Regulation of Collagen Production by the β-Adrenergic System

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ABSTRACT The suppression of collagen production by increasing the cyclic (c)AMP content of cultured cells was examined vis-à-vis the β-adrenergic system. Cultured human fetal lung fibroblasts incubated for 6 h with the β-agonists isoproterenol or epinephrine produced ~30% less collagen per cell than in the absence of the hormones. To demonstrate that the β-agonists were operating by their interaction with the β-receptor to stimulate adenylate cyclase to increase the intracellular content of cAMP, *d*- and *l*-isoproterenol were incubated separately with the cultured cells. Only *l*-isoproterenol increased intracellular cAMP and decreased collagen production. While 20 nM *l*-isoproterenol was effective, the *d*-isomer was ineffective even at 2μM. An increase in cAMP from 40 to 73 pmol/mg protein was effective in suppressing collagen production; increasing the cAMP content to much higher levels had little additional effect on collagen production. 3-Isobutyl-1-methylxanthine, an analog of theophylline that inhibits phosphodiesterase, potentiated the effect of isoproterenol in suppressing collagen production. Further support for the concept that isoproterenol suppressed collagen production by acting through the β-receptor was provided by the finding that only the *l*-isomer of propranolol, a β-blocker, was effective in blocking both the increase in intracellular cAMP and the suppression of collagen production caused by isoproterenol. These results demonstrate that collagen production in human fibroblasts can be regulated by the β-adrenergic system and indicate that when the cAMP content is increased beyond a threshold value, collagen production is suppressed. Since collagen production is sensitive to the small changes of cAMP content of cells brought about by β-stimulation in cultured cells, the results point to a possibly important mechanism for the regulation of collagen production in the body.

INTRODUCTION

Collagen is the major extracellular protein in connective tissues and is present to varying extents in all organs of the body (1–3). In most organs, fibroblasts are the major collagen-producing cells. Normally, fibroblasts from tissues such as skin and lung rigidly control the quantity of collagen they produce at 2–4% of their total protein production (4–6). It is known, however, that collagen production by fibroblasts can be influenced by numerous environmental factors, including hormones, metabolites, and pharmacologic agents (7–10). In addition, recent studies have shown that prostaglandins and cholera toxin dramatically elevate fibroblast cyclic AMP content and suppress collagen production in fibroblasts (11).

In vivo, the cyclic AMP content of cells is controlled, in part, by the β-adrenergic system (12). Since collagen production by fibroblasts has been shown to be inversely correlated with the cyclic AMP content of the cells, it is important to establish whether collagen can be influenced by changes in cyclic AMP achieved through β-agonist activity. In this context, fibroblasts exposed to a β-agonist would be expected to have elevated cyclic AMP levels and thus produce less collagen than they are capable of producing. However, if β-agonist activity were diminished or removed, more collagen would be produced. To evaluate this hypothesis, we have investigated effects of β-agonists, a β-blocker, and phosphodiesterase inhibition on collagen production by human lung fibroblasts.

METHODS

Cell cultures. Diploid fibroblasts derived from a fetal human lung (HFL-1, American Tissue Culture Collection No. CCL153) were stored frozen at passage five until used. The cells were maintained and subcultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Colorado

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Serum Co., Denver, Colo.), penicillin 100 U/ml, streptomycin 100 μg/ml (both from Grand Island Biological, Grand Island, N. Y.), and 0.06% glutamine as previously described (6, 11, 13). Cells detached by exposing the cultures to trypsin (0.25%, Grand Island Biological) for 15 min were counted with a hemocytometer.

Incubation of cells with hormones. To evaluate the effect of β-agonists on collagen production, cells were plated at 10^6 cells/100-mm culture plate and maintained in growth medium for 5 d until the cells had become confluent. The Dulbecco's medium was removed, and the plates were incubated for 6 h in the presence of labeling medium (Hank's balanced salt solution [National Institutes of Health media unit] containing 16 μCi [^{14}C]proline [265 mCi/mmol, Schwarz/Mann, Orangeburg, N. Y.], 40 μg/ml ascorbate, and 40 μg/ml β-aminopropionitrile). Parallel plates were incubated for 6 h in labeling medium alone, with labeling medium plus 0.2 μM *d, l*-isoproterenol, or with labeling medium plus 200 μM epinephrine. To evaluate the effect of β-agonists on intracellular cyclic AMP levels, identical incubations were carried out, but 2 mM [^{12}C]-proline was substituted for the [^{14}C]proline.

To evaluate the stereospecificity of β-agonists on collagen production and cyclic AMP levels of cultured fibroblasts, incubations were carried out as described above in the presence of *d*- or *l*-isoproterenol in concentrations from 2 nM to 20 μM. In additional studies, parallel cultures were incubated with 2 μM *d, l*-isoproterenol in the presence of the *d*- or *l*-isomers of the β-blocker propranolol, in concentrations from 20 nM to 2 μM.

To evaluate the effect of phosphodiestrerase inhibition in potentiating β-agonist-induced suppression of collagen production, plates were incubated as described above in the presence of 40 μM 3-isobutyl-1-methylxanthine (IBMX)[1], a theophylline analog, together with *d*- or *l*-isoproterenol 20 nM to 2 μM. The effect of propanolol on collagen production by cells treated with isoproterenol plus IBMX was evaluated by incubating plates in labeling medium alone or with various combinations of *d, l*-isoproterenol (2 μM), IBMX (40 μM), and *d, l*-propranolol (2 μM).

Quantification of collagen production. Following a 6-h incubation with labeling medium in the presence or absence of hormones as described above, collagen production was measured in combined cell layers plus medium using methods described previously (6, 11). Collagen production[2] was expressed in two ways: (*a*) as a percentage of total protein production; and (*b*) as an absolute amount of collagen produced. The first method used purified Clostridial collagenase digestion to quantify the [^{14}C]proline incorporated into collagen compared with the [^{14}C]proline incorporated into total protein (6, 11, 14). Briefly, the method consisted of scraping the cells from a single culture dish into a plastic tube, using the medium to rinse the plate. The sample was heated to 100°C for 15 min to destroy proteolytic enzymes, sonicated, and dialyzed to remove the unincorporated [^{14}C]proline. The nondialyzable labeled protein was then lyophilized and resuspended in 2.2 ml of 5 mM $CaCl_2$, 2.5 mM *N*-ethyl maleimide and 10 mM Tris-HCl, pH 7.5. A l-ml aliquot was incubated with 50 U of purified bacterial collagenase (Advanced Biofactures, Lynbrook, N. Y., type III) for 12 h at 20°C in a dialysis bag. The sample was dialyzed against 10 ml H_2O, and the radioactivity in both the dialysate and dialysand was determined. The percentage of collagen in the sample was calculated as the amount of radioactivity sensitive to collagenase divided by the total radioactivity in the sample, multiplied by a correction factor to convert the amount of [^{14}C]proline digested to [^{14}C]-procollagen sensitive to collagenase (6). Evaluation of this fraction of hydroxy[^{14}C]proline digested with collagenase was used to estimate the efficiency of collagenase digestion (6). The second method used a combination of collagenase digestion and quantification of the specific activity of the intracellular pool of free [^{14}C]proline to determine the moles of [^{14}C]proline incorporated into collagen per cell per unit time (6).

Quantification of intracellular cyclic AMP. To determine the intracellular concentration of cyclic AMP, cells were scraped in 5 ml of 10% trichloroacetic acid. After centrifugation, duplicate samples of supernate were taken for determination of cyclic AMP using a protein-binding assay (15). Protein was determined by the method of Lowry et al. (16).

Statistical methods. The data are presented as the mean ±SE. Differences in collagen production in various experimental conditions were evaluated using the Wilcoxon, Mann-Whitney Rank Sum test for nonparametric analysis, accepting $P < 0.05$ as significant (17).

RESULTS

The β-agonists isoproterenol and epinephrine suppressed collagen production in human lung fibroblasts by 30% ($P < 0.01$) (Fig. 1A). The suppression of colla-

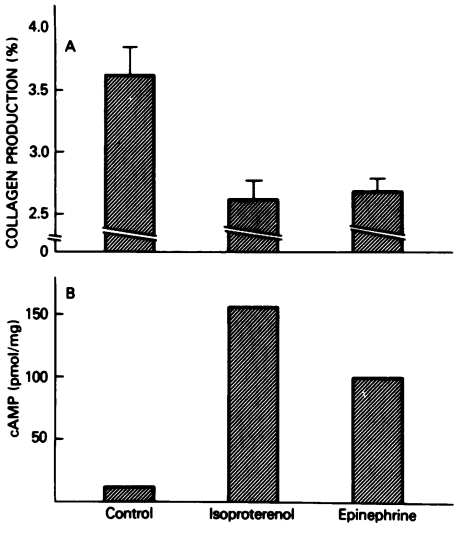

FIGURE 1 The effect of the β-agonists isoproterenol and epinephrine on collagen production and cAMP content of human lung fibroblasts. Cells were incubated for 6 h alone or in the presence of isoproterenol (2 μM) or epinephrine (200 μM). (A) Collagen production expressed as a percentage of total protein production. Each bar represents the average of six culture plates; the vertical lines indicate the standard error of the mean. Percent collagen production was significantly reduced in the presence of isoproterenol ($P < 0.05$) or epinephrine ($P < 0.05$). (B) cAMP content of cultures parallel to those in (A); each bar represents an average of two plates. Half the range averaged < 6.5% of the mean. The data are expressed as picomoles cAMP per milligram cell protein.

[1] *Abbreviations used in this paper*: c, cyclic; IBMX, 3-isobutyl-1-methylxanthine.

[2] Fibroblasts actually produce collagen in a precursor form termed "procollagen" or "pre-procollagen" (1, 2). The methods used here quantify procollagen production (6), but for convenience the term "collagen" is used to describe the macromolecule.

gen production correlated with an increase in the intracellular content of cyclic (c)AMP (Fig. 1B). To determine if the suppression of collagen production relative to total protein production was specific for collagen, the specific activity of the intracellular pool of [^{14}C]proline was used to calculate the number of moles of proline incorporated into intact collagen and into total, nondialyzable cellular protein. Treatment of the cultures with isoproterenol or epinephrine did not affect the cell number or the specific activity of intracellular proline (not shown). The β-agonist did not affect the amount of proline incorporated into total cellular protein (Fig. 2A). However, the absolute amount of proline incorporated into collagen was significantly decreased in cells treated with either isoproterenol or epinephrine ($P < 0.01$) (Fig. 2B).

To determine if the β-agonists were operating through an interaction with β-receptors, a comparison was made between *d*- and *l*-isoproterenol in suppressing collagen production and in increasing the intracellular content of cAMP (Fig. 3). The lowest concentration of *l*-isoproterenol that was effective in decreasing collagen production was 20 nM, a concentration that also raised the intracellular cAMP content of the fibroblasts. In contrast, *d*-isoproterenol had no effect on collagen production and little effect on cAMP even at 2 μM.

Further support for a specific interaction between isoproterenol and the β-receptor in modulating collagen production was obtained by testing the ability of the β-blocker propranolol (18) to inhibit the effect of isoproterenol on suppressing collagen production. Cells incubated with 2 μM *d*, *l*-isoproterenol demonstrated a reduced production of collagen from 3.8±0.1 to 2.6±0.2% (Fig. 4A). When the cells were incubated with 2 μM *d*, *l*-isoproterenol and 20 nM *l*-propranolol, collagen production was not suppressed and remained at ~ 3.7±0.1% ($P < 0.5$ compared with control). Higher concentrations of *l*-propranolol up to 2 μM were similarly effective in preventing the suppression of collagen production by isoproterenol. In contrast, the *d*-

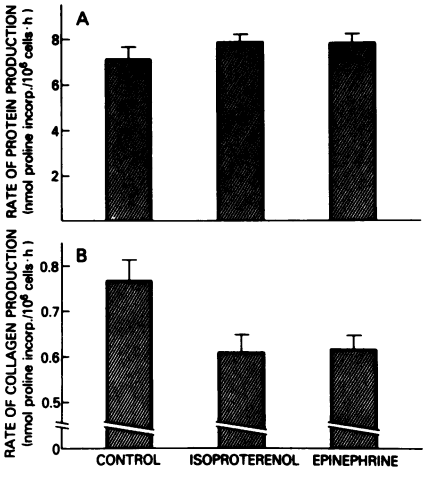

FIGURE 2 The effect of the β-agonists isoproterenol and epinephrine on total protein production and collagen production of human lung fibroblasts. Incubation conditions were identical to those described in Fig. 1. Both overall protein production and collagen production were determined using methods which measure the specific activity of the tracer isotope [^{14}C]proline within the cultured cells, thus accounting for possible differences in tracer specific activity caused by the presence of a β-agonist (6). (A) Rate of total protein production expressed as nanomoles proline incorporated into protein per 10^6 cells per hour. Neither isoproterenol nor epinephrine caused changes in total protein production by the cultured fibroblasts ($P < 0.3$, both comparisons). (B) Rate of collagen production expressed as nanomoles proline incorporated into collagen per 10^6 cells per hour. In the presence of isoproterenol or epinephrine, the rate of collagen production was significantly suppressed ($P < 0.05$, both comparisons). For the control cells, this is equivalent to 5×10^5 procollagen chains/cell per h. In both panels, the bars represent the average of six culture plates; the vertical lines represent the standard error of the mean.

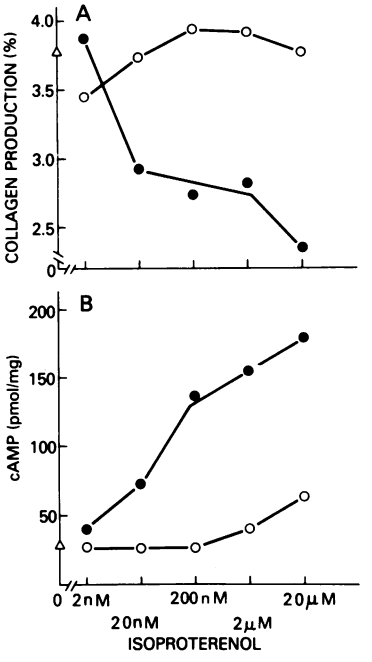

FIGURE 3 The effect of *d*- and *l*-isoproterenol on collagen production and cAMP levels of cultured human fibroblasts. Culture conditions were similar to those in Fig. 1. (A) Collagen production expressed as a percentage of total protein production in the presence of increasing levels of *d*-isoproterenol (○) or *l*-isoproterenol (●). Each data point represents an average of two culture plates; the average level of collagen production found in cells cultured in the absence of isoproterenol is also indicated (△). (B) cAMP levels in parallel cultures expressed as picomoles cAMP per milligram cell protein; each data point represents an average of two culture plates. ○, cAMP in the presence of *d*-isoproterenol ●, cAMP in the presence of *l*-isoproterenol. The average level of cAMP in cells cultured in the absence of isoproterenol is also indicated (△). For the cAMP determination, half the range averaged <6.5% of the mean.

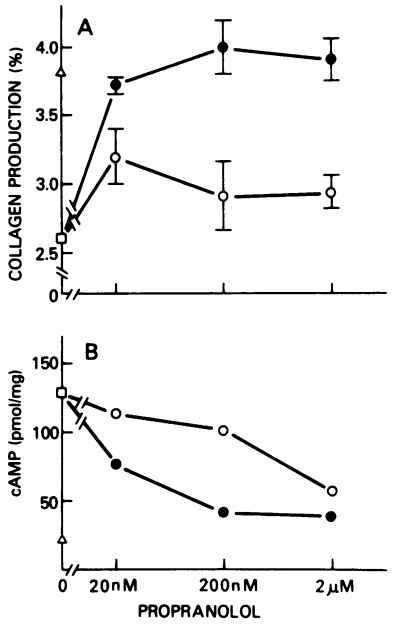

FIGURE 4 The effect of *d*- and *l*-propranolol on the cAMP levels and isoproterenol-induced suppression of collagen production of human fibroblasts. Culture conditions were similar to those in Fig. 1. (A) Collagen production expressed as a percentage of total protein production in the presence of 2 μM *d*, *l*-isoproterenol plus increasing levels of *d*-propranolol (○) or 2 μM *d*, *l*-isoproterenol plus increasing levels of *l*-propranolol (●). Each data point represents an average of four culture plates. The average collagen production in the absence of isoproterenol or propranolol is indicated (△). As shown in Fig. 1, collagen production in the presence of *d*, *l*-isoproterenol but not propranolol is suppressed (□) ($P < 0.05$). In the presence of *d*, *l*-isoproterenol, increasing levels of *d*-propranolol had no effect on the collagen production ($P < 0.2$, all levels of *d*-propranolol); however, increasing levels of *l*-propranolol did have an effect compared with no propranolol ($P < 0.05$ at all levels) and compared with the effect of *d*-propranolol) ($P < 0.05$). (B) cAMP levels in parallel cultures expressed as picomoles cAMP per milligram cell protein; each data point represents an average of two culture plates. For the cAMP determinations, half the range averaged <8% of the mean. The average cAMP levels in the absence of isoproterenol and propranolol (△), in the presence of isoproterenol (□), and in the presence of isoproterenol and increasing levels of *d*-propranolol (○) or *l*-propranolol (●) are shown.

isomer of propranolol was not effective in preventing the suppression of collagen production by isoproterenol. Isoproterenol in the presence of 2 μM *d*-propranolol still suppressed collagen production to 2.9±0.1% from the control value of 3.8±0.1% ($P < 0.05$). In parallel with these observations, isoproterenol increased intracellular cAMP content from 23 to 130 pmol/mg protein (Fig. 4B). Propranolol by itself had no significant effect on cAMP (see below), but the addition of as little as 20 nM *l*-propranolol simultaneously with isoproterenol partially inhibited the β-agonist-induced accumulation of cAMP to 66 pmol/mg. In contrast, the *d*-isomer of propranolol had little effect in reducing the level of cAMP even at 200 nM.

Further support for a central role of cAMP in suppressing collagen production in fibroblasts was demonstrated by studying the effect of IBMX, an inhibitor of phosphodiesterase, on collagen production and cAMP levels. Cultures of fibroblasts were incubated in the presence of 0.02–2 μM *d*, *l*-isoproterenol with or without 40 μM IBMX. The presence of IBMX caused cAMP content to increase beyond what it would have been with isoproterenol alone (Fig. 5). For example, in the presence of 20 nM *d*, *l*-isoproterenol (a concentration that had little effect of its own, Fig. 3) and 40 μM IBMX, the cAMP content was increased and the percent collagen production was decreased compared with the effect of this concentration of isoproterenol alone. In fact, at every concentration of isoproterenol tested, IBMX potentiated the effect of the β-agonist on cAMP content

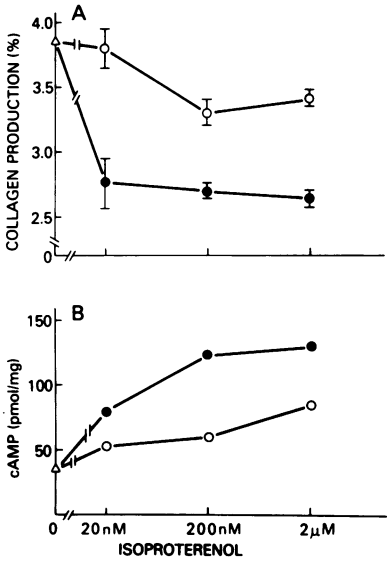

FIGURE 5 The potentiation of β-agonist-induced suppression of fibroblast collagen production cAMP levels by phosphodiesterase inhibition. Culture conditions were similar to those described in Fig. 1. (A) Collagen production expressed as a percentage of total protein production. Shown are control cultures (△) and cultures incubated in the presence of increasing levels of *d*, *l*-isoproterenol (○) or *d*, *l*-isoproterenol plus 40 μM IBMX (●). Each data point represents an average of three culture plates. At 0.2 μM, isoproterenol significantly suppressed collagen production compared with control cultures ($P < 0.05$ at all levels). With the addition of IBMX, significant collagen suppression was seen at all isoproterenol levels ($P < 0.05$ compared with controls, all levels) and IBMX potentiated the suppression observed with isoproterenol alone ($P < 0.05$, all levels of isoproterenol). (B) cAMP levels in parallel cultures are expressed as picomoles cAMP per milligram cell protein; each data point represents an average of two culture plates. Half the range averaged <10% of the mean. △, cAMP levels in control cultures ○, cAMP levels in the presence of *d*, *l*-isoproterenol; ●, cAMP levels in the presence of isoproterenol plus IBMX.

and collagen production. The added effect of IBMX on the isoproterenol suppression of collagen production was also evaluated in the presence and absence of propranolol (Fig. 6). Collagen production was reduced from 3.3±0.1% to 2.1±0.1% by isoproterenol and IBMX ($P < 0.01$). Propranolol partially blocked this suppression of collagen production (2.8±0.1%, $P < 0.01$ compared with isoproterenol plus IBMX), but did not cause collagen production to return to normal. This was likely due to the fact that propranolol was not able to block the effect due to IBMX alone (2.7±0.1%, $P < 0.01$ compared with control), a finding consistent with IBMX serving to maintain the level of endogenous cAMP through its inhibition of phosphodiesterase and not through an effect on the β-receptor.

DISCUSSION

Human lung fibroblasts rigidly control their collagen production both during their growth to confluency and over many population doublings (6). However, collagen production in these cells (19), as well as in human skin fibroblasts (11), is sensitive to agents which increase intracellular cAMP content. The present study demonstrates that the specific interaction of β-agonists for the fibroblast β-receptor can be used to elevate intracellular cAMP and decrease collagen production. Changes in cAMP content of cells by as little as a factor of two are effective in suppressing collagen production (Figs. 3 and 5). Increasing the cAMP content by an order of magnitude or more (11, 19) has no additional effect on the suppression of collagen production. Since only the *l*-isomer of isoproterenol was effective, and since the effect could be blocked by the *l*-isomer of propranolol, a β-blocker, the interaction appears to be specific for the β-receptor-adenylate cyclase system. Thus, collagen production in cultured human lung fibroblasts can be regulated by the β-adrenergic system operating through relatively small changes in intracellular cAMP.

The mechanism for increased cAMP in suppressing collagen production is unknown. However, since a variety of agents which operate through different mechanisms to raise cAMP all suppress collagen production (11, 19), cAMP apparently plays a central role. Support for the hypothesis that the effect of isoproterenol on collagen production is expressed through intracellular cAMP content was the finding that the effect was potentiated by IBMX, a theophylline analog which inhibits phosphodiesterase and therefore acts to maintain levels of intracellular cAMP (20). Although *l*-propranolol was able to block the effect of *l*-isoproterenol, it was not able to block the effect due to IBMX itself. This finding is consistent with IBMX serving to maintain a higher intracellular cAMP content through its inhibition of phosphodiesterase, and further demonstrates the importance of cAMP in the suppression of collagen production.

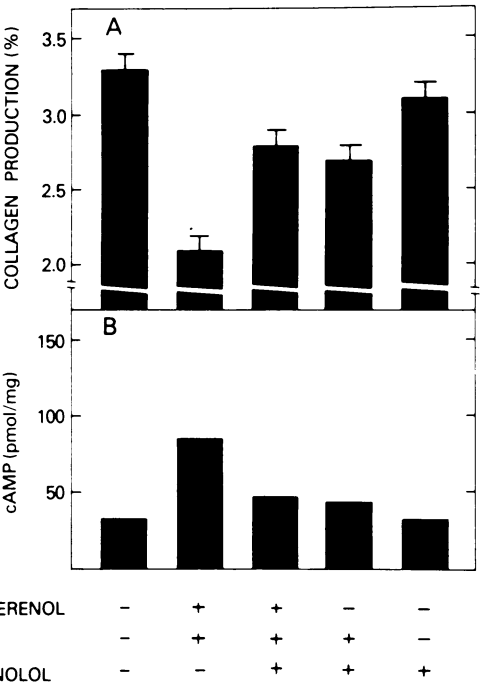

FIGURE 6 The combined effects of a β-agonist, a β-blocker, and a phosphodiesterase inhibitor on collagen production and cAMP levels of cultured human fibroblasts. Culture conditions were similar to those described in Fig. 1. (A) Collagen production in the presence or absence of *d*,*l*-isoproterenol (20 nM), *d*,*l*-propranolol (200 nM), and IBMX (40 μM). Collagen production is expressed as a percentage of total protein production. Each bar represents the average of six cultures; vertical lines represent the standard errors of the mean. Compared with controls, collagen production was significantly suppressed by isoproterenol plus IBMX ($P < 0.01$), isoproterenol plus IBMX plus propranolol ($P < 0.01$), IBMX plus propranolol ($P < 0.01$), but not by propranolol alone ($P < 0.3$). (B) cAMP levels in parallel cultures expressed as picomoles cAMP per milligram cell protein; each data point presents an average of two culture plates. For the cAMP determinations, half the range averaged <4% of the mean.

Since collagen production can be specifically suppressed in cultured diploid fibroblasts by β-adrenergic agents, it is reasonable to hypothesize that fibroblasts in vivo may also be affected by β-adrenergic activity. In this context, collagen production in the body would be in part under the control of endogenous β-adrenergic activity and affected fibroblasts would produce less collagen than they are capable of synthesizing. Since collagen production appears to be inversely correlated with intracellular cAMP content, agents which affect cAMP even in physiological conditions would be able to specifically influence collagen production. If this is the case in vivo, it points to the possibility of a new therapy for fibrotic conditions such as keloids, progressive systemic sclerosis, or idiopathic pulmonary fibro-

sis. In such disorders, collagen production might be suppressible by pharmacological concentrations of β-agonists such as isoproterenol, or phosphodiesterase inhibitors such as IBMX.

The finding that the β-blocker *l*-propranolol can block the effect of β-agonists on collagen production not only supports the conclusion that the β-adrenergic receptor-adenylate cyclase system regulates collagen production, but also may have important pharmacological consequences for the regulation of collagen production in the body. All of the β-blocking agents that have been used clinically, including propranolol, practolol, metoprolol, timolol, and atenolol, have been reported to cause fibrosis (21–26). Since the concentration of propranolol used in these experiments was similar to that which is effective in vivo (27), the observations showing that collagen production by cultured fibroblasts can be regulated by the β-adrenergic system may have physiological consequence. If the β-adrenergic system normally suppresses collagen production by fibroblasts, then when β-blockers are administered to susceptible individuals, certain of their fibroblasts may not possess adequate alternative mechanisms to regain control over collagen production. This situation, if unchecked, may lead to an overproduction of collagen per cell, eventually resulting in fibrosis in the area so affected.

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