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TNFα receptor expression in rat cardiac myocytes: TNFα inhibition of L-type Ca^{2+} current and Ca^{2+} transients

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Abstract Tumor necrosis factor-α (TNFα) is a potentially powerful anti-neoplastic agent; however, its therapeutic usefulness is limited by its cardiotoxic and negative inotropic effects. Accordingly, studies were undertaken to gain a better understanding of the mechanisms of TNFα-mediated cardiodepression. Single cell RT-PCR, [^{125}I]TNFα ligand binding and Western immunoblotting experiments demonstrated that rat cardiac cells predominantly express type I TNFα receptors (TNFRI or p60). TNFα inhibited cardiac L-type Ca^{2+} channel current (I_{Ca}) and contractile Ca^{2+} transients. Thus, it is possible that the negative inotropic effects of TNFα are the result of TNFRI-mediated blockade of cardiac excitation-contraction coupling.

Key words: TNFα receptor; Cardiac Ca^{2+}; Inotropy; Single cell RT-PCR; Rat cardiac myocyte

1. Introduction

The cytokine, tumor necrosis factor-α (TNFα), is responsible for many cardiotoxic and negative inotropic effects of septic shock, tissue transplant and HIV infection [1–3]. The cardiotoxic effects of TNFα have limited its usefulness as a therapeutic agent in human cancer therapy and as an anti-viral agent [4]. Hypotension resulting from the depressant effect of TNFα on the heart constitutes a major dose-limiting toxic effect of TNFα observed in phase I clinical trials [4]. TNFα levels in blood are elevated with congestive heart failure [5] and viral myocarditis [6] and may promote negative inotropy in those conditions. TNFα levels are elevated in human serum during septic shock and are correlated with the characteristic hypotension (systolic blood pressure < 90 mmHg) of the disease as well as with diffuse tissue injury, multiple-system organ failure and death resulting from severe sepsis [7]. In animal model systems, TNFα has a negative inotropic effect on cardiac tissue [8–11]. TNFα blocks spontaneous contractions of cultured murine cardiomyocytes [12] and reduces the size of Ca^{2+} transients in adult feline cardiomyocytes [13]. The mechanism by which TNFα exerts cardiodepressant effects is not known primarily because cardiac TNFα receptors have not been characterized and the signal transduction cascade(s) responsible for the acute effects of TNFα have not been elucidated. In this study, we have identified the type I TNFα receptor (TNFRI, p60) on adult rat cardiac myocytes. Further, we provide evidence suggesting that TNFα, presumably acting via the TNFRI, exerts its negative inotropic effect on cardiac cells in part by inhibiting the L-type Ca^{2+} channel of the cardiac cell surface membrane.

2. Materials and methods

2.1. Cell and microsome preparation

Acutely dissociated adult rat ventricular myocytes were prepared from adult (200–350 g) Sprague–Dawley rat hearts by enzymatic dissociation as described previously [14]. The Tyrode's solution used for cell isolation contained (mM): NaCl 140, KCl 5.4, $MgCl_2$ 0.5, $CaCl_2$ 1.0, HEPES 10, and NaH_2PO_4 0.25 (pH 7.3). The adult rat myocytes were 'Ca^{2+} tolerant' in that they did not supercontract in the presence of 1 mM Ca^{2+} and they were responsive to electrical field stimulation. For single cell RT-PCR, cells were plated on laminin-coated (50 μg/ml) glass coverslips and cultured 18–24 h in DMEM/F12 medium supplemented with 10% fetal calf serum and antibiotics, as previously described [15]. Rat cardiac, skeletal and liver microsomal fractions were obtained by utilizing methods described previously for canine membranes [16]. Isolated membranes were used for the Western immunoblotting and [^{125}]TNFα ligand blotting experiments (see below). Protein concentration was determined by the Lowry method [17].

2.2. Ca^{2+} current (I_{Ca}) and Ca^{2+} transient measurements

Calcium currents (I_{Ca}) were recorded from single cells patch-clamped in the whole-cell configuration as previously described [18–20]. The effects of TNFα on the Ca^{2+} transients and their comparison with the TNFα inhibition of the I_{Ca}, were examined in 13 cells electrically paced at 0.3 Hz for their responses to a level of recombinant murine TNFα (rmTNFα; 18.000 U/ml; Genzyme, Cambridge, MA) and other conditions (e.g. 5 min incubation) used in the L-channel work. Fluorescent measurements were performed as previously described [20]. For each of the 13 cells studied, 5 transients were analyzed immediately prior to TNFα addition and were compared to 5 transients exhibited by the cells 4.5–5 min later. In these experiments, 10 mM BSA was present either as a control or as a TNFα diluent to prevent TNFα from sticking to the chamber surfaces.

2.3. Whole tissue and single cell RT-PCR

Expression of the TNFRI gene in the rat heart was determined by both single cell RT-PCR and amplification of cDNA reverse transcribed from whole tissue mRNA. Total RNA from adult rat ventricular tissue was isolated by acid/guanidinium thiocyanate/phenol/chloroform extraction and quantified by ultraviolet absorption at 260 nm as described [15,21]. Aliquots of total RNA were electrophoresed on 1% agarose gels followed by ethidium bromide staining to rule out significant RNA degradation. Poly(A+) RNA was isolated by oligo(dT) cellulose chromatography (Promega). Purified transcript was reverse transcribed and subjected to PCR. Template cDNA was obtained from reverse transcription of the DNAse-treated (RNAse-free) poly(A)$^+$ RNA modified from [22,23]. Briefly, poly(A)$^+$ RNA (5 μg) was reverse transcribed with random hexamer primers (100 pmol/reaction), dNTPs and avian myeloblastosis reverse transcriptase (AMVRT; Promega), extracted with phenol/chloroform, ethanol-precipitated at −70°C and

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solubilized in water. PCR primers and annealing temperatures were selected utilizing Oligo 4.0 Primer Analysis software (National Biosciences Inc., Plymouth, MN)). Sense and antisense primers for TNFRI were 5′-GCTCCTGGCTCTGCTGAT-3′ and 5′-AACATTTCTTTC-CCACAT-3′, respectively. Primers for atrial natriuretic factor (ANF) were 5′-AGCGGGGGCGGCACTTAG-3′ (sense) and 5′-CTCCAAT-CCTGTCAATCC-3′ (antisense). Primers were synthesized in the SDSU Microchemical Core Facility.

Aliquots of the cDNA template were added to the primers (100 pmol), dNTP (200 μM of each dATP, dCTP, dTTP, dGTP) with $MgCl_2$ (2 mM) and *Taq* polymerase (1.25 U; Boehringer-Mannheim) and subjected to 30 cycles of PCR (94°C (1 min), 55°C (1 min), 72°C (1 min) followed by 3 min at 72°C) using a programmable thermal cycler (PTC-100; MJ Research Inc., Watertown, MA). PCR products were ethanol precipitated, electrophoresed through a 2% agarose gel and visualized by ethidium bromide staining. Gels were photographed and the negative image of the gel was used for densitometry (Molecular Dynamics). The remainder of the PCR product was ethanol precipitated and digested with *Eco*RI and *Xho*I, isolated following electrophoresis from a 2% LMP Agarose gel and the 288 bp fragment was subcloned into pBluescript (KS+) (Stratagene) and sequenced by the dideoxy chain-termination method (Sequenase).

2.5. Single cell RT-PCR

Expression of TNFRI was determined by RT-PCR of RNA harvested from individual adult cardiac myocytes which were obtained by enzymatic dissociation of adult rat hearts as described [18–20]. Following overnight culture, cells were rinsed thoroughly to remove cell debris and incubated in Ca^{2+}-free Tyrode's to minimize spontaneous myocyte contractility which would interfere with seal formation and could hamper the extraction of cytoplasmic contents. Extraction of RNA from the isolated cells was performed essentially as O'Dowd and co-workers described for chick ciliary ganglion cells [24]. Fire-polished, baked pipettes with a 5–10 MΩ resistance were filled with sterile Ca^{2+}-free Tyrode's solution. Individual cells were patch clamped in the whole cell configuration [18–20]. After detection of whole cell currents, negative pressure (suction to 9 ml mark on 10 ml syringe for 2–5 min) was applied to the pipette and a fraction of the cell's cytoplasmic contents were aspirated into the patch pipette tip which was then broken against the side of a 0.5 ml sterile microcentrifuge tube. Approximately 2 μl of the pipette's contents were expelled and rapidly mixed into 7.5 μl of ice-cold first strand cDNA synthesis mixture containing dNTPs (1 mM), random hexanucleotide primers (10 μM), RNAse (50 U) in 2.5× reverse transcription buffer, and DNAse I (10 U). First strand cDNA was synthesized by addition of 0.5 μl of AMVRT (20 U), followed by incubation at 42°C for 1 h. The reaction was terminated by incubating at 90°C for 10 min. cDNA from first strand synthesis reaction was subjected to PCR as described above for cardiac tissue cDNA amplification utilizing rat TNFRI-specific primers.

2.6. Western blotting

Cardiac, skeletal and liver microsomal proteins were separated by SDS-PAGE, and then electrotransferred to nitrocellulose membranes and receptor protein expression was evaluated by Western blot analysis as described [25] with modifications. Protein samples were solubilized either in a reducing (0.125 M Tris-base pH 6.8, 280 mM SDS, 20% glycerol, 2% β-mercaptoethanol (β-ME)) or in a non-reducing (without β-ME) SDS buffer. Samples (~40 μg) were electrophoresed on an 10% SDS-polyacrylamide gel [26] using a mini-PROTEAN II (Bio-Rad, Hercules, CA) apparatus with electrophoresis buffer (25 mM Tris-base, 192 mM glycine, 3 mM SDS). The transfers were performed at a constant 63 V for 16 h at 4°C in transfer buffer (12.5 mM Tris-base, 96 mM glycine, 20% methanol). The blots were blocked with 3% nonfat dry-milk in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20) solution for 1 h. Subsequently, the membranes were incubated overnight in TBST containing 3% nonfat dry-milk and the primary antibody (100 ng/ml monoclonal anti-human TNFRI IgG) (Genzyme). Membranes were washed in TBST (2 × 15 min) and incubated with biotinylated goat anti-mouse IgG (1 μg/ml) for 1 h. Following thorough washing and incubation with an avidin-alkaline phosphatase conjugate (1 μg/ml), receptor protein was visualized with alkaline phosphatase substrates (*p*-nitro blue tetrazolium chloride (NBT; 30 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP; 15 mg/ml)). Immunoblots were analyzed by scanning laser densitometry (Molecular Dynamics, Sunnyvale, CA) and molecular weights of the immunoreactive microsomal proteins were calculated from the prestained protein standards by ImageQuant (Molecular Dynamics).

2.6. [^{125}I]TNFα ligand blotting

Cardiac membrane proteins were also electroblotted and processed for [^{125}I]TNFα binding [27,28]. TNFα was iodinated by the Chloramine-T method [27,28]. Identical results were obtained using either [^{125}I]rhTNFα (1500 Ci/mmol obtained from NEN/DuPont) or the [^{125}I]rmTNFα labeled by the Chloramine-T method. Membrane proteins were transferred to nitrocellulose and blocked with 3% non-fat dry-milk as described above using non-denaturing conditions for the initial electrophoretic separation of microsomal membrane protein. Ligand blots were performed as described in [28] with modifications. Briefly, membranes were incubated overnight in 500 pM [^{125}I]TNFα with or without 50 nM unlabelled TNFα in TBST with 3% nonfat dry-milk. Blots were washed in TBST (4 × 15 min), air-dried and incubated on a storage phosphor screen (Molecular Dynamics) for 48–72 h, scanned on a PhosphorImager (Molecular Dynamics) and analyzed using ImageQuant. Liver and skeletal microsomal membrane proteins were electroblotted and probed with ^{125}I-TNFα at the same time as the cardiac membranes and under identical conditions for comparison purposes.

3. Results and discussion

3.1. L-Type Ca^{2+} channel currents

TNFα caused a significant and rapid inhibition of the L-type Ca^{2+} channel current (I_{Ca}) in myocytes patch-clamped in the whole cell configuration (Fig. 1A). As seen in Fig. 1B, the *I–V* plot of the Ca^{2+} currents recorded corresponds to that of high threshold L-type Ca^{2+} channels, and it is clear that TNFα exerts a proportional inhibitory action at all potentials. The *I–V* plot does not indicate the presence of any other current components that might contribute to the effect on I_{Ca} that we are attributing to TNFα. Significantly, there was a partial recovery of the inward Ca^{2+} current after TNFα washout, suggesting that a key second messenger may either have been removed by the washout or was turned over rapidly by the cell during that time period. TNFα inhibition of the I_{Ca} was dose dependent (Fig. 1C), displaying half-maximal inhibition at appproximately 6400 U/ml. Evidence for a specific receptor-mediated mechanism for TNFα action on the L-channel was suggested by neutralizing the inhibitory effects of TNFα with the soluble receptor fragment (sTNFRI). In one experiment, 9000 U/ml TNFα inhibited the I_{Ca} by 30%, while pretreatment of a cell with 4 μg/ml sTNFRI limited the TNFα inhibition to 6.6%. sTNFRI competes with the membrane-bound form of the native receptor to modulate the effects of TNFα and has been used in other cell types to implicate the TNFRI in mediating TNFα action [29–31].

3.2. Calcium transients

The L-type Ca^{2+} channel is an essential trigger for cardiac EC coupling. It has been demonstrated that Ca^{2+} entry via the cardiac L-channel regulates the amount of Ca^{2+} released from the cardiac sarcoplasmic reticulum (SR) membranes as the rate of SR Ca^{2+} release is dependent on the peak I_{Ca} amplitude [32]. Since TNFα exhibited a significant inhibitory action on the cardiac L-type channel, TNFα may interfere with the Ca^{2+}-induced Ca^{2+} release (CICR) mechanism of EC coupling [33] by inhibiting the entry of 'trigger Ca^{2+}' into the cell via the L-channel and reducing the amount of Ca^{2+} released from cardiac SR membranes. Accordingly, we examined actions of

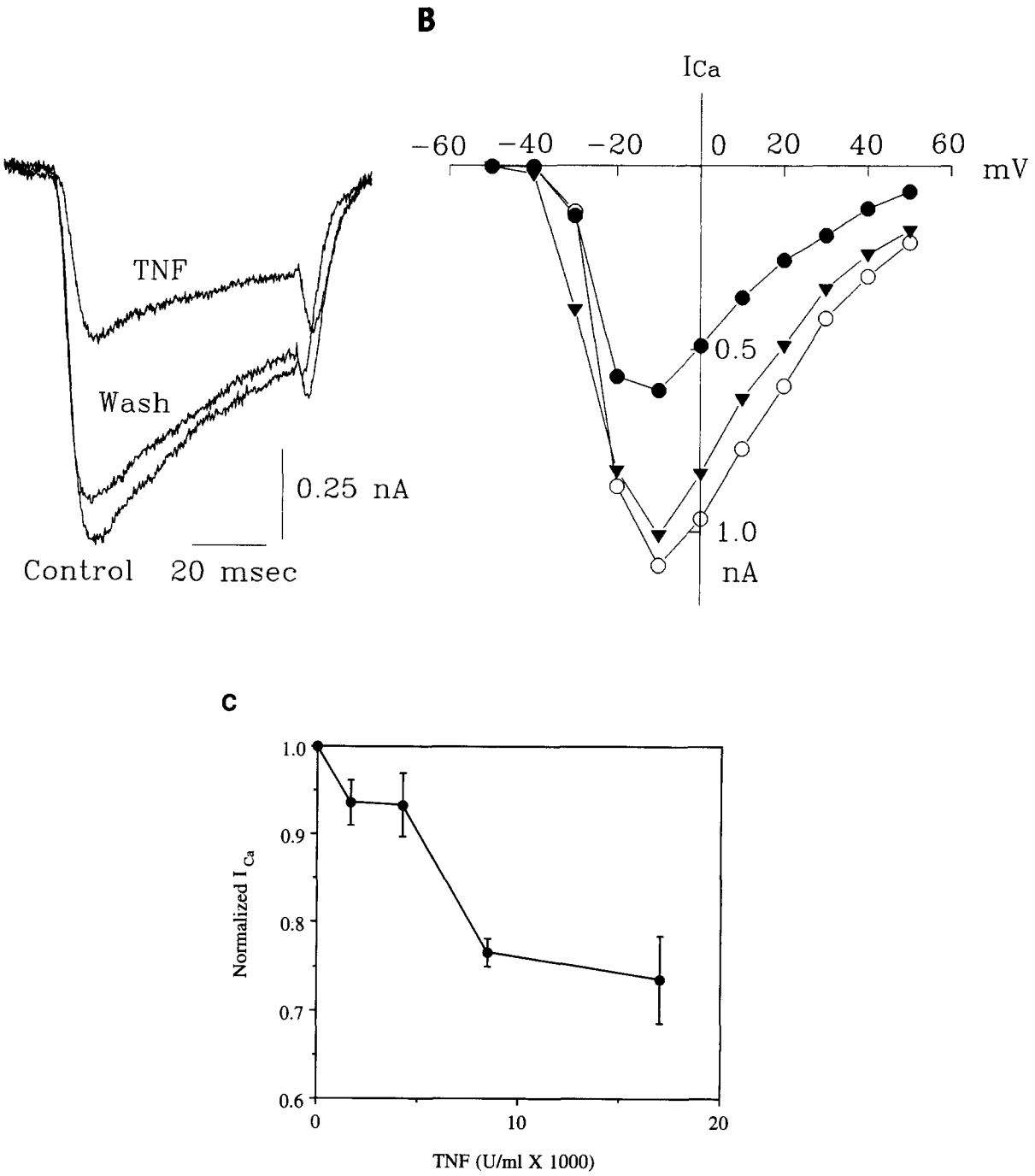

Fig. 1. Tumor necrosis factor-α (TNFα) inhibits cardiac L-type channel Ca^{2+} current. (A) L-type channel recordings from a whole cell patch- clamped adult rat cardiac myocyte representing one of the largest responses to TNFα (45% inhibition of inward Ca^{2+} current (I_{Ca})). The myocyte was treated for 5 min at room temperature with recombinant murine TNFα (18,000 U/ml). There was a partial recovery after washout. Control experiments using fatty acid free BSA (data not shown) failed to show a nonspecific protein effect on the channel in the range of protein used in the TNFα experiments. (B) Current–voltage relationship for I_{Ca} in the same cell prior to TNFα application (open circles), 5 min after TNFα application (closed circles) and after washout (closed triangles). (C) Average dose-dependent effect of TNFα on L-type channel current in adult cardiac myocytes demonstrated half-maximal inhibition with 6400 U/ml. Holding potential −50 mV, pulses to 0 mV.

TNFα on the Ca^{2+} transients exhibited by acutely dissociated, electrically paced (0.3 Hz) adult rat cardiomyocytes. Fig. 2 shows a representative cell in which TNFα (0.6 nM, 18,000 U/ml) caused a progressive decrease in the amplitude of the Ca^{2+} transients, reaching 43% of control amplitude after 5 min. Of the 13 cells studied with this concentration of TNFα, the peak systolic indo-1 ratios recorded 4–5 min after TNFα addition exhibited magnitudes of 46 ± 24% (S.D.) of control values. In most of cells studied, the transients reached steady state levels after 5 min but sometimes took up to 15 min. As can be

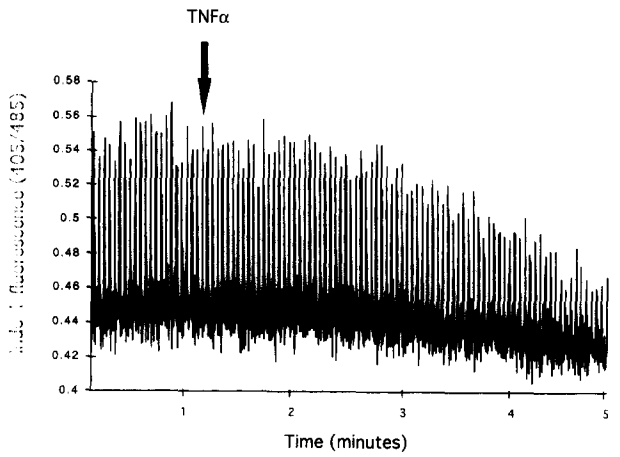

Fig. 2. Tracing showing inhibition of cardiac Ca^{2+} transients by TNFα. Indo 1 Ca^{2+} transients of an adult rat ventricular myocyte are shown. These and all subsequent Ca^{2+} transient measurements were made at room temperature from the cytoplasmic regions of individual cells electrically paced at 0.3 Hz. At the indicated times, 18000 U/ml recombinant murine TNFα was added together with 10 μM BSA while indo-1 fluorescence was continuously monitored. The $[Ca^{2+}]_i$ is expressed as the ratio of indo-1 fluorescence emission at 405 and 485 nm. Control experiments (not shown) established that the reduction in transients was not due to a side effect of the 10 μM BSA used to prevent TNFα from sticking to the chamber surfaces.

seen in Fig. 2, the diastolic (resting) Ca^{2+} level was not affected by TNFα. Importantly, the time course of TNFα action on the peak Ca^{2+} transients was similar to that exhibited by TNFα on the L-type Ca^{2+} currents in that by 5 min both were significantly affected by TNFα. Commonly, the Ca^{2+} transients were more strongly inhibited than the L-channel Ca^{2+} currents, suggesting that the effect of TNFα on L-channel Ca^{2+} current only partially accounts for the inhibition of intracellular Ca^{2+} release. Our data are consistent with a previous report utilizing feline cardiomyocytes [13] which demonstrated that the negative inotropic effect of TNFα is exerted by an inhibition of the Ca^{2+} transients and the EC coupling mechanism. Their report for feline cardiac cells utilizing 200 U/ml human TNFα showed that the Ca^{2+} transients were reduced by TNFα but the L-type Ca^{2+} channel currents were unaffected, suggesting that TNFα exerts its effects downstream from the L-channel in the EC coupling process. In contrast, our data with rat myocytes suggest that TNFα-inhibition of the I_{Ca} can account for a significant proportion of the EC coupling deficit exhibited by TNFα-treated cardiac cells.

3.3. TNFα receptor expression on isolated cardiac membranes

The ability of TNFα to inhibit I_{Ca} and Ca^{2+} transients of single rat cardiac myocytes (Figs. 1A and 2, respectively) plus the ability of sTNFRI to block TNFα's effects on the L-channel was consistent with TNFα exerting its negative inotropic effects by interaction with a specific membrane-bound receptor on the cardiac myocyte. Despite clear implications of a role for the cytokine in cardiac function, studies examining the expression of TNFα receptors by cardiac cells are lacking. Two TNFα receptors, type I (TNFRI, 60 kDa) and type II (TNFRII, 80 kDa) have been identified [34] and are expressed in several non-muscle tissues [35–37]. Investigations of signaling pathways mediating cytotoxic effects of TNFα in non-cardiac cells have revealed a function for the TNFRI [3,38–40] and that the major role of TNFRII is to recruit TNFα for the TNFRI by a process termed 'ligand passing' [41].

Expression of the receptor protein on cardiac cell membranes was assessed initally by immunoblotting and ligand blotting methods. TNFRI protein was identified with an anti-human TNFRI monoclonal antibody following transfer from a reducing SDS-PAGE. As expected, a single 60 kDa molecular weight band corresponding to TNFRI was obtained (Fig. 3A, lane 2) demonstrating that TNFRI is expressed in the cardiac myocyte. Although greater than the predicted size of the rat TNFRI as determined by cDNA sequence (48 kDa) [42], the results are consistent with the M_r for TNFRI after glycosylation [28,42,43]. In contrast to rat cardiac membranes, TNFRI expression in rat skeletal muscle membrane was low (Fig. 3A, lane 3). The apparent differential expression between muscle types is intriguing and may be confirmed in future studies by comparing TNFRI and transcript levels as well as tissue responsiveness to TNFα.

Cardiac membrane proteins were also electroblotted and processed for $[^{125}I]$TNFα binding. Fig. 3B (lane 2) shows that rat cardiac membranes possess a prominent $[^{125}I]$TNFα-binding protein with M_r of 60 kDa. Identity of the $[^{125}I]$TNFα binding protein as TNFRI was confirmed by Western blot analysis (Fig. 3B, lane 1) performed on the same blot used for the $[^{125}I]$TNFα ligand blotting. The TNFα concentrations used for both ligand blotting and the physiological experiments (0.5–0.6 nM) described above are similar to the K_d range (0.3 nM) for TNFα binding to the TNFRI [34]. Both ligand blots and immunoblots show the presence of higher M_r bands on this non-reducing gel. High molecular weight bands are commonly observed in studies characterizing TNFα receptors [43,44] and may be due to the formation of disulfide-linked TNFα receptor aggregates between the cysteine-rich extracellular domains [28] which are particularly apparent under the non-reducing electrophoresis conditions used in the $[^{125}I]$TNFα binding experiments. The appearance of faint bands near 80 kDa in both lanes 1 and 2 is likely an artifact of the non-reducing gel as described above since the monoclonal antibody to TNFRI exhibits no cross-reactivity to the type II receptor. The specificity of TNFα binding was demonstrated in control experiments using 100-fold excess of cold TNFα which completely blocked $[^{125}I]$TNFα binding (Fig. 3B, lane 3). In contrast to cardiac membranes, rat skeletal muscle membranes do not possess appreciable levels of functional receptor as judged by their lack of $[^{125}I]$TNFα binding (Fig. 3B, lane 4), an observation which confirms the low level of protein expression seen in Western blots (Fig. 3A, lane 3). Importantly, the data in Figure 3B also indicate that within the limits of detection, cardiac muscle membranes express principally TNFRI, since TNFα binding was weak in the 80 kDa range characteristic of TNFRII. Our $[^{125}I]$TNFα binding methods were capable of detecting both subtypes of the receptor if present in a particular tissue. For example, we observed at least two prominent $[^{125}I]$TNFα binding proteins in rat liver membranes (Fig. 3B, lane 5) with M_r values of 60 kDa and 80 kDa, corresponding to both TNFRI and TNFRII subtypes, respectively. Liver membranes exhibited an additional TNFα-binding protein with an M_r of 35 kDa. This protein is of a size corresponding to the extracellular fragment of the native TNFRI receptor (sTNFRI) which is often shed and retains its ability to bind $[^{125}I]$TNFα [45].

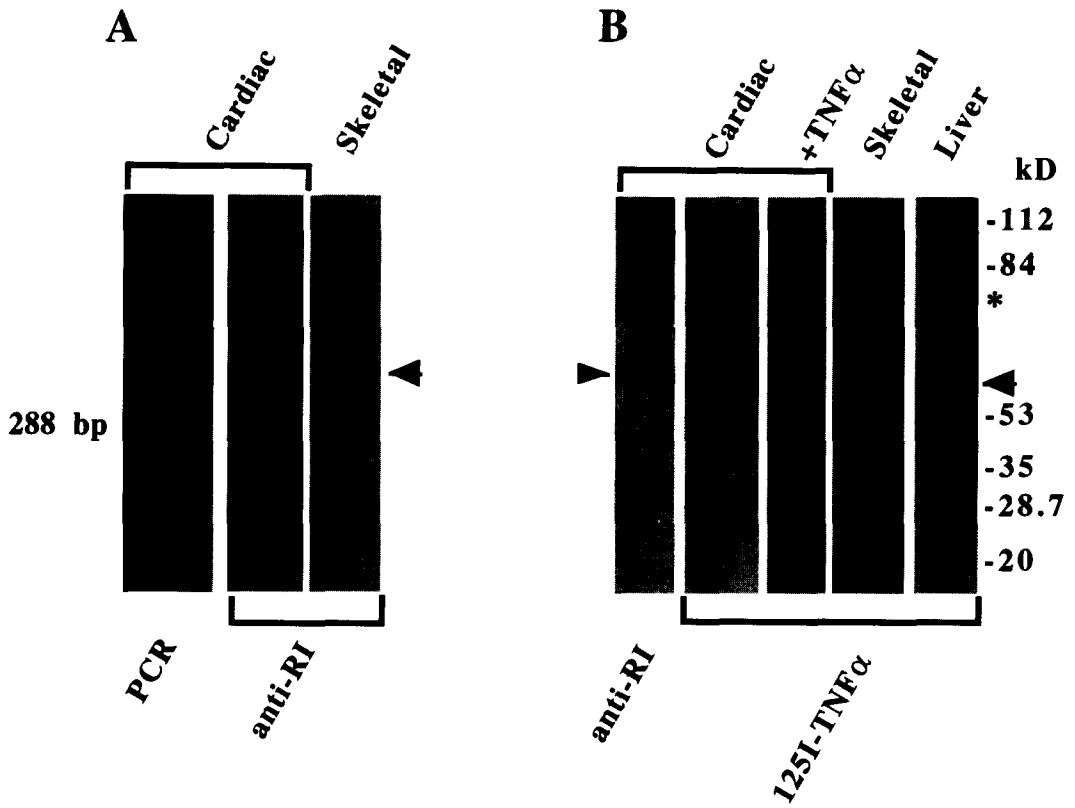

Fig. 3. Expression of the TNFα type I receptor (TNFRI) on cardiac membranes. (A) Lane 1 is a 288 bp RT-PCR product amplified from reverse transcribed adult rat ventricular poly(A)$^+$ RNA [15,21]. The dideoxy sequenced (see text) 288 bp product corresponds to an extracellular portion of TNFRI. Lanes 2 and 3 are Western blots [25] from a reducing SDS-PAGE of rat cardiac membranes [16] probed with a monoclonal anti-human TNFRI IgG (anti-RI). A 60 kDa receptor (arrowhead) was identified in cardiac but not in skeletal muscle membranes. (B) Western immunoblot (lane 1) and [^{125}I]TNFα ligand blots (lanes 2–5) from a non-reducing SDS-PAGE of rat membranes. A 60 kDa [^{125}I]TNFα-binding protein (arrowhead) was identified in cardiac but not in skeletal muscle membranes. Specificity of the binding protein for TNFα was determined by effective competition of [^{125}I]TNFα with a 100-fold excess of unlabelled TNFα (+TNFα). Western blot analysis (lane 1) of the same piece of nitrocellulose used in lane 2 of this non-reducing gel confirmed the 60 kDa binding protein as TNFRI. Rat liver membranes (lane 5) clearly expressed two [^{125}I]TNFα-binding proteins with M_r of 60 kDa (arrowhead) and 80 kDa (asterisk) in contrast to the predominant 60 kDa protein in the rat heart. The minor band at 35 kDa may correspond to the soluble TNF receptor (sTNFRI) as described in the text.

3.4. Single cell RT-PCR

We initially determined expression of the TNFRI gene in the rat heart by RT-PCR following total RNA extraction from cardiac tissue. The predicted 288 bp product was obtained (Fig. 3A, lane 1). Sequencing [46] of the PCR product (Table 1) confirmed amplification of the extracellular domain of the rat TNFRI and demonstrated that this segment of the rat cardiac TNFRI shares 100% identity with the nucleotide sequence of the TNFRI obtained from the rat gliomal cell line [42]. These results provide evidence that the TNFRI gene is transcriptionally active in adult rat heart tissue. It is not clear, however, that the ventricular myocytes themselves are the source of the TNFRI message amplified in Fig. 3A, lane 1. Although the heart consists primarily of ventricular myocytes, other cell types are present in heart tissue such as fibroblasts, endothelial cells, neurons and infiltrating macrophages. These non-muscle cell types could be the source of the TNFRI message detected by RT-PCR shown in Fig. 3A, lane 1. We hypothesized that the receptor expression detected by RT-PCR was located on ventricular myocytes since the negative inotropic effects of TNFα on cardiac Ca^{2+} currents and transients were direct and rapid (Figs. 1,2).

To confirm that the myocytes themselves expressed the receptor, we performed single cell RT-PCR from mRNA extracted from the cytoplasm of individual myocytes. This technique has been successfully used to examine cell-specific gene expression without potential contamination of RNA from other cell types and without amplifying genomic DNA. [47]. Single cell PCR is advantageous over other methods which utilize whole tissue RNA to demonstrate gene expression and is particularly useful when the level of the message in question is not abundant, as is the case for TNFα receptors. Although the PCR method employed here cannot be used to accurately quantify differential gene expression, it can clearly indicate whether or not a particular gene is transcribed in the cell type of interest. In Fig. 4 (lane 2), a 288 bp PCR product was successfully amplified from a single cell, indicating that adult ventricular myocytes express the TNFRI. To eliminate the possibility that a PCR product might be derived from DNA of degenerating cells in the culture dish, several cell-conditioned media samples were subjected to RT-PCR. An example of this important control experiment is shown in Fig. 4 (lane 3) and clearly shows the lack of PCR product. Typically 3–5 media controls were performed for each experiment in which cytoplasmic contents were aspirated. All of the media controls were free of any PCR product. Of the 28 cells from which we api-

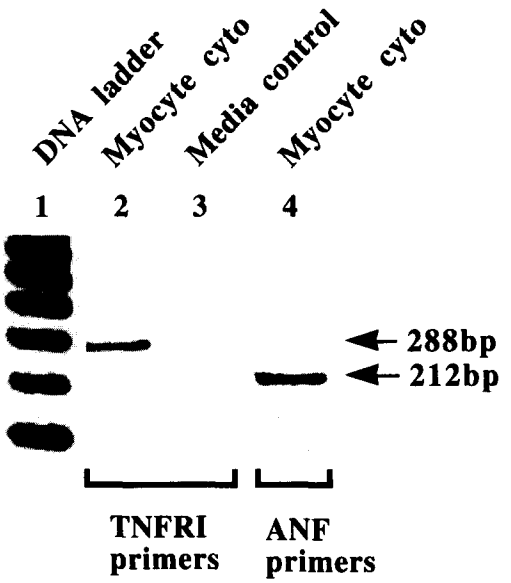

Fig. 4. Expression of TNFRI in cardiac myocytes by single cell RT-PCR. Total RNA from an adult rat ventricular myocyte was reverse transcribed and the resulting cDNA then run in a PCR reaction with sense and antisense primers for rat TNFRI resulting in a 288 bp product (lane 2). Lane 3 is a media control in which 2 μl of cell-conditioned media was aspirated for first strand cDNA synthesis and processed in parallel with the experimental lane (lane 2) containing cytoplasmic RNA. Lane 4 is a 212 bp RT-PCR product amplified using primers for rat atrial natriuretic factor (ANF). An 100 bp DNA ladder is shown in lane 1 with the lowest band representing 100 bp DNA. PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining. The figure is an inverted image of the original.

rated cytoplasmic contents, we were able to successfully amplify 10 PCR products. In other control experiments, we examined the expression of atrial natriuretic factor (ANF), a myocyte-specific gene. Using rat ANF primers (see section 2), we observed a characteristic 212 bp ANF gene product (Fig. 4, lane 4).

The presence of the TNFRI message detected by single cell PCR is in agreement with Fig. 3B (lane 2) in which we identified an [^{125}I]TNFα binding protein in rat cardiac membranes with an M_r consistent with the TNFRI (60 kDa).

4. Conclusions

These experiments provide the first direct evidence for the presence of TNFα receptors on rat cardiac myocyte membranes. Expression of the 60 kDa TNFRI and the receptor-mediated blockage of the cardiac L-channel and intracellular Ca^{2+} transients suggest that the negative inotropic effects of TNFα may be mediated by the TNFRI. The TNFα-mediated inhibition of cardiac Ca^{2+} fluxes likely accounts for the depressant actions of TNFα on cardiac contractility by adversely affecting cardiac EC coupling. Since the cardiotoxic effects of TNFα have limited its usefulness as a potent anti-neoplastic agent, and since several clinical conditions, including sepsis and ischemia, are complicated by TNFα's cardiodepressant effects, an understanding of TNFα's receptor-mediated signaling pathway(s) may be important in mitigating the cytokine's adverse effects. Signaling pathways coupled to TNFRI in non-muscle cells include cascades involving the sphingomyelinase system [48], phosphatidylcholine-specific phospholipase C [39], phospholipase A_2 [49], nitric oxide synthase [50], and their respective second messengers. We are currently investigating the participation of these pathways in mediating responses to the cardiac TNFRI.

5. Note of added proof

Since this manuscript was submitted, a paper appeared which demonstrates that the TNFRI (and not the TNFRII) is the principal receptor that mediates the inhibitory effects of TNFα on cat myocyte cell shortening parameters [51]. This finding compliments our present work in which we have identified the source of the EC-coupling deficits caused by TNFα. In the Torre-Amione et al. report, immunocytochemical staining of

Table 1
Sequence of rat cardiac myocyte TNFRI

	Sense Primer →
Rat gliomal:	GC TCCTGGCTCT GCTGATGGGG ATACACCCAT CAGGGGTCAC CGGACTGGTT
Rat cardiac:	GC TCCTGGCTCT GCTGATGGGG ATACACCCAT CAGGGGTCAC CGGACTGGTT
Gliomal:	CCTTCTCTTG GTGACCGGGA GAAGAGGGAT AATTTGTGTC CCCAGGGAAA
Cardiac:	CCTTCTCTTG GTGACCGGGA GAAGAGGGAT AATTTGTGTC CCCAGGGAAA
Gliomal:	GTATGCCCAT CCAAAGAATA ATTCCATCTG CTGCACCAAG TGCCACAAAG
Cardiac:	GTATGCCCAT CCAAAGAATA ATTCCATCTG CTGCACCAAG TGCCACAAAG
Gliomal:	GAACCTACTT GGTGAGTGAC TGTCCAAGCC CAGGGCAGGA AACAGTCTGC
Cardiac:	GAACCTACTT GGTGAGTGAC TGTCCAAGCC CAGGGCAGGA AACAGTCTGC
Gliomal:	GAGGTGTGTG ATAAAGGCAC CTTTACAGCT TCGCAGAACC ACGTCAGACA
Cardiac:	GAGGTGTGTG ATAAAGGCAC CTTTACAGCT TCGCAGAACC ACGTCAGACA
Gliomal:	GTGTCTCAGT TGCAAGACAT GTCGGAAAGA AATGTT
Cardiac:	GTGTCTCAGT TGCAAGACAT GTCGGAAAGA AATGTT
	← Antisense primer

The 288 bp PCR product from the extracellular domain of the TNFRI in rat cardiac membranes was 100% identical to the TNFRI extracellular domain sequence identified in rat gliomal cells.

human cardiac tissue suggested a myocyte localization of the TNFRI, and Northern blot analysis of human cardiac tissue showed the presence of the TNFRI transcript. However, since the Northern blot would have included RNA from non-myocytes as well as from myocytes in the cardiac tissue, it is not certain that the TNFRI came from ventricular myocytes. On the other hand, our single cell RT-PCR data (Fig. 4) unequivocally localize the TNFRI transcript to rat cardiac myocytes.

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