Elevated Myocardial Cytosolic Calcium Impairs Insulin-Like Growth Factor-1-Stimulated Protein Synthesis in Chronic Renal Failure

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Abstract. Rats and humans with chronic renal failure (CRF) are reported to have resistance to recombinant human insulin-like growth factor-1 (rhIGF-1). Because basal cytosolic calcium ([Ca\(^{2+}\)], a second messenger, may be increased in CRF, this study was conducted to examine whether elevated basal [Ca\(^{2+}\)], may cause resistance to IGF-1. Cardiomyocytes from four groups of rats were studied: untreated CRF, CRF with parathyroidectomy (PTX), CRF with the calcium channel blocker felodipine (F), and sham operation of the kidney (SO). CRF was created by ligation of two-thirds of the left renal artery and contralateral nephrectomy. Rats from each group were pair-fed the same diet for 20 to 22 d. Basal [Ca\(^{2+}\)], in cardiomyocytes (nM) in the CRF rats (102.0 ± 2.8; SEM), was significantly higher than in each of the CRF-PTX, CRF-F, and SO groups (65.2 ± 1.9, 63.8 ± 2.6, and 63.5 ± 2.0, respectively; \(P < 0.01\)). rhIGF-1 increased cardiomyocyte [Ca\(^{2+}\)], in all four groups of rats. The rise in [Ca\(^{2+}\)], was significantly diminished in the CRF rats (\(P < 0.05\)) and did not differ among the CRF-PTX, CRF-F, and SO rats. Protein synthesis after incubation with 0, 50, 100, 200, or 400 ng/ml rhIGF-1 was lower in cardiomyocytes from CRF rats than in each of the other three groups (\(P < 0.05\)) and was significantly less in the CRF-F rats compared with SO animals. IGF-1 receptor mRNA and IGF-1 receptor number and affinity were not different among the four groups. These findings suggest that cardiomyocytes from CRF rats display elevated basal [Ca\(^{2+}\)], and attenuated rhIGF-1-induced increase in [Ca\(^{2+}\)]; basal protein synthesis is decreased, and IGF-1-stimulated protein synthesis is impaired; elevated basal [Ca\(^{2+}\)], seems to contribute to this diminished response to rhIGF-1.

Recent evidence has demonstrated that there is resistance to insulin-like growth factor-1 (IGF-1) in experimental animals and humans with chronic renal failure (CRF)(1–3). There is an attenuation in the IGF-1-induced stimulation of protein synthesis and inhibition of protein degradation in skeletal muscle and also an impairment in the IGF-1-induced suppression of plasma amino acids, insulin, and C-peptide. Among the known mechanisms for IGF-1 resistance in CRF are defects in the phosphorylation and activity of the intrinsic tyrosine kinase of the IGF-1 receptor (2) and plasma inhibitors of IGF-1 (4,5).

Basal cytosolic calcium ([Ca\(^{2+}\)],) is elevated in a number of cell types in experimental rats or humans with CRF (6). Cytosolic calcium serves as a second messenger in the signal transduction system for many biologic functions of the cell (7).

For calcium to act as such, the ratio of the calcium signal (i.e., the acute increase in [Ca\(^{2+}\)], induced by an agonist) and the background calcium (basal levels of [Ca\(^{2+}\)],) must be sufficiently great (8). Elevated basal levels of [Ca\(^{2+}\)\(^{-}\)], decrease the signal-to-background ratio, and this is believed to attenuate the biologic response to the agonist. Indeed, several lines of evidence provide support for the thesis that an elevation in basal levels of [Ca\(^{2+}\)\(^{-}\)], causes cell dysfunction in CRF. First, [Ca\(^{2+}\)], is elevated in many cell types in CRF, and these cells have altered function (6). Second, if basal [Ca\(^{2+}\)], is maintained at normal levels in CRF rats, many of their cell functions remain normal (9–13). Third, animals with phosphate depletion and normal renal function have elevated basal levels of [Ca\(^{2+}\)], (14–16). These animals display a number of metabolic and functional derangements that are similar to those observed in CRF, despite the absence of CRF (15–17).

Because IGF-1 induces a transient increase or oscillation of [Ca\(^{2+}\)], in certain cells and because this IGF-1-induced change in [Ca\(^{2+}\)], has been considered to mediate some of the actions of IGF-1 (18–27), we hypothesized that elevation of basal [Ca\(^{2+}\)], in CRF may contribute to IGF-1 resistance. To test this hypothesis, we examined whether in cardiomyocytes of CRF rats: 1. basal [Ca\(^{2+}\)], is indeed elevated, and the increment in [Ca\(^{2+}\)], in response to IGF-1 is attenuated; 2. protein synthesis, with or without IGF-1 stimulation, is impaired; and 3. normal-
ization of basal and IGF-1-stimulated [Ca^{2+}], by either parathyroidectomy or treatment with a calcium channel blocker, can increase the reduced level of protein synthesis toward normal, both without IGF-1 stimulation and in response to treatment with IGF-1.

**Materials and Methods**

**Reagents**

Felodipine was kindly provided by Dr. Mark Page (Astra Hässle AB, Mölndal, Sweden). [13C]-Leucine (168 Ci/mmol) was purchased from DuPont NEN (Boston, MA). 131I-IGF-1 (2000 Ci/mmole) was obtained from Amersham Life Sciences (Arlington Heights, IL). Reagents for electrophoresis and protein measurements were purchased from Bio-Rad Laboratories (Hercules, CA). Recombinant human insulin-like growth factor-1 (rhIGF-1) was kindly provided by Dr. Hans-Peter Guler (Chiron Therapeutics, Emeryville, CA). Anti-sense RNA probes for detection of IGF-1 receptor mRNA were graciously provided by Dr. Dereck LeRoith (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless indicated otherwise.

**Animals**

Male Sprague Dawley rats (Charles River Breeding Laboratories, Wilmington, MA), each weighing 210 to 230 g, were studied. Animals were fed Purina Laboratory Rodent Diet (Purina Mills, St. Louis, MO) containing 24% protein and tap water, the NaHCO₃ was added instead to the food of the group 4 (CRF) rats. Group 1, 2, and 3 rats were pair-fed the same quantity of rat chow that was ingested by the group 4 (CRF) rats. Group 1, 2, and 3 rats were pair-fed the drinking water, the NaHCO₃ was added instead to the food of the untreated CRF rats. Group 2, 3, and 4 rats were pair-fed the drinking water of the untreated CRF rats contained 80 mM NaHCO₃ to prevent acidosis.

**Preparation of RNA**

Total RNA was extracted from the heart by the guanidine thiocyanate isolation method of Chomczynski and Sacchi (29) with minor modifications. The heart was removed and flushed with ice-cold BSS, pH 7.20, to wash out the blood. Approximately 150 mg of ventricular tissue was then homogenized in 1.5 ml of ice-cold 4 M guanidine thiocyanate buffer, pH 7.0, with a Polytron homogenizer (Brinkman Instruments, Westbury, NY). One volume of the homogenate was thoroughly mixed with 0.1 vol of 2 M sodium acetate, pH 4.0, 1 vol of phenol, and 0.2 vol of chloroform/isooamyl alcohol (49:1). The mixture was centrifuged at 16,000 × g for 30 min at 4°C, and the clear supernatant was precipitated with 1.0 vol of isopropanol at −70°C for 60 min. After centrifugation at 14,000 × g for 10 min at 4°C, the resultant pellet was washed in ice-cold 75% ethanol, air-dried, and resuspended in deionized formamide. The quantity of RNA was determined by ultraviolet absorbance at 260 nm; the quality of the samples was evaluated by visualizing the 18S and 28S ribosomal RNA after 3 μg of total RNA was resolved in a 1% agarose/formaldehyde gel.

**Riboprobe Preparation**

The antisense cRNA probe used for the identification of IGF-1 receptor mRNA has been described previously (2,30). The probe for the IGF-1 receptor was synthesized and labeled with 50 μCi (10 mCi/ml) [α-32P]UTP (ICN Biomedicals, Costa Mesa, CA) and 0.48 nmol of unlabeled UTP by using Sp6 RNA polymerase with Riboprobe Gemini II System (Promega, Madison, WI) according to the manufacturer’s instructions. After transcription, 1 μg of DNAse I, 8 μg of tRNA, and 1 μl of 200 mM vanadyl-ribonucleoside complex were added. The mixture was incubated for 15 min at 37°C and extracted with 0.5 vol of phenol and 1 vol of chloroform. The antisense cRNA probe for the rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Ambion, Austin, TX) was synthesized and labeled by the same procedures and with the same RNA polymerase but with 5 μCi (10 mCi/ml) of [α-32P]UTP and 1.38 nmol of unlabeled UTP (2).

**Solution Hybridization/RNase Protection Assay**

This assay was performed as described by Lowe et al. (30) with minor modifications as described previously (2). Fifteen-microgram samples of total RNA was suspended in 29 μl of hybridization buffer containing 20 mM Tris, pH 7.50, 1.0 mM ethylenediaminetetraaceta- te, pH 8.0, 400 mM NaCl, 0.1% sodium dodecyl sulfate, and 75% formamide. After addition of 1 μl of probe mixture containing 32P-labeled antisense cRNA probes for IGF-1 receptor (4 × 10⁵ cpm) and GAPDH (1 × 10⁵ cpm), the samples were heated to 90°C for 5 min and then incubated for 16 h at 60°C. Single-stranded RNA was digested by addition of 270 μl of digestion buffer containing 10 mM Tris, pH 7.60, 5 mM ethylenediaminetetraacetate, pH 8.0, 300 mM NaCl, 40 μg/ml RNase A, and 2.0 μg/ml RNase T1 in a 1-h incubation at 30°C. After treatment with 0.6% sodium dodecyl sulfate and 50% solution, further processed for extraction of total RNA or for preparation of membrane fractions or single-cell cardiomyocyte suspensions (see below). Only the CRF rats that had a serum creatinine concentration of 1.10 mg/dl or greater and had survived the 20 to 22 d of pair feeding were included in the studies. Tail-cuff BP was measured by the IITC Mark 12 photoelectric/oscillometric tail-cuff system (IITC Life Sciences, Woodland Hills, CA) according to the manufacturer’s instructions. Serum creatinine was measured by using the Sigma kit (Sigma-Aldrich Corp.). Serum calcium and phosphorus were measured with an AutoAnalyzer (Technicon Instrument Corp., Tarrytown, NY).
µg of proteinase K for 15 min at 37°C, the protected hybrids were extracted with phenol/chloroform and precipitated with 2.5 vol of ethanol by using 8 µg of tRNA as a carrier. After a wash with 75% ethanol, the samples were resuspended in loading buffer (80% formamide, 0.2% xylene cyanol, 0.2% bromphenol blue), denatured by heating for 3 min at 95°C, and chilled on ice. The protected fragments were separated on an 8% polyacrylamide/8 M urea denaturing gel. The dried gels were subjected to autoradiography, and band signal intensity was quantified by scanning densitometry of autoradiograms obtained from several exposures of original gels. Results were normalized to GAPDH expression.

Preparation of Membrane Fractions from Heart

Approximately 1 g of heart tissue was homogenized in 6 ml of ice-cold buffer A, pH 7.40, containing 50 mM Hepes, 1.25 mM NaCl, 1.2 mM MgSO4, 5 mM KCl, 1.2 mM CaCl2, 3 mM sodium azide, 10 U/ml bacitracin, 2 µM leupeptin, 2 µM pepstatin, 1 mM phenylmethylsulfonyl fluoride, and aprotinin equivalent to 1 trypsin inhibitor unit/ml buffer). The homogenate was centrifuged at 10,000 × g for 10 min. The resultant supernatant was centrifuged at 100,000 × g for another 20 min. The pellet was resuspended with 0.6 ml of buffer B (buffer A supplemented with 0.5% Triton X-100). The protein content was measured according to Bradford (31). All procedures were performed at 0 to 4°C. Heart samples from each CRF rat and its pair-fed control were processed at the same time on the same day and at the same time with cold (0 to 4°C) buffer B to obtain comparable receptor yields.

Receptor-Ligand Binding Assay

Hormone binding assays were carried out in soft polyethylene microcentrifuge tubes in duplicates or triplicates as reported previously (2). One hundred micrograms of membrane protein were incubated in buffer C (buffer A supplemented with 0.1% Triton X-100), in a total volume of 200 µl, with 50 pM [3H]-labeled IGF-1 in the presence of different concentrations (0 to 25 nM) of unlabeled IGF-1. After incubation for 16 h at 4°C, the binding reaction was stopped by adding 100 µl of ice-cold 0.3% γ-globulin and 300 µl of 25% polyethylene glycol 8000 in buffer C. The tubes were incubated for 15 min on ice and then centrifuged for 10 min at 14,000 × g. After the resultant pellet was washed twice with 500 µl of cold 12.5% polyethylene glycol 8000, the tips containing the pellet were cut off and counted in a gamma counter (Packard Instrument Co., Meridian, CT).

Nonspecific binding was estimated in the presence of 50 nM unlabeled IGF-1, and the specific binding was calculated as the difference between each individual binding value and the average value for nonspecific binding. The binding data were analyzed by Scatchard plots (32) by using the LIGAND program to fit the binding curve and to calculate the dissociation constant (Kd) and the maximal number of binding sites (Bmax).

Preparation of Single-Cell Cardiomyocyte Suspensions

Cardiomyocytes were isolated by using a modification of the methods of De Young et al. (33) and Haworth et al. (34). The rat was anesthetized with an intramuscular injection of ketamine (100 mg/kg body wt) and xylazine (3 mg/kg body wt). With the abdominal cavity exposed, the rat received a heparin injection (500 to 600 U/kg body wt) into the abdominal vena cava. After 15 s, the animal was decapitated, and the blood was collected into a heparin tube. The thoracic cavity was then opened, and the heart was immediately removed and placed into cold (4°C) BSS, pH 7.20, containing (in mM): 130 NaCl, 3.0 KCl, 1.2 KH2PO4, 1.0 MgSO4, 1.25 CaCl2, 10 Na-Hepes, and 10 glucose. The heart was perfused by means of the aorta with oxygenated calcium-Joklik medium, pH 7.20, containing Joklik medium, 1.25 mM CaCl2, 10 mM Na-Hepes, and 10 mM glucose for 2 min at 37°C. The heart was then perfused with 55 ml of circulating oxygenated calcium-free Joklik medium (same as calcium-Joklik medium, except 10 mM KCl is used instead of 1.25 mM CaCl2) for an additional 3 min at 37°C. Thereafter, 128 U/ml collagenase type II (Worthington Biochemicals, Freehold, NJ), 290 U/ml of hyaluronidase, and 0.1% bovine serum albumin (BSA) were added to the perfusion media. After 30 to 35 min, CaCl2 (1.25 mM) was added to the perfusate, and the perfusion was continued for another 5 min. The heart was then removed from the perfusion system, and the atria were cut off and discarded. The ventricles were cut into four pieces and digested for an additional 10 min in the same medium in a 37°C shaking water bath (120/min) with continuous gassing of the medium with 100% oxygen. The cell suspension was filtered through a 250-µm mesh Nitex screen (Tetko, Elmsford, NY) and washed once in calcium-Joklik medium supplemented with 1% BSA, pH 7.40, at 20°C. The cells were pelleted at 100 × g for 2 min at 20°C for subsequent purification.

Purification of the myocytes was carried out according to procedures reported previously (33) by using a 45% Percoll step gradient medium. Briefly, 1 vol of the cell suspension in calcium-Joklik medium (4 × 107/ml) was gently mixed with 3 vol of 60% Percoll gradient medium, which was prepared freshly by mixing three parts of Percoll (Pharmacia Biotech AB, Uppsala, Sweden) and two parts of 2.5% calcium-Joklik medium, pH 7.40, at 20°C. The mixture was then centrifuged at 500 × g for 3 min using a TJ-6 swinging bucket rotor (Beckman Instruments, Palo Alto, CA). The nonviable cell layer, which lay on the top of the gradient medium, and the supernatant were discarded. The pellet was washed twice with calcium-Joklik medium supplemented with 1% BSA at 20°C. The morphology of the purified myocytes was evaluated under light microscopy. The viable and undamaged cells were rod-shaped with clear striations and noncontractile in the presence of 1.25 mM calcium, as reported previously (33). The viability of the cells, which varied between 80 to 90% in different preparations, was determined by the trypan blue exclusion method (33).

Measurement of Cytosolic Calcium

Cytosolic calcium ([Ca2+]i) in cardiomyocytes was measured as Fura 2-acetoxymethyl ester (Fura-2 AM) induced fluorescence as described by Smogorzewski et al. (28). Purified cardiomyocytes were resuspended in calcium-Joklik medium with 1% BSA. An aliquot (7.5 ml) of the cell suspension (5 × 10⁴ cells/ml) was loaded with Fura-2 AM by incubating the cells with 2 µM Fura-2 AM in a shaking water bath for 40 min at 37°C. The cells were then purified over a 45% Percoll step gradient as described above, washed, and resuspended in 2 ml of calcium-free Joklik medium with 1% BSA. An aliquot (0.1 ml) of this suspension was added to a spectrophotometer cuvette containing 1.9 ml of a medium, pH 7.4, consisting of (in mM): 120 NaCl, 10 NaHCO3, 3.0 KCl, 1.2 KH2PO4, 1.0 MgSO4, 1.25 CaCl2, 10 Na-Hepes, and 10 glucose. The fluorescence of the mixture in the cuvette was measured as reported previously (28) with a fluorometer (model LS-5B; Perkin-Elmer, Norwalk, CT) that was equipped with a magnetic stirrer and temperature regulator. The excitation wavelengths were 340 and 380 nm, and the emission wavelength was 510 nm. The time course of changes in fluorescence intensities was recorded on a strip chart recorder (Perkin-Elmer). Calculation of [Ca2+]i was made using the Grynkiewicz equation (35), and the dissociation constant for calcium-Fura-2 AM was assumed to be 225 nM (28). From the same cell suspension from each rat, three aliquots of 0.1 ml were each used...
for measurement of basal levels of \([Ca^{2+}]_i\), and another three aliquots of 0.1 ml were each used for determining the effect of 400 ng/ml IGF-1 or 3 \(\mu M\) ATP on \([Ca^{2+}]_i\).

**Measurement of Protein Synthesis in Cardiomyocytes**

Total protein synthesis in cardiomyocytes was measured by the incorporation of \([^{14}C]\)leucine into cellular protein (36,37). Purified cardiomyocytes were resuspended in calcium-Joklik medium with 1% BSA to a concentration of about 3 \(\times\) 10^5 cells/ml. An aliquot (0.2 ml) of the cell suspension was added to 1 ml of fresh calcium-Joklik medium supplemented with \([^{14}C]\)leucine (1 mCi/ml) and with or without different concentrations of rhIGF-1 (see Results). The cells were incubated on a shaking platform in a 37°C incubator (5% CO\(_2\)) without different concentrations of rhIGF-1 (see Results). The cells were incubated on ice for 30 min and centrifugation for 10 min at 2000 \(\times\) g (4°C). The TCA-insoluble precipitate was washed three times with ice-cold 10% TCA, dissolved in 180 \(\mu\)l Solvable (DuPont NEN Research Products), and neutralized by 10 \(\mu\)l of 1.0 M NaOH; the radioactivity was determined by a liquid scintillation counter (model 2200CA; Packard Instrument Co.). The \([^{14}C]\)leucine incorporated into the newly synthesized protein during the incubation period was expressed as the radioactivity in the TCA-insoluble fraction after subtracting the TCA-insoluble counts at zero time and corrected for the number of viable cells. All assays were performed in triplicate.

**Statistical Analyses**

All statistical analyses were performed by using StatView or Statwork™ software (BrainPower, Inc., Calabasas, CA). Evaluation of statistically significant differences among the four groups was carried out by one-way ANOVA. Linear regression analyses were also used. The variance of the values is given as the SEM. Statistical significance is taken as \(P < 0.05\).

**Results**

**General Characteristics of the Four Groups of Rats**

Table 1 indicates the body weights and plasma concentrations of creatinine, calcium, and phosphorus in the four groups. After 3 wk of pair-feeding, the body weights in each group of CRF rats were significantly lower than that of the SO rats (253 ± 6.2 g; \(P < 0.05\) for each comparison). The gain in body weight was also significantly lower in each group of CRF rats. The serum creatinine in each of the three groups of CRF rats was significantly greater compared with the SO rats (\(P < 0.05\)). There was no significant difference in body weights, body weight gains, or serum creatinine levels among the CRF-PTX, CRF-F, and CRF rats. The serum calcium and phosphorus concentrations tended to be slightly lower in the CRF-PTX, CRF-F, and CRF rats, but the values for either mineral were not significantly different among the four groups of rats.

Figure 1 depicts the tail systolic BP during the 3 wk of pair feeding. Before surgery, there was no difference in BP among the four groups of rats. The systolic BP in the SO rats remained stable throughout the 3 wk of pair feeding. One week after surgery, the systolic BP of the untreated CRF rats was 158 ± 6.5 mmHg and was significantly higher than the levels in the SO rats (128 ± 3.4 mmHg, \(P < 0.05\)). The systolic BP of these CRF rats during the second and third weeks after surgery remained significantly greater than in the SO rats and did not differ from the levels obtained 1 wk after surgery. During the pair feeding period, the BP values in the CRF-PTX and CRF-F rats were not different from the CRF rats and were each significantly higher than in the SO rats (Figure 1).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>(n)</th>
<th>SO Rats</th>
<th>CRF-PTX Rats</th>
<th>CRF-F Rats</th>
<th>CRF Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight (g)</td>
<td>22</td>
<td>253 ± 6.2</td>
<td>216 ± 10.4(^e)</td>
<td>211 ± 8.5(^e)</td>
<td>209 ± 9.7(^e)</td>
</tr>
<tr>
<td>Gain in body weight (g/d)(^d)</td>
<td>22</td>
<td>0.9 ± 0.3</td>
<td>−1.0 ± 0.5(^f)</td>
<td>−1.1 ± 0.4(^f)</td>
<td>−1.4 ± 0.4(^f)</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>22</td>
<td>0.38 ± 0.02</td>
<td>1.20 ± 0.03(^e)</td>
<td>1.20 ± 0.03(^e)</td>
<td>1.19 ± 0.03(^e)</td>
</tr>
<tr>
<td>Serum calcium (mg/dl)</td>
<td>18</td>
<td>10.32 ± 0.14</td>
<td>9.44 ± 0.56</td>
<td>9.49 ± 0.38</td>
<td>9.56 ± 0.46</td>
</tr>
<tr>
<td>Serum phosphorus (mg/dl)</td>
<td>18</td>
<td>8.99 ± 0.32</td>
<td>8.08 ± 0.60</td>
<td>8.07 ± 0.58</td>
<td>8.04 ± 0.45</td>
</tr>
</tbody>
</table>

\(^a\) Data are the mean ± SEM.
\(^b\) Measurements were made after pair feeding the rats for 20 to 22 d after surgery. SO, sham-operated; CRF-PTX, chronic renal failure with parathyroidectomy; CRF-F, chronic renal failure with felodipine.
\(^c\) Number of rats in each of the four groups on which the measurement was made.
\(^d\) Gain in body weight designates the difference between the body weight measured on the day of surgery and the final body weight obtained on the day of killing, 20 to 22 d after surgery.
\(^e\) \(P < 0.01\), different from SO rats.
\(^f\) \(P < 0.001\), different from SO rats.
Figure 1. The time course of systolic BP of the four groups of rats. BP was measured at the indicated times immediately before surgery (presurgery) and after surgery as described in Materials and Methods. Each data point represents the mean ± SEM of values obtained in 12 rats from each group. ●, untreated chronic renal failure (CRF) rats; ○, sham-operated (SO) rats; ■, CRF-parathyroidectomized rats; □, CRF-felodipine rats. *P < 0.05 versus SO rats. Statistical calculations were carried out by ANOVA.

In preliminary studies, incubation of cardiomyocytes with rhIGF-1 at concentrations ranging from 50 to 400 ng/ml induced a dose-dependent, transient increase in [Ca$^{2+}$], that typically was maximum at 15 to 30 min and that was maximal at concentrations of 300 or 400 ng/ml (data not shown). Thus, to achieve a maximal response in [Ca$^{2+}$], a rhIGF-1 concentration of 400 ng/ml was added to the media, and the change in [Ca$^{2+}$] was recorded 25 min after the addition of rhIGF-1.

Addition of 400 ng/ml rhIGF-1 stimulated an increase in [Ca$^{2+}$] in cardiomyocytes in all four groups of rats (Figure 2, A and B). The net increase and the percentile increase in [Ca$^{2+}$] were quite different among the four groups. rhIGF-1 increased [Ca$^{2+}$] by 30.3 ± 1.8 nM (Figure 2B) or 47.6 ± 2.1% above basal levels in the SO rats (differs from no change, P < 0.001). In contrast, the rise in [Ca$^{2+}$], above baseline in the untreated CRF rats, although significant (P < 0.02), was markedly attenuated (8.2 ± 2.2 nM or 8.2 ± 2.5%; change in [Ca$^{2+}$] in CRF versus pair-fed SO rats, P < 0.01) (Figure 2B). The increases in [Ca$^{2+}$], in response to stimulation with 400 ng/ml IGF-1 in the CRF-PTX and CRF-F rats were also significant (27.8 ± 9.3 nM and 42.6 ± 14.2%, P < 0.025; and 32.6 ± 8.4 nM and 50.5 ± 12.3%, P < 0.01, respectively). These values were each significantly greater than the respective increases observed in the untreated CRF rats (P < 0.01 for each comparison) and were not different from the values from the SO rats (Figure 2B).

Effects on rhIGF-1 on Protein Synthesis

The dose–response relationship of rhIGF-1 and protein synthesis in cardiomyocytes is shown in Figure 3, A and B. Freshly prepared cardiomyocyte suspensions were incubated for 2 h with different concentrations of rhIGF-1, ranging from 50 to 400 ng/ml, or without rhIGF-1. Cell suspensions obtained from each rat were studied at each of these concentrations of rhIGF-1. The basal rate of protein synthesis, without rhIGF-1 added, in cardiomyocytes from SO rats was 898 ± 24 cpm/10$^4$ cells per h; the addition of various concentrations of rhIGF-1 to the media induced a dose-dependent increase in the rate of protein synthesis (P < 0.001). The basal rate of protein synthesis was significantly lower in the cardiomyocytes from untreated CRF rats (638 ± 51 cpm/10$^4$ cells per h) as compared with SO rats (P < 0.05) (Figure 3A). Furthermore, the response of protein synthesis in the CRF cardiomyocytes to 100, 200, or 400 ng/ml rhIGF-1 was also significantly diminished (Figure 3, A and B). The dose–response curve was markedly flattened in the untreated CRF rats compared with the SO rats, although there was a linear correlation between the
protein synthesis rate and the rhIGF-1 concentrations in the CRF rats ($r = 0.923$, $P = 0.026$).

Compared with the untreated CRF rats, the cardiomyocytes from the CRF-PTX rats and CRF-F rats displayed significantly higher basal levels of protein synthesis (842.695 and 807.624 cpm/10⁴ cells per h, respectively; $P < 0.05$ for each comparison with CRF rats). Moreover, the protein synthesis rate at each concentration of rhIGF-1 was significantly greater in the CRF-PTX rats and the CRF-F rats when each group was compared with the CRF rats (Figure 3A). On the other hand, the rate of protein synthesis in the cardiomyocytes from the CRF-F rats after incubation with 100, 200, or 400 ng/ml rhIGF-1 was significantly lower than in the SO rats (Figure 3B). The protein synthetic rate was significantly greater at rhIGF-1 concentrations of 400 ng/ml as compared with when there was no rhIGF-1 in the incubation media, in the SO rats ($P < 0.001$), the CRF-PTX rats ($P < 0.005$), the CRF-F rats ($P < 0.0025$), and the untreated CRF rats ($P < 0.01$).

**IGF-1 Receptor mRNA, Number, and Binding Affinity in Cardiomyocytes**

IGF-1 receptor mRNA in cardiomyocytes, measured by solution hybridization/RNase protection assays, and the IGF-1 receptor number and binding affinity in cardiomyocytes, measured by receptor-ligand binding assays, in the four groups of rats are shown in Figure 4 and Table 2, respectively. There were no differences in the IGF-1 receptor mRNA or in the IGF-1 receptor number or binding affinity among the four groups of rats.

**Discussion**

Resistance to IGF-1 has been observed in both experimental animals and humans with chronic renal failure. Although the mechanisms that contribute to this resistance are incompletely understood, they appear to be multifactorial. Evidence suggests
that there are at least two plasma inhibitors, one of which has a molecular weight of about 45,000 D and another that is sufficiently small to be readily removed by hemodialysis (4,5). Among the larger-sized inhibitors of IGF-1, elevated IGF binding proteins have been considered to be strong candidates (5). We previously found that IGF-1 resistance in skeletal muscle of rats with CRF is not due to decreased IGF-1 receptor number or binding affinity (2). Instead, both a diminished phosphorylation of the tyrosine kinase intrinsic to the beta subunit of the IGF-1 receptor and an attenuated activity of the activated (phosphorylated) tyrosine kinase were observed (2). These findings suggest that causes of resistance to IGF-1 in CRF are complex and involve defects in the signal transduction system for IGF-1. We considered the possibility that altered [Ca\(^{2+}\)], may contribute to IGF-1 resistance in CRF because IGF-1 stimulates a rise in [Ca\(^{2+}\)], (18–27), an increase in [Ca\(^{2+}\)], may stimulate protein synthesis (8), basal [Ca\(^{2+}\)], is reported to be elevated in CRF (6), and, in other systems, elevated [Ca\(^{2+}\)], is associated with impaired cellular responses to various stimuli (6).

Cardiomyocytes were selected for this study for the following reasons: 1. IGF-1 may be an important regulator of protein synthesis in adult cardiomyocytes (37,38); 2. IGF-1 increases [Ca\(^{2+}\)], in cardiomyocytes from adult rats (18), possibly by increasing formation of inositol 1,4,5-triphosphate (39); and 3. basal [Ca\(^{2+}\)], is reported to be abnormally elevated in cardiomyocytes of CRF rats (40).

In this study of cardiomyocytes from rats with experimental CRF, we provide the first evidence that there is resistance to IGF-1 in these cells and that an abnormally increased cytosolic calcium is associated with impairment in the actions of exogenous IGF-1 on protein synthesis and, hence, is a contributor to IGF-1 resistance. The present findings indicate that in cardiomyocytes from rats with CRF, as compared with SO pair-fed control rats, basal [Ca\(^{2+}\)], is increased and the increase in [Ca\(^{2+}\)], in response to rhIGF-1 is markedly attenuated; protein synthesis both without and with rhIGF-1 added to the cardiomyocyte suspensions is greatly reduced. When CRF rats were treated with parathyroidectomy or the calcium channel blocker felodipine, cardiomyocyte basal [Ca\(^{2+}\)], was normal as was the rise in [Ca\(^{2+}\)], in response to stimulation with rhIGF-1. This normal basal [Ca\(^{2+}\)], was associated with significantly greater protein synthesis, both in the basal state and in response to rhIGF-1 stimulation, as compared with the CRF rats that were not subjected to any intervention.

These results indicate that elevated basal cytosolic calcium in cardiomyocytes of rats with CRF is associated with both the impaired basal protein synthesis and the resistance to the actions of IGF-1 in protein synthesis in these cells. It is pertinent that although normalizing basal [Ca\(^{2+}\)], in cardiomyocytes from the CRF rats by PTX or felodipine increased the IGF-1-stimulated protein synthesis rate, these maneuvers did not increase protein synthesis to the control levels. This was not an unexpected finding, because previous research has indicated that there are several mechanisms for IGF-1 resistance in CRF (2,41), and lowering basal [Ca\(^{2+}\)], may correct only one of these causes. Indeed, we have previously shown in skeletal muscle that impaired phosphorylation of the β-subunit of the IGF-1 receptor and reduced phosphorylation of insulin-related substrate 1 by tyrosine kinase are causes of the IGF-1 resistance (2).

In many cells, the transient increase in [Ca\(^{2+}\)], induced by an agonist must be of sufficient magnitude for calcium to function as a second messenger (8). To attain this magnitude, the background cytosolic calcium (i.e., the basal levels of [Ca\(^{2+}\)],) must be maintained at relatively low levels. Also, the stimulus provided by the agonist must be strong (8). Therefore, in situations in which the basal levels of [Ca\(^{2+}\)], are elevated, the signal-to-background ratio will tend to be smaller than in conditions in which the basal levels of [Ca\(^{2+}\)], are normal. Under these circumstances, the biologic response to the agonist may be reduced. Indeed, Fadda and coworkers have reported that the diminished insulin secretion in CRF rats is related to the increased basal [Ca\(^{2+}\)], in the islets of Langerhans of these rats (10). Therefore, the ratio of the magnitude of the rise in [Ca\(^{2+}\)], to the basal [Ca\(^{2+}\)], after exposure of the islets to glucose is smaller in the CRF rats than in the normal animals (42). This, in turn, led to the release of a smaller quantity of insulin. When basal [Ca\(^{2+}\)], in the islets of the CRF rats was reduced to normal, the magnitude of insulin release in response to glucose stimulation increased (42). In the present study, we also found that normalization of the basal [Ca\(^{2+}\)], in cardiomyocytes of CRF rats was not only associated with an increased calcium signal induced by IGF-1, but also with an improved cellular response to stimulation of IGF-1 as indicated by enhanced protein synthesis.

The cellular mechanism by which elevated basal [Ca\(^{2+}\)], is associated with reduced basal protein synthesis and a rise in [Ca\(^{2+}\)], is associated with increased protein synthesis in cardiomyocytes is unclear. [Ca\(^{2+}\)], is a multifactorial intracellular messenger with large numbers of effects on physiologic processes (43). Depletion of [Ca\(^{2+}\)], is associated with inhibition of protein synthesis in several cell lines (43), whereas a rise in [Ca\(^{2+}\)], is associated with an increase in synthesis of specific proteins and cell proliferation (26,27,44,45) in addition to many other actions (9–16,26,44,45). Further research will be necessary to elucidate whether, in cardiomyocytes, there is a direct cause and effect relation between basal [Ca\(^{2+}\)], and the incremnet in [Ca\(^{2+}\)], and the rates of protein synthesis. Nonetheless, these observations support the thesis that, in CRF, elevated basal [Ca\(^{2+}\)], may contribute to cellular malfunction by decreasing the calcium signal.

It is not likely that the results of this study are attributable to differences in nutritional status. One rat from each of the SO, CRF-PTX, and CRF-F groups was pair-fed to a CRF rat, and the final body weights as well as the weight gain during the study were similar in the three groups of CRF rats (Table 1). Differences in hypertension-induced cardiac hypertrophy and injury also would not seem to account for these results. The BP in the three groups of CRF rats were similar. Indeed, the dose of felodipine administered to the CRF-F rats was selected to be sufficiently low so as to avoid reducing the BP in these animals (Figure 1, see Materials and Methods). There were no differences in the magnitude of renal function in the three CRF
Table 2. IGF-1 receptor number and binding affinity in hearts from the four groups of rats

<table>
<thead>
<tr>
<th>Group</th>
<th>$B_{\text{max}}$ (nmol/mg protein)</th>
<th>$K_d$ (nM)</th>
</tr>
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<tbody>
<tr>
<td>SO rats</td>
<td>8.96 ± 1.90</td>
<td>1.75 ± 0.77</td>
</tr>
<tr>
<td>CRF-PTX rats</td>
<td>9.32 ± 2.06</td>
<td>1.65 ± 0.82</td>
</tr>
<tr>
<td>CRF-F rats</td>
<td>8.69 ± 1.58</td>
<td>1.72 ± 0.53</td>
</tr>
<tr>
<td>CRF rats</td>
<td>9.16 ± 1.95</td>
<td>1.68 ± 0.77</td>
</tr>
</tbody>
</table>

a $^{125}$I-IGF-1 binding to the membrane fraction from the hearts of rats was determined in the absence or presence of unlabeled rhIGF-1 (0.1 to 25 nM) as described in Materials and Methods. Each value represents the mean ± SEM of separate experiments performed in duplicate or triplicate in six rats from each group. IGF-1, insulin-like growth factor-1; rhIGF-1, recombinant human IGF-1.

The LIGAND program was used to fit the displacement binding curves and to compute the characteristic parameters of the IGF-1 receptor. The $B_{\text{max}}$ and $K_d$ were calculated from the rhIGF-1 displacement binding data.

groups (as indicated by the serum creatinine) or in the serum calcium or phosphorus levels (Table 1) or degree of acidemia in all four groups; the CRF-PTX rats received sufficient NaHCO$_3$ to prevent acidemia (28), and the three groups of CRF rats were given sufficient NaHCO$_3$ to prevent acidemia (2). Our studies also showed similar IGF receptor mRNA levels (Figure 4) and similar receptor numbers and binding affinity values (Table 2) in the four groups of rats. These findings indicate that the enhanced stimulation of protein synthesis by rhIGF-1 in the CRF-PTX and CRF-F rats is not attributable to altered IGF-1 receptor number or binding affinity.

It is of interest that protein synthesis in the cardiomyocytes from untreated CRF rats was impaired even when IGF-1 was not added to the media. These findings are consistent with our previous observations and those of others that unstimulated protein synthesis is decreased in skeletal muscle and liver of rats with CRF (2,46–50). The finding that lowering basal [Ca$^{2+}$]$_i$ to normal values in the CRF cardiomyocytes was associated with a basal protein synthetic rate that was not different from the SO control rats is consistent with the possibility that elevated basal [Ca$^{2+}$]$_i$ has a pervasive inhibitory effect on protein synthesis in CRF. If elevated basal [Ca$^{2+}$]$_i$ impairs protein synthesis in other tissues, this phenomenon may be an important contributor to protein malnutrition in CRF. This is potentially an important question because protein malnutrition is a common complication of CRF and is a strong risk factor for morbidity and mortality in this condition. Thus, the possibility that lowering basal [Ca$^{2+}$]$_i$ in other tissues may increase protein synthesis and increase body protein mass would appear to be a matter that merits further investigation.

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