β-Adrenergic Agonist Hyperplastic Effect Is Associated with Increased Fibronectin Gene Expression and Not Mitogen-Activated Protein Kinase Modulation in C_2C_{12} Cells (44495)

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Abstract. **β-Adrenergic agonists (β-AA) enhance protein accretion in skeletal muscles. This stimulation is characterized by increased protein synthesis, increased expression of myofibrillar protein genes and a depression in protein degradation in animals, and increased proliferation and DNA synthesis in muscle cells *in vitro*. The mechanism or signal path in muscle whereby β-AA would elicit these physiological effects upon binding to the G protein–coupled β-adrenergic receptor (β-AR) is unclear. C_2C_{12} myoblasts were used to determine β-AR ligand binding characteristics, cyclic AMP synthesis in response to isoproterenol (ISO) stimulation, and effects of ISO on DNA synthesis, mitogen activated protein kinase (MAPK), and fibronectin (FN) gene expression. Results showed that C_2C_{12} cells possess β-AR which are specific, saturable, and of high affinity (K_d = 0.2 n*M*). Forskolin and ISO stimulated cAMP production by ≈ 20-fold ($P < 0.001$) and 17-fold ($P < 0.001$), respectively. ISO and the cAMP analog, 8-bromo-cAMP (8-BC) stimulated DNA synthesis in proliferating cells by 150% ($P < 0.05$) and 200% ($P < 0.01$), respectively, without modulating MAPK activity, whereas addition of fetal bovine serum to culture resulted in a 500% increase ($P < 0.01$) in DNA synthesis and MAPK activation. DNA synthesis in C_2C_{12} cells treated with ISO, 8-BC, or FBS was abolished in the presence of 25 μ*M* PD098059, an MAPK-kinase inhibitor, suggesting that an MAPK-dependent pathway is likely involved in C_2C_{12} proliferation. During cAMP elevating agent stimulation, basal MAPK activity may be sufficient, in the presence of other putative signaling molecules, to support proliferation in these cells. ISO or 8-BC treatment increased FN mRNA by three- and seven-fold, respectively, in growing C_2C_{12} cells implying a connection between increased DNA synthesis and FN gene expression.** [P.S.E.B.M. 2000, Vol 223]

Oral administration of selected β-adrenergic agonists (β-AA) to rodents and livestock increases skeletal muscle mass and diminishes body fat depots (1–5). Likewise, β-AA increase protein accumulation in cultured myotubes (6, 7) and depress fat accumulation in adipose cells in culture (8). These β-AA effects can be reversed with the β-antagonist propranolol (6, 8). In growing pigs fed adequate dietary protein, β-AA enhance skeletal muscle mass *via* hypertrophy as evidenced by parallel changes in skeletal muscle mass and protein and RNA content, with increased RNA/DNA and protein/DNA ratios (1). This hypertrophy is typified by enhanced expression of skeletal muscle myofibrillar genes (α-actin; myosin light chains) (9, 10), increased muscle protein synthesis (1, 3, 5), and attenuation of muscle protein degradation (3, 5, 11). An increase of total semitendinosus DNA, an indication of *de novo* DNA synthesis and hyperplasia, was noted in one study with sheep fed the β-AA cimaterol (12). This increase in DNA in postnatal-differentiated skeletal muscle was likely due to proliferation of satellite cells (13).

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The membrane-intracellular signaling cascade initiated by β-AA binding to the β-adrenergic receptor (β-AR) of muscle cells that results in enhanced skeletal muscle protein accretion is not well understood (3, 5). In contrast, the signaling pathway responsible for diminished fat deposition by β-AA has been documented. When β-AA bind to membrane β-AR in adipose cells, the G_s protein → adenylyl cyclase → cAMP → protein kinase A pathway is activated resulting in increased lipolysis and depressed lipogenesis (3, 14, 15).

Ractopamine [a β-AA] and the cAMP analog dibutyryl-cAMP increase protein synthesis in L_6 myoblasts (7). Both dibutyrlyl-cAMP and forskolin appeared to increase mitogen-activated protein kinase (MAPK) activity in L_6 myoblasts (7). The MAPkinases are critical components in cell surface receptors-*ras* signal transduction pathways that are involved in transcriptional regulation of cell growth and development (16, 17). Addition of β-AA isoproterenol and ractopamine to chick breast muscle satellite cells enhanced the cell proliferation rate (18); this effect was blocked by the β-antagonist propranolol suggesting involvement of functioning β-AR (18). Basic fibroblast growth factor (bFGF) promotes proliferation of skeletal muscle (19), chick breast and porcine satellite cells (18, 20), and other cell lines (21, 22). Recent work has indicated that cell surface heparan sulfate proteoglycans, such as fibronectin (FN), are involved in bFGF cell signaling and appear also to play a role in proliferation of satellite cells (23, 24). Others have reported that β-AA/cAMP elevating agents stimulate gene expression of the extracellular protein fibronectin via cAMP-PKA mediated events (25–27). Thus, we hypothesized that the β-AA effect on muscle cell proliferation/growth may be mediated by direct or indirect β-AR → Heterotrimeric G proteins → MAPK activation and/or *via* cAMP-stimulated FN gene expression.

Experiments described here used C_2C_{12} myoblasts to study β-AR function and associated signal transduction. We first examined ligand binding characteristics of C_2C_{12} β-AR, followed by cAMP response to ISO stimulation and finally examined the effect of ISO on DNA synthesis, MAPK involvement, and fibronectin mRNA abundance/gene expression.

Materials and Methods

Materials. [γ^{32}P]ATP (3000 Ci/mmole) and [^{3}H]CGP12177 (42.5 ci/mmole) were purchased from DuPont NEN (Boston, MA); fetal bovine serum (FBS) from Hyclone Laboratories Inc. (Logan, UT); Dulbecco's modified Eagle's medium (DMEM), Phosphate-Buffered Saline (PBS), antibiotic-antimycotic (ABAM) and gentamicin from Gibco BRL (Grand Island, NY); cAMP assay kits from Diagnostic Products Corporation (Los Angeles, CA); BCA protein assay kits from Pierce (Rockford, IL); extracellular signal-regulated kinase (ERK)-2 polyclonal antibody from Santa Cruz Biotechnology (Santa Crus, CA); PD 098059 from Calbiochem (LaJolla, CA); [^{3}H]thymidine (1MCi/ml) from ICN Pharmaceutical (Irving, CA); scintillation cocktail (Sentiserve, SX18-4) and Whatman GF/F filters from Fisher Scientific (Pittsburgh, PA); tissue culture plates and dishes from Corning Glass Works (Corning, NY); isoproterenol, 100X pen/strep, HEPES, MOPS, insulin, 4-(3-butoxy-4-methoxy-benzyl) imidazolidin (cAMP phosphodiesterase inhibitor), myelin basic protein (MBP), forskolin (F-8668), phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and 8-bromo cAMP from Sigma Chemical Co. (St. Louis, MO). Fibronectin cDNA was a gift from Dr. K. Yamada (NDIR, NIH, Bethesda, MD). The S14 protein cDNA was a gift from Dr. P.E. Ray (Children's National Medical Center, Washington, DC).

C_2C_{12} Culture. Mouse C_2C_{12} myoblasts (obtained from the American Type Culture Collection; ATTC, Rockville, MD) were cultured in DMEM containing 10% FBS and 0.5% ABAM, 0.1% gentamicin or 1% pen-strep-fungisome mixture and grown in a humidified incubator under an atmosphere of 95% air and 5% CO_2 at 37°C to confluency. Fresh medium was supplied every 48 hr. Upon confluence (4 days) differentiation was induced by switching cells to DMEM containing 2% FBS and 10^{-6} *M* insulin (differentiation medium) for 72 hr before returning cells to the growth medium (DMEM + 10% FBS) for 24 hr.

Membrane Preparation. Cell membranes were prepared essentially as described by Hausdorff *et al.* (28). Medium was aspirated from differentiated cells, and monolayers were washed with ice-cold PBS, harvested, and resuspended in 5-m*M* Tris (pH 7.4) 2-m*M* EDTA, approtinin (20 μg/ml), leupeptin (20 μg/ml) buffer. Cells were lyzed with a polytron homogenizer (four bursts for 5 sec at maximum setting) on ice. Lysates were centrifuged at 200*g* for 20 min at 4°C to remove organelles and unbroken cells; supernatants were centrifuged at 40,000*g* for 20 min at 4°C. The resulting pellets were washed once with 5 m*M*-Tris (pH 7.4) 2 m*M*-EDTA buffer, and re-suspended in 10 m*M* Tris-HCl (pH 7.4), 5 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, and 10% glycerol. Aliquot samples were taken for protein content determination (29). Membrane suspensions were rapidly frozen in liquid nitrogen and maintained at – 80°C for use within 1 week.

β-Adrenergic Receptor Radioligand Binding Assays. Assays were conducted using the hydrophilic nonselective β-adrenergic antagonist [^{3}H]CGP12177 (30). Membrane suspensions were incubated at 30°C for 2 hr in a shaking water bath with increasing concentrations (0.45 n*M*–15.6 n*M*) [^{3}H]CGP12177 (specific activity 42.5 Ci/mmole) with or without a 1000-fold, molar excess of unlabeled CGP12177. Incubations were stopped by addition of ice-cold PBS, followed by rapid vacuum filtration of the suspension through GF/F glass fiber filters. Filters were then washed twice with ice-cold PBS and placed in scintillation vials containing scintillation cocktail. After gentle shaking for 20 min, the vials were counted for ^{3}H activity with a scintillation counter.

β-Adrenergic Stimulated cAMP Accumulation. Medium was aspirated from differentiated cells, and monoloyers were washed with PBS. Monolayers were then overlaid with PBS (37°C) containing the following treatments: ISO at 10^{-5}, 10^{-7}, and 10^{-9} *M*; forskolin (positive control) at 5×10^{-5} *M*, and negative control (no treatments). A cAMP phosphodiesterase inhibitor (10^{-6} *M*) was added to all incubations to prevent enzymatic degradation of cAMP. Culture dishes were placed in a humidified incubator under an atmosphere of 95% air and 5% CO_2 at 37°C for 5–10 min. After the incubation, aliquots of incubation medium were quickly removed, chilled, and stored at –20°C for cAMP determination (within 3 days). Cells were washed and frozen for later protein determination (29). The cAMP assays were done as prescribed by the manufacturer of the cAMP assay kit; this procedure is patterned after a procedure described by Tovey *et al.* (31). The procedure entails a competition of ^{3}H cAMP and free cAMP for cAMP binding protein, separation of free (unbound) cAMP by adsorption onto dextran-coated charcoal, followed by counting of bound ^{3}H cAMP by liquid scintillation. Preliminary work showed that stimulation of the β-AR adenylyl-cyclase system in C_2C_{12} myoblasts in the presence of 4-(3-butoxy-4-methoxy-benzyl) imidazolidin (a phosphodiesterase inhibitor) resulted in cAMP accumulation within the cells coupled with some accumulation in the incubation media (PBS). Thus, cAMP production was determined as the sum of assayable cAMP in the PBS and within the cells. Time course studies showed a linear cAMP production response up to 10 min; others (32) have used 10 min incubations to assess cAMP production in L_6 cells.

DNA Synthesis. Mitogenesis was measured in subconfluent C_2C_{12} myoblasts by [^{3}H]thymidine incorporation. Cells were serum-starved overnight and then treated with one of the following agents diluted in serum-free DMEM containing either 0 or 25 μ*M* PD098059; ISO (10^{-5} *M*), 8-bromo-cAMP (8-BD; 10^{-3} *M*) (33), 10% FBS (positive control), and no treatment (negative control). Cells and treatments were incubated for 18 hr as described for C_2C_{12} growth. After this initial incubation, 1 μm Ci/ml [^{3}H] thymidine was added to the cells, and dishes were incubated for an additional 4 hr. All incubations were terminated by aspirating culture medium, washing cells with cold PBS, followed by addition of ice-cold 10% trichloroacetic acid (TCA). TCA-treated cells were washed with ice-cold water and solubilized in 0.5 *M* NaOH at 37°C. Upon solubilization, appropriate aliquot samples were removed and transferred to scintillation vials. Scintillation cocktail was added, and ^{3}H activity was quantitated by liquid scintillation.

MAPK Assay. Subconfluent C_2C_{12} cells were serum-starved overnight and then treated with one of the following agents diluted in serum-free DMEM containing either 0 or 25 μ*M* PD0980059: ISO (at 10^{-9}, 10^{-7}, 10^{-5} *M*; 8-BC, 10^{-3} *M*, 10% FBS (positive control), no treatment (negative control). After a 10-min incubation, medium was aspirated, cells were washed with cold PBS and lyzed in: 20 m*M* HEPES (pH 7.5) with 10 m*M* EGTA, 40 m*M* glycerophosphate, 1% NP-40, 2.5 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 2 m*M* sodium vanadate, 1 m*M* phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. Lysates were scraped into Eppendorf tubes and centrifuged. The clarified lysates were recovered and immunoprecipitated with ERK-2 antibody for 1 hr at 4°C, and immunocomplexes were recovered using protein G-sepharose. Pellets were washed three times with PBS supplemented with 1% NP-40 and 2 m*M* sodium vanadate, once with 0.5 *M* LiCl in 100 m*M* TRIS (pH 7.5), and once in kinase reaction buffer containing 12.5 m*M* MOPS (pH 7.5), 12.5 m*M* glycerophosphate, 7.5 m*M* $MgCl_2$, 0.5 m*M* EGTA, 0.5 m*M* sodium fluoride, and 0.5 m*M* sodium vanadate. MAPK (ERK) activities were determined using its substrate MBP. Kinase reactions were carried out in kinase reaction buffer containing 1 μCi of [^{32}P] ATP/reaction, 20 μ*M* unlabeled ATP, and MBP at 30°C for 30 min (34). Reactions were halted by the addition of 5X Laemmli buffer; samples were then boiled and electrophoresed in a polyacrylamide gel (12% total monomer). Phosphorylated MBP was visualized by autoradiography and quantitated with a densitometer.

Northern Analysis. Fibronectin mRNA abundance was determined in C_2C_{12} myoblasts grown in the presence of ISO (10^{-5} *M*) or 8-BC (10^{-3} *M*). Total RNA was extracted from subconfluent cells using guanidine thiocyanate-phenol-chloroform (35), and total RNA (15 μg/sample) was subjected to denaturing formaldehyde-1% agarose gel electrophoresis. Following electrophoresis, RNA was transferred to nylon filters (34). Filters containing RNA were prehybridized in buffer containing 50% formamide, 0.1% SDS, 5X SSPE, 100 μg/ml sheared salmon DNA, and 2X Denhardt's solution for 2 hr at 37°C. cDNA probes were labeled with [^{32}P] ATP using 5′ end labeling procedures (34). A ^{32}P-labeled fibronectin cDNA probe (2.2-kb EcoR1 fragment of a human fibronectin cDNA clone, pFH154) and nylon filters were then incubated in hybridization buffer for 18 hr. Following hybridization, filters were washed twice in 6X SSPE, 0.1% SDS at room temperature for 15 min followed by two additional washes in 1X SSPE, 0.1% SDS for 15 min at 37°C. Filters were dried and exposed to Kodak XAR-2 film at –70°C for 3 days. Equal RNA loading was confirmed with the S14 protein cDNA probe (data not shown) (36).

Statistical Analyses. Results were analyzed statistically using ANOVA (37). Scatchard analyses of ligand binding data were conducted as previously outlined (38). Comparisons between treatment means, where applicable, were achieved with the student-Neuman-Keul's test. Data were plotted using Cricket graph programs that do not show SD in bar graphs less than 10% of mean.

Results

Specific binding of the radioligand [^{3}H]CGP12177 to C_2C_{12} membrane preparations was saturable, reversible (Fig. 1) and of high affinity (K_D = 0.2 ± 0.035 n*M*) as

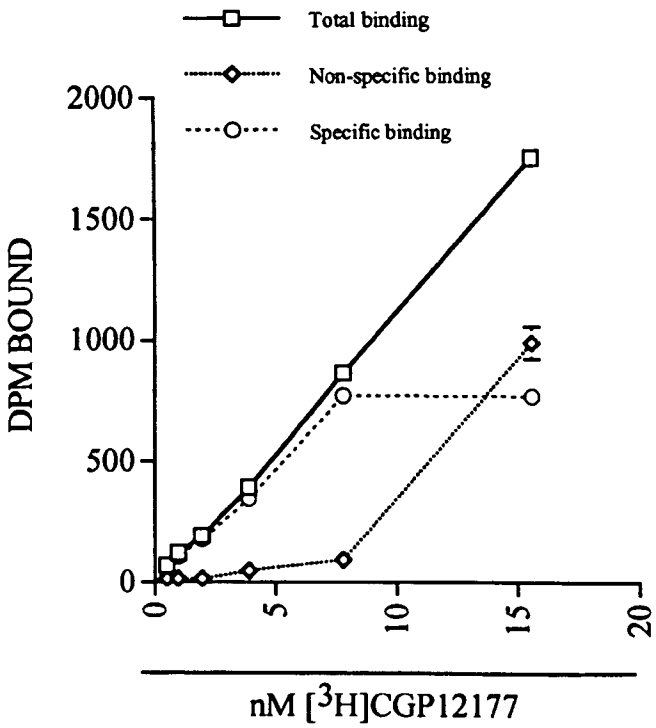

Figure 1. Binding of [^{3}H]CGP12177 to β-AR in C_2C_{12} cell membrane preparations. Forty μg membrane protein/ml harvested from differentiated C_2C_{12} cells were incubated with increasing concentrations of [^{3}H]CGP12177, with or without a 1000-fold concentration of unlabeled CGP12177 at 30°C for 2 hr. C_2C_{12} cells were propagated and differentiated as described under the Materials and Methods section. The Y axis range is 0–400 fMole/mg protein. Each data point represents a mean ± SD of three independent experiments. (SD less than 10% not plotted).

determined by the Scatchard Plot analysis of values ranging from origin to 10 n*M* CGP12177.The Scatchard plot for specific binding was linear, and the regression had a correlation coefficient of > 0.96. Regression analysis and tests for linear fit showed no evidence for curvilinearity to suggest a two-affinity site β-adrenergic receptor. Nonspecific binding was ≈ 10% of specific binding below 10 n*M* CGP12177 (Fig. 1), and the B_{max} was found to be 150 ± 10.4 fmole/mg protein.

Since the binding data only suggested the presence of β-AR, receptor functionality was evaluated by measuring cAMP release upon β-AA to β-AR binding. To this end, differentiated C_2C_{12} cells were treated with ISO or forskolin (control). Forskolin and ISO stimulated cAMP release by 20-fold ($P < 0.001$) and 17-fold ($P < 0.001$), respectively, compared with negative controls (Fig. 2). Additionally, ISO-stimulated cAMP release was also dose-dependent (Fig. 2), suggesting the presence of functional β-AR in C_2C_{12} cells.

Changes in DNA synthesis in growing C_2C_{12} myoblasts upon treatment with ISO, 8-BC, and FBS were measured. Results (Fig. 3A) showed ISO induced a 150% increase in DNA synthesis ($P < 0.05$). The stimulatory effect of ISO was also mimicked by the cAMP analog, 8-BC, which resulted in a 200% ($P < 0.01$) increase in DNA synthesis compared with control (Fig. 3A). A 500% increase in DNA synthesis ($P < 0.01$) was obtained upon serum (FBS) addition to proliferating cells (Fig. 3A). DNA synthesis in cells treated with either ISO, 8-BC, or FBS was greatly inhibited (90%–98%) by the MAPK kinase inhibitor PD098059 (Fig. 3B).

Activation of the MAPK pathway has emerged as important for cell growth upon stimulation of G protein–coupled receptors (16, 17, 39). Thus, the potential role of the MAPK signaling pathway to elicit growth effects in C_2C_{12} myoblasts upon treatment with ISO, 8-BC, and FBS was determined. A more than eight-fold increase in MAPK activation was observed when cells were treated with 10% FBS (Fig. 4); however, neither ISO nor 8-BC stimulated MAPK activation (Fig. 4). Serum-induced MAP-kinase activation was completely blocked by MAP-kinase inhibitor PD098059; MAPK activity appeared dampened by PD098059 in cells treated with ISO, 8-BC, or the no-treatment control (Fig. 4) suggesting that proliferative ac-

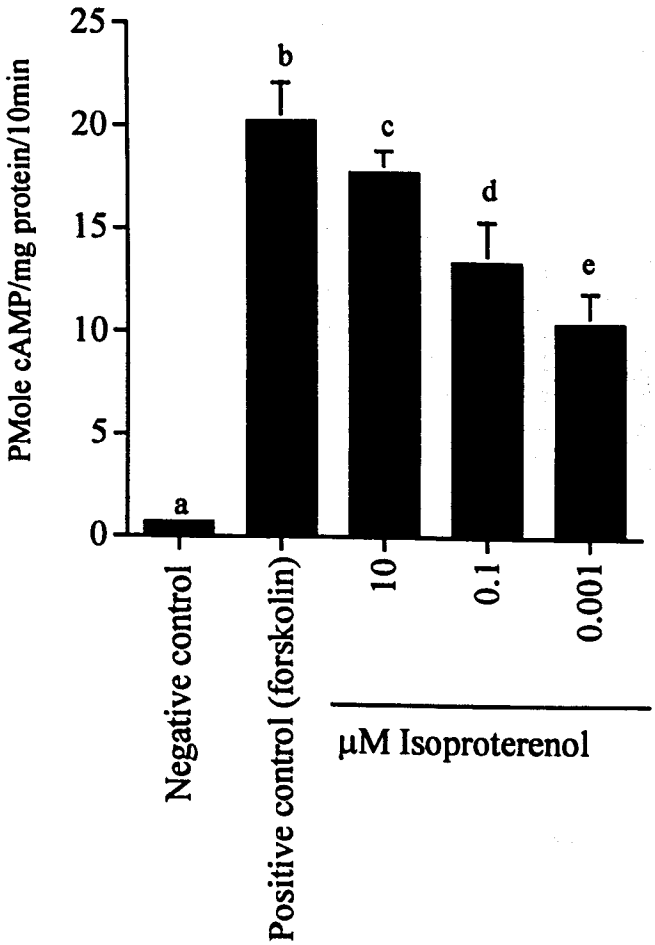

Figure 2. Isoproterenol dose-dependent cAMP production in differentiated C_2C_{12} cells. C_2C_{12} cells were propagated and differentiated as described under the Materials and Methods section. Cells were treated with either forskolin (50 μ*M*) or isoproterenol (10, 0.1 or 0.001 μ*M*) final concentrations. Monolayers were then incubated at 37°C for 10 min, and cAMP was quantified as described under the Materials and Methods section. Each data point represents mean ± SD for three independent experiments. Means (positive control, ISO at 0.001, 0.1, 10 μ*M*) not sharing common letter were different $P < 0.05$.

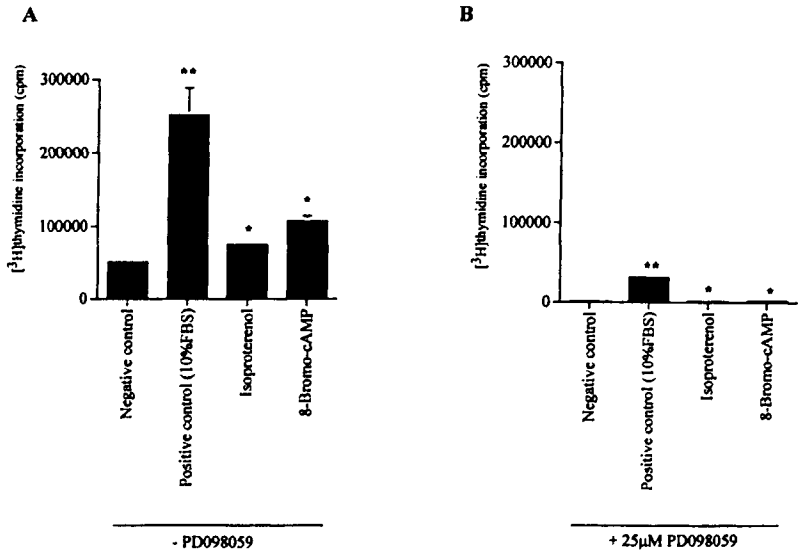

Figure 3. (A) [^{3}H] Thymidine incorporation studies in C_2C_{12} cells. Cells were plated in DMEM containing 10% FBS, 1% pen/strep/fungisome mixture. At subconfluence, cells were serum-starved overnight and then treated with either 10 μ*M* ISO; 1 m*M* 8-BC; or 10% FBS for 18 hr. After incubation, 1 μCi/ml [^{3}H] thymidine was added to the cells for an additional 4-hr period and treated as described under the Materials and Methods section. * $P < 0.05$; ** $P < 0.01$ significant difference from negative control. (SD less than 10% not plotted.) (B) [^{3}H] Thymidine incorporation studies in C_2C_{12} cells. Cells were plated in DMEM containing 10% FBS, 1% pen/strep/fungisome mixture. Cells were serum-starved overnight and then treated with either 10 μ*M* ISO; 1 m*M* 8-BC; or 10% FBS in the presence of 25 μ*M* PD098059 (MAP-kinase kinase inhibitor) for 18 hr. After incubation, 1 μCi/ml [^{3}H] thymidine was added to the cells for an additional 4-hr period and treated as described under the Materials and Methods section. * $P < 0.05$; ** $P < 0.01$ significant difference from negative control. (SD less than 10% not plotted.)

tivity of C_2C_{12} cells is MAPK-dependent. Additions of ISO or 8-BC to growing C_2C_{12} cells induced a three-fold and eight-fold increase, respectively, in FN mRNA abundance (Fig. 5).

Discussion

Initial work with growing animals provided evidence that feeding or administration of β-AA resulted in skeletal muscle hypertrophy characterized by increased tissue protein/DNA, increased abundance of mRNA for myofibrillar proteins, increased protein mass accretion, increased skeletal muscle protein synthesis, and decreased protein degradation (1–3, 5, 9–11). In addition, β-AA have also been noted to enhance proliferation of chick and porcine skeletal muscle satellite cells (18, 20). However, an understanding of the signaling pathway, from β-AA through β-AR within skeletal muscle that may account for the observed physiological effects of β-AA is incomplete. We have used the murine skeletal muscle cell line, C_2C_{12} to study β-AA–mediated events in skeletal muscle further. Our hypothesis was, at least in the case of proliferating skeletal muscle cells in culture, that the signal path from the G-protein–coupled β-adrenergic receptor leads directly or indirectly to mitogen-activated protein kinase.

Initial experiments were conducted to detect, characterize, and show functionality of β-AR in C_2C_{12} myoblasts. Present data on nonspecific binding, K_D and B_{max} in C_2C_{12} myoblasts (based on ligand concentrations up to 10 n*M*) are consistent with previous reports on β-AR ligand binding characteristics (40–42). The K_D (0.2 ± 0.035 n*M*) and B_{max} (150 ± 10.4 fmole/mg protein) values reported herein indicate the presence of a typical β-AR. Further, addition of the β-AA ISO to culture medium resulted in cAMP production and release into the medium demonstrating presence of a functional β-AR as shown with other muscle cell lines (32).

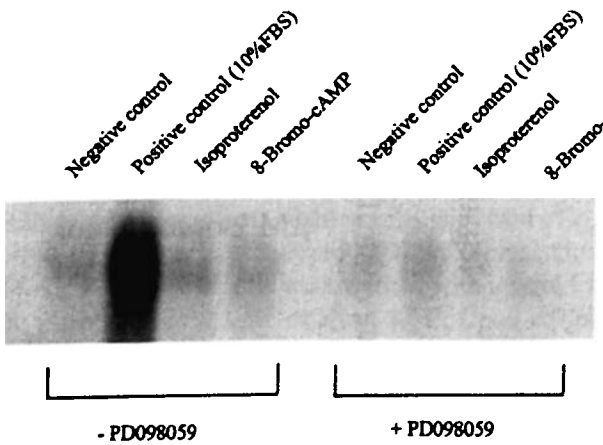

Figure 4. Isoproterenol or 8-bromo-cAMP does not modulate MAPK activity in C_2C_{12} cells. Data shown represent two separate experiments conducted in triplicate with a total of six observations. Differentiated C_2C_{12} cells were serum-starved overnight and stimulated with the following agents (10 μ*M* ISO; 1 m*M* 8-BC; 10% FBS) with or without PD098059 (MAP-kinase kinase inhibitor) for 10 min at 37°C. After incubation, MAPK assays were performed as described in the Materials and Methods section.

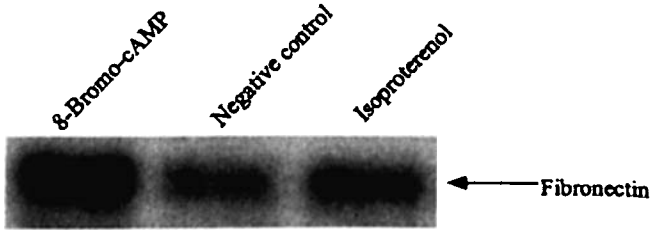

Figure 5. Northern analysis: Isoproterenol or 8-bromo-cAMP increased fibronectin mRNA production in C_2C_{12} cells. Data shown represent two separate experiments conducted in triplicate with a total of six observations. Proliferating cells were serum-starved overnight and treated with either 1 μ*M* ISO or 1 m*M* 8-BC at 37°C for 18 hr. Total RNA were extracted as described under the Materials and Methods sections, and 15 μg were electrophoresed, transferred to nytran, and hybridized with a human fibronectin probe.

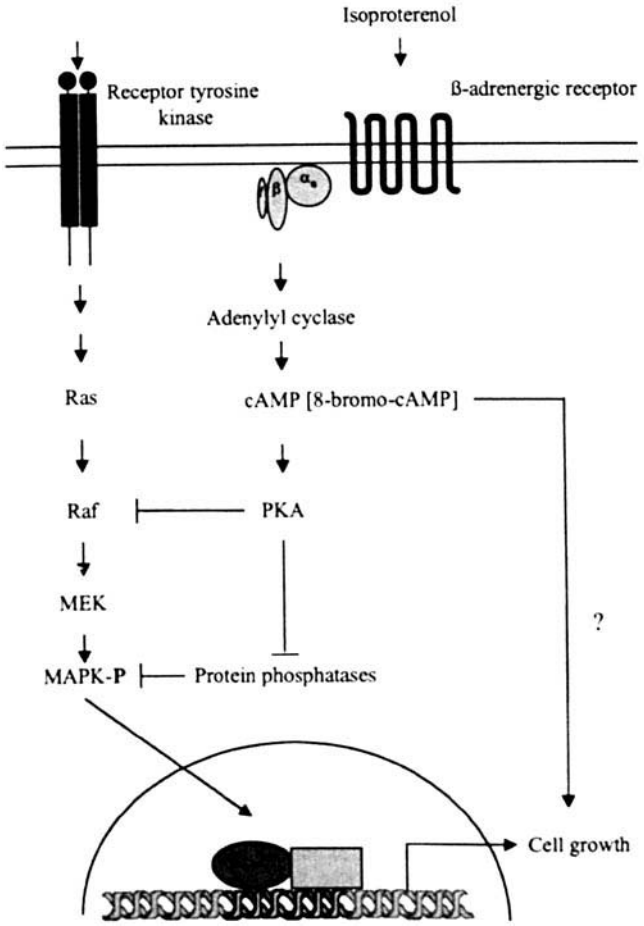

Figure 6. Cross-talk between membrane receptor tyrosine kinase and β-adrenergic receptor intracellular signaling pathways. PKA, protein kinase A; MEK, *mitogen-activated extracellular regulated* kinase or MAPK/ERK kinase; MAPK-P, mitogen-activated protein kinase in the activated (phosphorylated) form. PKA can inhibit the *ras-raf*-MAPK path of *raf* and stimulate MAPK activation by inhibiting protein phosphatases.

We have no ready explanation for the steep increase in nonspecific ligand binding at > 15 n*M*.

We also demonstrated that ISO and 8-BC stimulated DNA synthesis in proliferating C_2C_{12} cells, but we were unable to document a stimulation of MAPK above basal levels by cAMP-elevating agents. In our hands, addition of serum to proliferating C_2C_{12} cells resulted in a five-fold increase in DNA synthesis, a response greatly exceeding that noted for ISO of 8-BC, and an eight-fold increase in MAPK activity suggesting that activation of MAPK activity several-fold above basal levels was necessary for serum-induced, high rates of DNA synthesis. These results were not expected since others have reported strong MAPK activation by ISO, for example, in COS-7 cells (33) and a possible involvement, in the presence of insulin, of MAPK in cAMP-stimulated protein synthesis in L_6 myoblasts (7). Nevertheless, our studies did show clear involvement of MAPK in FBS-stimulated DNA synthesis in muscle cell cultures. Both DNA synthesis and MAPK activation of cells treated with ISO, 8-BC, or FBS were greatly reduced by the MAPK-kinase inhibitor PD098059. Our results indicate that in C_2C_{12} cells heterotrimeric G protein–coupled receptors, as stimulated by β-AA, and cAMP are involved in cell proliferation, but a link to MAPK activation was not observed (16, 17, 39).

Our results with C_2C_{12} cells can be interpreted to indicate that ISO may act in concert with other signaling molecules without augmenting basal MAPK activity. Similar contradictory observations have been described by others. For example, in smooth muscle cells, platelet-derived growth factor (PDGF-BB) acted as a weaker activator of MAPK yet as a stronger mitogen compared with the PDGF-AA isoform. In fact, PDGF-AA stimulated both MAPK isoforms (p42 and p44) two-fold compared with the PDGF-BB isoform but was a less effective mitogen of smooth muscle cells (43).

Guanine nucleotide–binding, protein-coupled receptors have been implicated in mitogen-activated protein kinase (MAPK) activation (16, 17, 39). MAP-kinases, also known as extracellular signal–regulated kinases (ERKs), are serine/threonine kinases that are rapidly activated upon stimulation of a variety of cell surface receptors (16). ERKs function to convert extracellular stimuli to intracellular signals regulating the expression of genes important for many cellular processes including cell growth and differentiation (16, 17). These kinases play a central role in mitogenic signaling, as impediment of their functions may prevent agonist-induced cell proliferation. Thus, MAPKs appear to be a critical component of growth-promoting pathways in C_2C_{12} cells as noted in our work with FBS. In contrast, our observation that ISO or 8-bromo-cAMP increased DNA synthesis 150% and 200%, respectively, beyond baseline without modulating MAPK activity further suggests that extensive MAPK activation may be unnecessary to obtain the observed stimulation of C_2C_{12} cell growth by β-adrenergic agonists. Indeed, the literature suggests (16, 44) the existence of ligand and cell type variations in MAPK activation. Figure 6 illustrates cross-talk between *ras* and PKA signaling pathways. PKA exerts both stimulatory and inhibitory effects on MAPKs. PKA inhibits *raf* to downregulate MAPK activation (16, 33). In addition, PKA may also inhibit certain protein phosphatases, causing an attenuation of dephosphorylation of active (phosphorylated) MAPKs thereby eliciting a stimulatory effect on MAPK (44). Thus, the final activation state of MAPK may be dependent on such contervaling processes in different cell lines treated with cAMP/PKA-enhancing agents.

Our work demonstrated a clear increase in FN gene expression (mRNA abundance) in C_2C_{12} cells upon treatment with ISO or 8-BC. An effect on FN gene expression has been noted with cAMP-elevating agents with various cell lines or systems (25–27, 45, 46). Previous studies have demonstrated that the FN gene promoter contains cAMP response elements at nucleotide positions – 170 (25) and – 260 and – 415 (45). Cell surface heparan sulfate proteoglycans (FN) are involved in bFGF signaling in MM14 skeletal muscle cells (19). The present data can be interpreted

broadly to suggest an association between extent of cAMP-induced DNA synthesis and FN mRNA abundance; for example, 8-BC had greater stimulatory effects on FN mRNA abundance and on ^{3}H-thymidine incorporation into DNA than was noted for ISO. Although this quantitative difference in response to ISO versus 8-BC is likely due to differences in the actual "effective dosage" between treatments, these data do indicate that a higher availability/concentration of cAMP resulted in more extensive DNA synthesis and greater FN mRNA abundances. From the above results, we have no evidence of any mechanistic link between β-AA/cAMP-induced increases in FN gene expression and cell proliferative activities.

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