ionophore did not substantially affect free proline trans-
port or metabolism. There is evidence to suggest that the free
amino acid pool is the immediate precursor for protein syn-
thesis and that there is rapid equilibration between amino
acids in the extracellular fluid and the intracellular pool (39,
40). To assure equilibration of the medium and internal pools,
unlabeled proline was added with the radiolabeled proline.
The fact that a linear rate of incorporation was attained in
the preliminary experiments suggests that equilibrium had
been reached (22). However, it has been suggested that mea-
surement of the aminoseryl tRNA pool specific activity is
required to calculate absolute rates of protein synthesis (41).
These workers proposed that measurement of procollagen
specific activity is a simpler approach (25, 26). We determined
the proline specific activity of newly synthesized proteins
secreted into the media. Procollagen represented ~60% of the
total radioactivity in media proteins. Furthermore, proteins
of a molecular mass of ~50 kDa were dialyzed. In A23187-
treated cells, the proline specific activity of the newly synthe-
sized proteins was increased from control values to an extent
similar to that of the specific activity of the free proline pool
(2.2 ± 0.3 versus 3.0 ± 0.3; p < 0.05). Therefore use of the
latter would result in valid conclusions under our experimen-
tal conditions.

Since the specific decrease in the net production of collagen
could have resulted from changes in biosynthesis, and/or
degradation, we measured [14C]hydroxyproline in the low
molecular weight (acid-soluble) fraction, which is derived from
collagen degradation. The percentage of low molecular
weight [14C]hydroxyproline compared with the total [14C]
hyroxyproline synthesized by normal human fetal fibroblasts
was 25%, in close agreement with the results obtained in
cultured human lung fibroblasts (13), explants of rabbit lung
parenchyma (42), and chick embryo bone (20). Although the
percentage of collagen degradation in A23187-treated cells
was 2-fold greater than control levels (59 ± 3% versus 25 ±
3%; p < 0.001), when the absolute rates of collagen degra-
dation were determined, there was an insignificant difference
between the two groups. Thus, the absolute rates calculated
by using the [14C]proline specific activities showed a marked
decline in collagen biosynthesis in human fetal fibroblasts
in the presence of A23187, whereas collagen degradation was
essentially unchanged (Fig. 4).

Since the stimulation of protein degradation in rat skeletal
muscle by A23187 was mediated by prostaglandin E2 (16), we
assessed the effects of prostaglandin synthesis inhibitors on
collagen production. Neither aspirin (2.1 ± 0.1 dpm/10^6 cells/
h) nor indomethacin (2.3 ± 0.6) has any effect on basal net
collagen production (1.9 ± 0.1), suggesting that prostaglandins
are not involved in the inhibition of collagen biosynthesis
induced by A23187 in human fibroblasts.

Inhibition of protein degradation was achieved by Roden-
mann and co-workers (16) with protease inhibitors in rat
muscle treated with A23187. Therefore, we examined the
effects of leupeptin (Table I) and pepstatin (data not shown),
inhibitors of proteases; these compounds prevented only in
part the inhibition of collagen production by the Ca2+ iono-
phore. These findings suggest that activation of lysosomal
proteases is not responsible for the decreased collagen pro-
duction induced by A23187.

**TABLE I**

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Collagen</th>
<th>Noncollagen protein</th>
<th>Relative rate of collagen production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm/10^6 cells/h</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9.7 ± 1.6</td>
<td>83.5 ± 14.6</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>Control + leupeptin</td>
<td>11.8 ± 1.6</td>
<td>118.8 ± 6.9</td>
<td>86 ± 4</td>
</tr>
<tr>
<td>A23187</td>
<td>2.1 ± 0.1</td>
<td>42.5 ± 2.4</td>
<td>46 ± 4</td>
</tr>
<tr>
<td>A23187 + leupeptin</td>
<td>3.5 ± 0.1</td>
<td>53.3 ± 1.3</td>
<td>59 ± 3</td>
</tr>
<tr>
<td>Control</td>
<td>19.3 ± 4.0</td>
<td>160.0 ± 51.1</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>Control + leupeptin</td>
<td>24.1 ± 0.4</td>
<td>230.7 ± 12.0</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>A23187</td>
<td>7.4 ± 0.6</td>
<td>134.4 ± 14.0</td>
<td>48 ± 3</td>
</tr>
<tr>
<td>A23187 + leupeptin</td>
<td>8.6 ± 0.4</td>
<td>135.6 ± 10.7</td>
<td>54 ± 4</td>
</tr>
</tbody>
</table>

* Control and 0.6 μM A23187-treated confluent cell cultures were incubated in the presence or absence of leupeptin (50 μM in Experiment 1 and 100 μM in Experiment 2).

**FIG. 4.** Effect of A23187 on the absolute rates of collagen biosynthesis and degradation. Samples of the experiment described in the legend to Fig. 2 were used. The absolute rates of collagen biosynthesis and degradation were calculated from the total incorporation of [14C]proline and the specific radioactivities of the free [14C]proline pools and are expressed as picomoles of proline incorporated per 10^6 cells/h. The rates of collagen degradation (black bars) were determined from the relative proportions of [14C]hydroxy-
proline in the high and low molecular weight fractions and expressed as picomoles of proline incorporation into collagens proteins that were degraded per 10^6 cells/h. The absolute rates of collagen biosynthesis (white bars) were calculated as the sum of degraded and undegraded newly synthesized collagen as described under "Experimental Procedures." Results are mean ± S.E. p < 0.001 for biosynthesis and was not significant for degradation.

Since tumor-promoting phorbol esters and Ca2+ mobilization
similarly affect many cellular functions (15), we evaluated
the effect of phorbol dibutyrate on collagen production. This
phorbol ester was found to selectively inhibit collagen pro-
duction by normal human fibroblasts (Table II).

We also found that Ca2+ mobilization induced by 0.1 nM
CCK was associated with inhibition of the relative rate of
collagen production from control levels (−32.3 ± 6.6; p < 0.001). Concentrations of CCK as low as 10^{-12} M have an
inhibitory effect on collagen production.

**Extent of Proline Hydroxylation in Newly Synthesized Collagen**—Since it has been suggested that impaired proline
hydroxylation could lead to both decreased synthesis (43) and
Evidence of toxicity was noted at concentrations of A23187 phorbr A23187 on the absolute and relative rates of collagen production of collagen simultaneously (19). As expected, the AF2 fibroblasts incubated in the presence of α,α'-dipyridyl, after the addition of fresh DMEM with 10% fetal calf serum, analysis in human fibroblasts of the effects of the Ca⁺ ionophore A23187 was exponential up to a concentration of 1.0 μM. Likewise, recovery growth patterns for control and experimental fibroblasts were nearly identical for up to 14 days (25 μCi in Experiment 1 and 40 μCi in Experiment 2). Phorbr dibutyryl was added to the incubations at the described concentrations 1 h prior to the addition of the isotope.

We have found no evidence of cellular toxicity in AF2 cells treated with 0.6 μM A23187 for 4 h. Following the exposure of fibroblasts to the Ca²⁺ ionophore (0.6 μM), the release of lactic dehydrogenase, intact cell counts, and subsequent cell growth were similar to that of control cells. In addition, cellular functions such as protein transport and collagen proline hydroxylation were not affected by A23187. Finally, the extent of noncollagen protein production was only moderately affected by the Ca²⁺ ionophore. However, some degree of toxicity was observed at higher concentrations of A23187 (1 μM).

The major mechanism for the decreased net production of collagen was decreased biosynthesis, rather than increased degradation (Fig. 4). We found no significant effect of A23187 on the absolute rate of collagen degradation in normal human fibroblasts. Rodemann and co-workers (16) showed increased protein turnover in rat skeletal muscle treated with A23187. The apparent discrepancy between this study and ours could be explained in several ways. It is conceivable that the A23187-induced proteolysis does not affect either collagen proteins or human fibroblasts. Although Rodemann and co-workers found that the overall rates of protein degradation (nmol tyr/mg) were increased by the Ca²⁺ ionophore, the percentage of protein degradation (protein degradation/protein synthesis + degradation) was only slightly increased (5-11%) from control values (as calculated from data in Ref. 16), indicating that protein biosynthesis (the sum of degraded and undegraded newly synthesized proteins) was increased as well in rat skeletal muscle treated with A23187.

We have found a small effect of A23187 upon collagen hydroxylation, which was incorporated to calculate the rates of collagen degradation. The minor reduction in proline hydroxylation is unlikely to explain the effects of A23187 on collagen synthesis since complete blockage of proline hydroxylation in human fibroblasts with α,α'-dipyridyl did not affect the relative rate of collagen production.

Interestingly, the effect of A23187 on collagen production has also been observed in our laboratory in cultured human myofibroblasts from Dupuytren's dermic nodules (44), in primary rat hepatocyte cultures, and in human episceral fibroblasts. These findings suggest that inhibition of collagen production by the Ca²⁺ ionophore A23187 occurs in other types of cells incremented in the pathogenesis of tissue fibrosis. In addition, we found that Ca²⁺ mobilization induced by CCK is also associated with inhibition of collagen production. CCK, a hormone present in the intestine and brain (36, 37), is found in plasma at concentrations used in our experiments (45). Although the potential physiological relevance of these observations remains to be evaluated, the association of Ca²⁺ fluxes with a selective decrease in collagen polypeptide synthesis supports the role of Ca²⁺ in the modulation of collagen production. Further studies are needed to assess whether our findings are related to mobilization of Ca²⁺ from intracellular organelles (15) and to the Ca²⁺ requirement in the translational phase of protein synthesis (46).

The tumor-promoting phorbol esters, which directly activate a protein kinase C (47), have effects similar to those induced by Ca²⁺ mobilization on cellular function (15). We found that phorbol dibutyrate markedly inhibits collagen production in cultured normal human fibroblasts, in agreement with the effects of phorbol esters on collagen production in chick embryo fibroblasts (48) and mouse epidermal cells (49). Thus, the similar effects of A23187 and phorbol esters on collagen production further support the proposed regulatory role of Ca²⁺ on collagen metabolism.

<table>
<thead>
<tr>
<th>Phorbol dibutyryl concentration¹</th>
<th>Collagen²</th>
<th>Noncollagen protein²</th>
<th>Relative rate of collagen production²</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td>dpm/10⁶ cells/h</td>
<td>%</td>
<td>dpm/10⁶ cells/h</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>16.3 ± 1.7</td>
<td>285 ± 0.2</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>1</td>
<td>8.7 ± 1.6</td>
<td>254 ± 45.6</td>
<td>55 ± 2</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>45.6 ± 15.5</td>
<td>554 ± 18.8</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>1</td>
<td>26.5 ± 0.8</td>
<td>638 ± 20.4</td>
<td>51 ± 4</td>
</tr>
<tr>
<td>20</td>
<td>12.9 ± 13.9</td>
<td>348 ± 57.9</td>
<td>46 ± 4</td>
</tr>
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</table>

¹ Confluent cell cultures were studied in the presence of 0.2 mM ascorbic acid, and proteins were labeled with [5-³H]proline (0.1 mM) for 3 h (25 μCi in Experiment 1 and 40 μCi in Experiment 2). Phorbr dibutyryl was added to the incubations at the described concentrations 1 h prior to the addition of the isotope.

² Determined from the ³H radioactivity incorporated into collagen-sensitive and -resistant proteins. Values are mean ± one-half the range of duplicate samples.

³ Calculated by the formula: [³H]collagen dpm/([³H]noncollagen dpm × 5.4) + [³H]collagen dpm), and expressed as percentage of control values.

Increased degradation (28) of collagen, we analyzed the extent of proline hydroxylation in cultured fibroblasts. Cells were incubated with [4-³H]proline and [¹⁴C]proline, which allowed us to measure the extent of proline hydroxylation and the net production of collagen simultaneously (19). As expected, the percentage of proline hydroxylation was normal in cells incubated in the presence of ascorbate. The extent of proline hydroxylation in fibroblasts incubated with 0.6 μM A23187 was ~10% lower than control values (41.0 ± 1.0 versus 45.2 ± 0.7%; p < 0.01). These parameters were also examined in AF2 fibroblasts incubated in the presence of α,α'-dipyridyl, an inhibitor of prolyl hydroxylase. Although newly synthesized peptidyl proline was unhydroxylated in the presence of this inhibitor, the relative rate of collagen production was essentially unchanged when compared to fully hydroxylated control cells (98 ± 13 versus 100 ± 7%; not significant).

**Effects of A23187 on Cell Viability—**Cellular toxicity was assessed in both ionophore-treated and control AF2 fibroblasts. The release of cellular lactate dehydrogenase, a good indicator of cell damage (32), was less than 4% of total cellular lactate dehydrogenase for both cells treated with 0.6 μM A23187 and for control cells for up to 4 h. Similar results have been shown for viable rat submandibular acinar cells exposed to up to 1.0 μM A23187 for 40 min (32). Also, at the end of incubation with the calcium ionophore, the number of intact cells was comparable to that of control cells (data not shown). Likewise, recovery growth patterns for control and experimental fibroblasts were nearly identical for up to 14 days after the addition of fresh DMEM with 10% fetal calf serum. In all three systems for evaluating cellular integrity, some evidence of toxicity was noted at concentrations of A23187 greater than 1.0 μM.

**DISCUSSION**

The study reported here represents the first comprehensive analysis in human fibroblasts of the effects of the Ca²⁺ ionophore A23187 on the absolute and relative rates of collagen synthesis and degradation.

We have found that A23187 decreases the rate of collagen production relative to total protein production in cultured AF2 cells (Fig. 2). This selective inhibition of collagen production by A23187 was exponential up to a concentration of about 3 μM (Fig. 3); 50% inhibition of basal collagen production was achieved with 0.6 μM A23187.
A similar modulatory role for intracellular cAMP on collagen metabolism in normal human fibroblasts has been reported (3, 12). Prostaglandin E_2_ and cholera toxin were associated with higher levels of cAMP and an increase in the percentage of intracellular procollagen degradation (13). 

β-Adrenergic agonists together with phosphodiesterase inhibitors, presumably mediated by cAMP, also decreased collagen production (50). Interestingly, β-adrenergic stimulation of amino acid and hexose transport, and endocytosis in mouse kidney cortex were associated with increased Ca^{2+} fluxes (30).

As for many other cellular functions (15), intracellular Ca^{2+} as well as cAMP seems to modulate collagen production. Either increased cAMP or Ca^{2+} mobilization inhibits collagen production to a significant extent in cultured cells. Our findings suggest a newly recognized mechanism for the control of collagen production in normal human fibroblasts and provide new insights toward the understanding and regulation of tissue fibrosis.

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