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RECEPTOR EXPRESSION IN RAT SKELETAL MUSCLE CELL CULTURES

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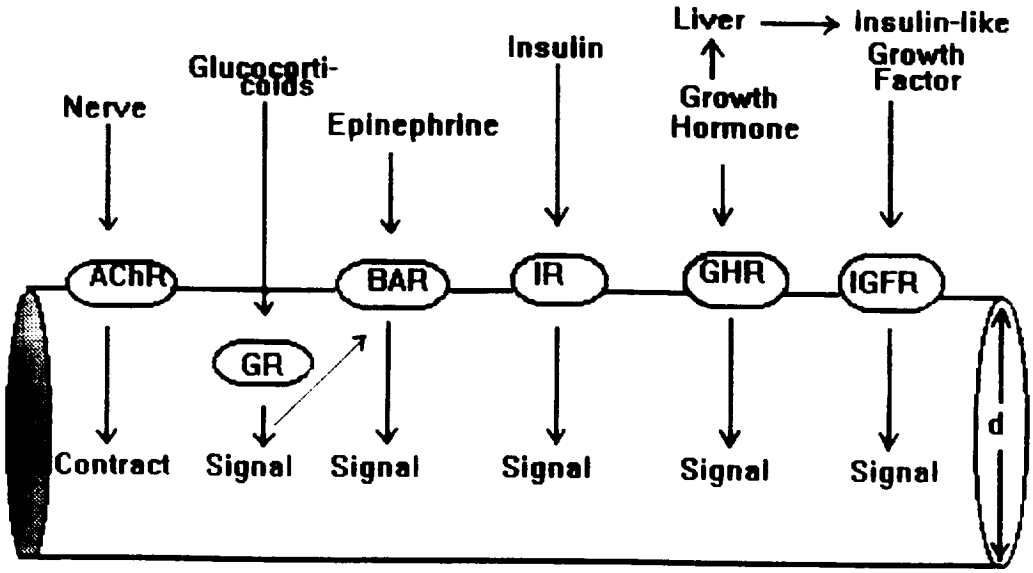
INTRODUCTION

One on the most persistent problems with long-term space flight is atrophy of skeletal muscles. Skeletal muscle is unique as a tissue in the body in that its ability to undergo atrophy or hypertrophy is controlled exclusively by cues from the extracellular environment. The mechanism of communication between muscle cells and their environment is through a group of membrane-bound and soluble receptors, each of which carries out unique, but often interrelated, functions. The primary receptors include acetyl choline receptors, beta-adrenergic receptors, glucocorticoid receptors, insulin receptors, growth hormone (i.e., somatotropin) receptors, insulin-like growth factor receptors, and steroid receptors.

This project has been initiated to develop an integrated approach toward muscle atrophy and hypertrophy that takes into account information on the populations of the entire group of receptors (and their respective hormone concentrations), and it is hypothesized that this information can form the basis for a predictive computer model for muscle atrophy and hypertrophy. The conceptual basis for this project is illustrated in the figure below. The individual receptors are shown as membrane-bound, with the exception of the glucocorticoid receptor which is a soluble intracellular receptor. Each of these receptors has an extracellular signalling component (e.g., innervation, glucocorticoids, epinephrine, etc.), and following the interaction of the extracellular component with the receptor itself, an intracellular signal is generated. Each of these intracellular signals is unique in its own way; however, they are often interrelated.



MODEL FOR MUSCLE ATROPHY/HYPERTROPHY

In summary, the presence of an active signal within a muscle cell depends on a number of levels, each of which is a potential site for regulation. These levels include the concentration or pulse frequency of the extracellular hormone, the number of receptors either on the surface or within the interior of the cells, whether or not the receptor-ligand interaction is coupled to the appropriate intracellular signal, and the extent to which individual signalling pathways interact with each other. The fact that interaction among pathways takes place is often referred to as receptor "cross talk". An example of the fact that receptors do not function independently of each other is as follows. An increase in muscle contraction via acetylcholine receptors and an increase in concentration of beta-adrenergic agonists (such as epinephrine) tend to lead to muscle hypertrophy, whereas an increase in glucocorticoid concentrations tend to lead to muscle atrophy. However, these are not independent events. A prolonged increase in contraction also results in a higher number of beta-adrenergic receptors and a lower number of glucocorticoid receptors. In addition, a prolonged decrease in contraction (such as that which occurs during weightlessness) results in a decreased number of beta-adrenergic receptors and an increased number of glucocorticoid receptors. Thus, not only is the number of receptors affected, but their capability to produce a signal is affected as well.

Materials and Methods

Rat Muscle Cell Cultures. Skeletal muscle tissue was excised from the hind limbs of newborn rats and placed in ice cold culture medium. The culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine and 1000 mg glucose/L (catalog #D5523, Sigma Chemical Co., St. Louis, MO), sodium bicarbonate buffer (3.7 g/L) and 13% horse serum (Gibco BRL Life Technologies, Grand Island, NY). Fungizone (250 µg/L; GIBCO BRL), Penicillin (100,000 U/L), and Gentamicin (20 µg/ml) were also added and the pH was adjusted to 6.8.

Working under sterile conditions, the muscle tissue was placed in warm phosphate buffered saline (PBS). After rinsing the tissue in PBS, it was minced with a scalpel. Muscle tissue was then placed in 50 ml conical tubes at approximately 10 g/tube, and 25 ml of a 2 mg/ml pronase enzyme solution in PBS (Catalog No. P6911, Sigma Chemical Co., St. Louis MO). The digestion procedure was carried out at 37°C in a rotary shaker at 175 rpm for approximately 45 min. The suspension was centrifuged at 1500 x g for 6 min, the protease solution was discarded, and myoblasts were harvested from the pellet by differential centrifugation. Briefly, PBS was added to the pellet to give a final volume of 30 ml, and the pellet was resuspended at top speed for 30 sec on a vortex mixer. The suspension was centrifuged at 400 x g for 3 min, and the supernatants were collected and saved. These steps were repeated two more times with the digested muscle pellet. The filtrate was then

centrifuged at 1500 x g for 6 min to collect the myoblasts. Cells were resuspended in culture medium. The cells were then plated in matrigel-coated 6 cm tissue culture dishes at a density of 1.8 x 10^5 cells/cm^2. On the day fusion was first observed, 10^{-6} M fluorodeoxyuridine was also added to inhibit DNA synthesis and thus overgrowth by fibroblasts.

Experimental Design. The purpose of this project is to measure the populations of beta-adrenergic receptors, glucocorticoid receptors, insulin receptors, insulin-like growth factor receptors and growth hormone receptors. To make these measurements, two different general approaches were taken. The beta-adrenergic receptor population was measured directly as described below, and culture samples for the others were collected and frozen for future analysis. In all, six experiments were carried out and samples collected. Since twenty-four culture wells are needed for each of the five receptors, a total of 720 samples were collected during the summer (i.e., 5 receptors x 6 experiments x 24 samples).

Beta-adrenergic Receptor Measurement. Cells in multiwell dishes were rinsed and incubated at room temperature for 15 min in buffer containing 0.1-3 nM (-)-[^{3}H]CGP-12177 (40-50 Ci/mmole) to determine total binding to beta-adrenergic receptors. Nonspecific binding was determined by measuring the amount of (-)-[^{3}H]CGP-12177 bound in the presence of 10^{-6} M (±)-propranolol. Myotubes from the rat skeletal muscle cell line L_6 were used as a positive control. These L_6 cells are grown in the same culture medium and exhibit a large population of beta-adrenergic receptors. The cell number in replicate cultures was determined by measuring the DNA content so the number of receptors per cell could be calculated. To determine the K_d and B_{max}, the least squares nonlinear curve fitting technique of the LIGAND computer program was used.

Sample Collection Protocol. For glucocorticoid receptor analysis, cultures were rinsed three times with cold phosphate buffered saline (PBS), and then homogenized 10 times with a tightly fitting Dounce homogenizer. Samples were either frozen directly at -70 C, or were centrifuged at 50,000 x g for 1 hr immediately prior to receptor analysis. Total binding was determined with 0-50 nM [^{3}H]dexamethasone, and nonspecific binding was determined with 0-50 nM [^{3}H]dexamethasone in the presence of 20 µM nonradioactive dexamethasone.

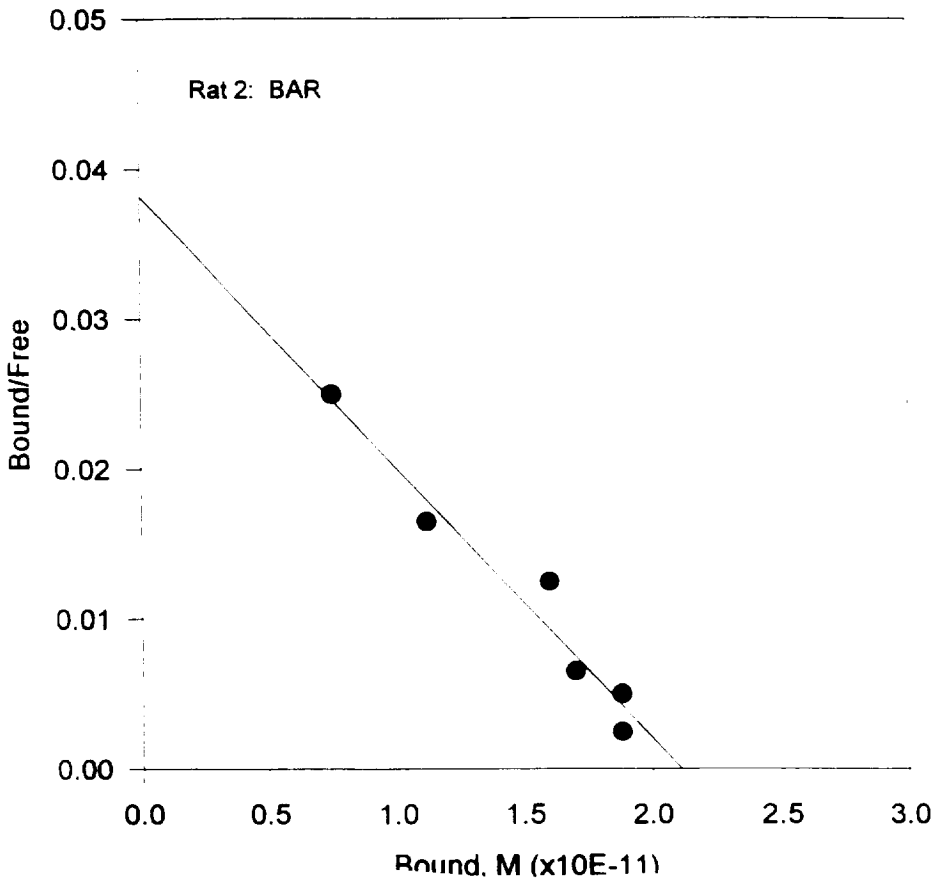
Cultures for insulin, IGF and growth hormone receptor measurements were rinsed three times with cold PBS, and scraped from the dishes into 2 mM EDTA, 1 mM HEPES, 0.3 mM PMSF, pH 7.4. Cells were collected by centrifugation at 24,000xg for 15 min, the cell pellet was resuspended in isotonic saline, and cells were homogenized 10 times with a loose fitting Dounce homogenizer. Samples were frozen at -70 C.

Results

Beta-adrenergic receptor population was quantified in rat primary cell cultures as described above. The receptor population was estimated to be 10.2 $\pm$ 1.2 million receptors per muscle cell. The K_d (i.e., the binding constant) was 0.47 $\pm$ 0.06 nM. An example of a saturation curve is shown at the top of the next page, and the transformation of these data to a Scatchard plot to determine receptor number is shown at the bottom of the next page. While the K_d is quite consistent with published values, it is difficult to compare the receptor population with published information. First, extensive literature searches have indicated that this receptor population has not previously been analyzed in muscle cell cultures. Second, values have been published for several other cell types (including heart cells, adipose cells and connective tissue cells), and the population ranges from approximately 5-15 million receptors per cell. Thus, the observed number of receptors is physiologically realistic.

In addition to the above results, two other lines of investigation were initiated during the summer, one of which was productive and the other was not. In anticipation of studying muscle atrophy and hypertrophy using a rotating wall bioreactor in the future, an attempt was made to grow an established muscle cell line under the same conditions as the rat primary cultures. Use of a cell line would be more adaptable and easier for shuttle flights than primary cultures. Unfortunately, these C_2C_{12} cells differentiated poorly and did not exhibit most of the characteristics of muscle cells. It was initially planned to compare the receptor populations between the cell line and the primary cultures; however, the decision was made to exclude the C_2C_{12} cell because of their poor quality. The second line of investigation was to attempt to grow primary cultures of muscle on micro-carrier beads in suspension. This was successful, with tissue differentiation and aggregation evident. Once a rotating bioreactor is available, experiments can be initiated to examine growth of cells on microcarrier beads under conditions that will simulate microgravity; furthermore, conditions can be established to analyze muscle cell receptor populations under conditions that will mimic either muscle atrophy or muscle hypertrophy.

This project was initiated to develop an integrated approach toward muscle atrophy and hypertrophy that takes into account information on the populations of the entire group of receptors (and their respective hormone concentrations), and it is hypothesized that this information can eventually form the basis for a predictive computer model for muscle atrophy and hypertrophy. This would be an extremely helpful approach in understanding the potential responses during tissue modeling experiments because the complex interactive effects from changing a single parameter could be predicted. We have made a few small steps in this new direction. At least we are certain of the two following things: 1) That receptors can be measured in muscle cells, and 2) that muscle cells will grow on microcarrier beads.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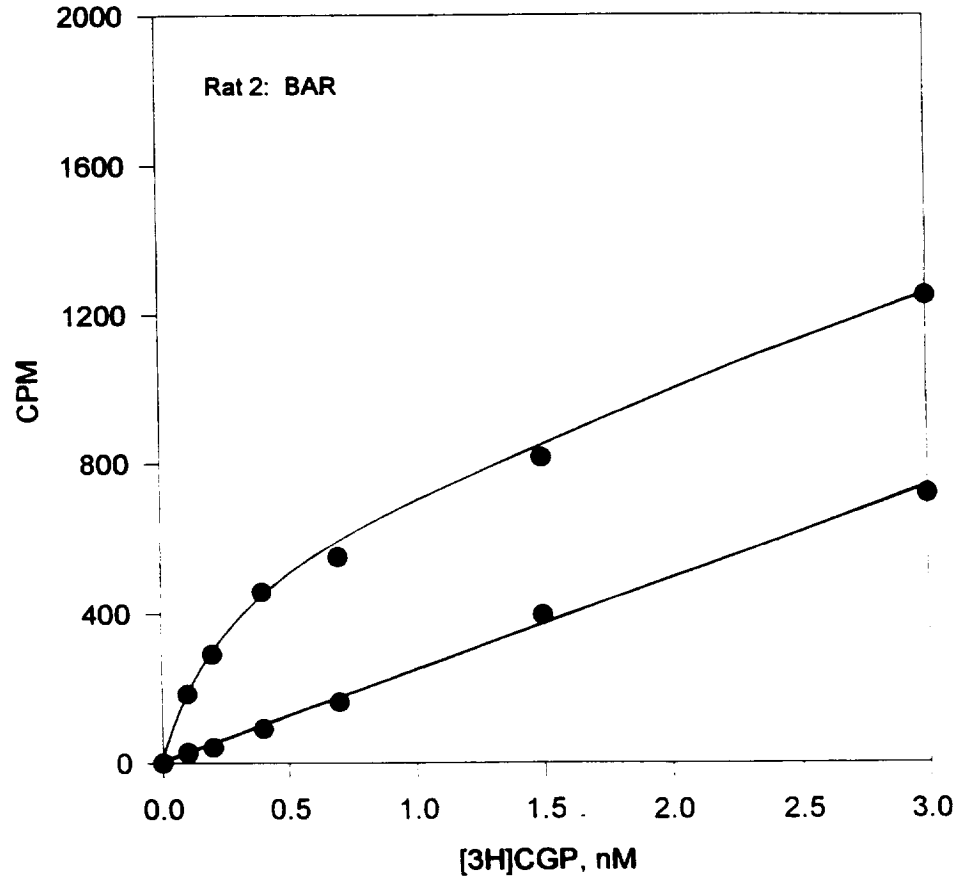
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