A monoclonal antibody against the immunodominant epitope of the ribosomal P2β protein of Trypanosoma cruzi interacts with the human β 1-adrenergic receptor

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A monoclonal antibody against the immunodominant epitope of the ribosomal P2β protein of *Trypanosoma cruzi* interacts with the human β1-adrenergic receptor

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Monoclonal antibodies were raised against a recombinant ribosomal P2β protein of *Trypanosoma cruzi*. One of these reacted with the C terminus of this protein (peptide R13, EEEDDDMGFGLFD) and epitope mapping confirmed that this epitope was the same as the one defined by the serum of immunized mice, and similar to the previously described chronic Chagas' heart disease (cChHD) anti-P epitope. Western blotting showed that the monoclonal antibody recognized the parasite ribosomal P proteins, as well as the human ribosomal P proteins. Electron microscopy showed that it stained different structures in parasite and human cells. Interestingly, surface plasmon resonance measurements indicated that the affinity for the parasite ribosomal P protein epitope (R13) was five times higher than for its human counterpart (peptide H13, EESDDDMGFGLFD). Since the human epitope contained an acidic region (EESDD) similar to the AESDE peptide recognized by cChHD patients in the second extra-cellular loop of the human β1-adrenergic receptor, the biological activity of the antibody was assessed on neonatal rat cardiomyocytes in culture. The monoclonal antibody had an agonist-like effect. These results, together with the fact that the monoclonal reacted in Western blots with the different isoforms of the heart β1-adrenergic receptor, confirm the possible pathogenic role of antibodies against the parasite ribosomal P protein based on their cross-reaction with the human β1-adrenergic receptor.

Key words: Autoimmunity / *Trypanosoma cruzi* / Ribosomal P protein / β1-adrenergic receptor

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1 Introduction

Chronic Chagas' heart disease (cChHD) is a frequent cardiomyopathy in South America, occurring in individuals suffering from chronic infection by the protozoan parasite *Trypanosoma cruzi* [1]. Histological examination of the heart reveals mononuclear cell infiltration, myocyte damage and fibrosis in the absence of parasites, although the presence of parasite DNA [2, 3] and parasite proteins [4] have been detected by, respectively, PCR amplification and immunohistochemistry. The origin of heart damage may be related to the immune response against the parasite that can, in turn, lead to an autoimmune attack of the heart tissue by cross reacting T and/or B cells.

It has been shown that antibodies from cChHD patients displayed β1-adrenergic and more recently M2 muscarinic effects on myocardium [4]. Although antibodies interfering with these receptors could not explain the whole symptomatic picture, they may be responsible, at least partly, for the electrocardiogram (ECG) abnormalities observed [4]. In fact, autoantibodies against the β1-adrenergic receptor were detected in sera from patients with idiopathic dilated cardiomyopathy and the target

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The first two authors contributed equally to this work.

Abbreviations: cChHD: Chronic Chagas' heart disease **ECG:** Electrocardiogram **MBP-TcP2β:** Recombinant maltose binding protein fused with ribosomal P2β protein of *Trypanosoma cruzi* **Huβ1AR:** Human β1-adrenoreceptor

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epitope was localized on the second extracellular loop of this receptor [5]. Evidence of a cross-reactivity between a β1-adrenergic receptor epitope in the same domain, namely peptide AESDE, and in the main epitopes of the C termini of the *T. cruzi* ribosomal P0, and the P1 and P2 proteins has also been determined [6, 7]. The epitope on these ribosomal P proteins is represented by a stretch of acidic amino acids [8]. This sequence is particularly conserved in evolution and a single amino acid differentiates the C terminus of the low molecular weight *T. cruzi* P1 and P2 proteins (EEEDDDMGFGLFD, peptide R13) from that of its human P counterparts (EESDDDMGFGLFD, peptide H13) (see [8] for an evolutionary account of the P protein system).

Interestingly, mice immunized with the recombinant TcP2β protein show changes in the ECG, namely an increase in cardiac frequency [9] and develop anti-P antibodies which recognize the C-terminal end of the parasite protein and possess functional autoreactivity with cardiomyocytes through recognition of the β1-adrenergic receptor [10].

To further substantiate the origin of functional anti-cardiac receptor antibodies in cChHD, mice were immunized with a recombinant protein corresponding to the ribosomal P2β protein of *T. cruzi* according to Sepulveda et al. [10]. Three mAb were obtained. Two of them reacted with the hinge region of TcP2β, while the third one reacted with the peptide R13 encompassing the C terminus of the parasite TcP2β molecule [7, 10]. The physicochemical properties of the latter antibody were analyzed and its ability to cross-react with homologous counterparts on mammalian cells was investigated. This antibody presented several features characteristic of cChHD anti-ribosomal P antibodies, including recognition of a polyanionic stretch, and the ability to cross-react with the β1-adrenergic receptor [7]. Structural analysis of this antibody may eventually lead to the development of specific pharmacological treatments directed to avoid receptor-mediated pathology in Chagas' heart disease.

2 Results

2.1 Characterization of mAb against ribosomal TcP2β protein

Three positive hybridoma supernatants reacted with recombinant maltose binding protein fused with ribosomal P2β protein of *T. cruzi* (MBP-TcP2β) and not with MBP. Of these, only one (mAb 17.2) reacted with the C terminus of TcP2β (sequence: AEEE/EEDDDMGFGLFD, peptides 24 and 25 in [10]). The two other clones (mAb 68.3 and mAb 40.14) obtained from two different fusions, reacted with the same epitope in the central part of the TcP2β protein (sequence: LVGG/ATRPNAATASAP, pep-

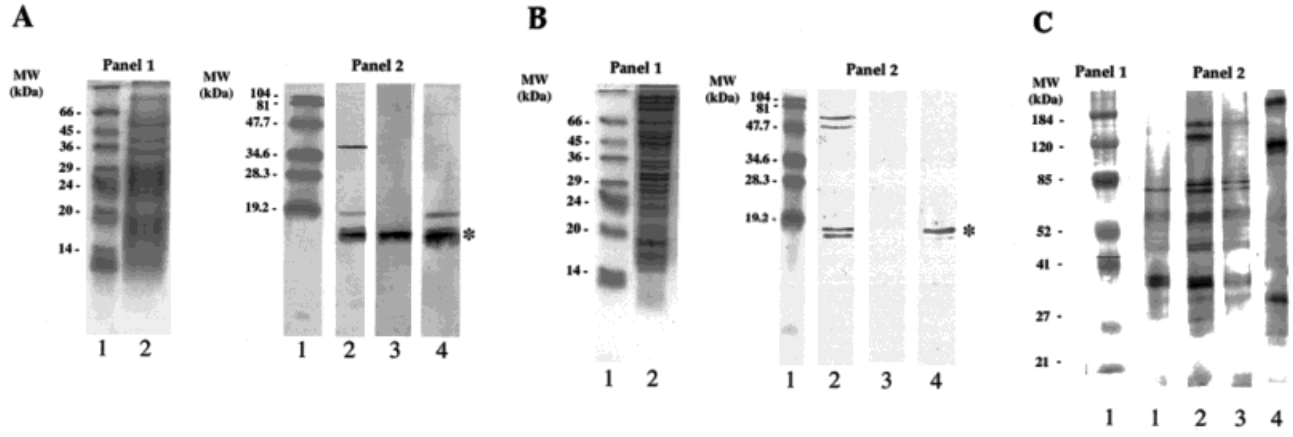

Fig. 1. Immunoblotting of ribosome preparations of *T. cruzi* (A) or HeLa cells (B) or rabbit cardiomyocyte membranes (C) with anti-TcP2 antibodies. Ribosome preparations of *T. cruzi* epimastigotes (A) or HeLa cells (B). Panel 1: Coomassie blue staining, lane 1: molecular weight markers, lane 2: *T. cruzi* (A) or HeLa cells (B) ribosomal preparations. Panel 2: Western blot. Samples applied to each well contained 40 μg protein, lane 1: molecular weight markers, lane 2: immunoblot with mAb 17.2, lane 3: immunoblot with mAb 68.3, lane 4: immunoblot with rabbit polyclonal anti-R-13 antibody. * Doublet bands of 13/15 kDa corresponding to ribosomal P1/P2 proteins. (C) Membrane proteins from rabbit heart. Panel 1: molecular weight markers. Panel 2: lane 1: immunoblot indicating the presence of nonspecific polypeptides revealed by the rabbit anti-mouse antibody in the absence of mAb 17.2, lane 2: immunoblot in the presence of mAb 17.2 showing two specific bands: one major at 120 kDa, one minor at 60 kDa, lane 3: effect of preincubation of mAb 17.2 with R13 peptide: the two bands of 60 and 120 kDa were no more recognized. Lane 4: immunoblot in the presence of mAb 68.3: no recognition of the specific 120 and 60 kDa bands.

tides 16 and 17 in [10]). The isotypes are IgG1 for the mAb 17.2 and IgG2a for the mAb 68.3 and 40.14 (not shown).

Immunoblotting confirmed that mAb 17.2 and mAb 68.3 reacted with the ribosomal proteins of *T. cruzi* (Fig. 1A, Panel 2, lanes 2 and 3). mAb 17.2 reacted with all the components of the ribosomal P protein system of *T. cruzi* [8]. This cross-reactivity spilled over to human ribosomal proteins since the mAb 17.2 recognized a polypeptide doublet in ribosomal preparations of HeLa cells (Fig. 1B, Panel 2, lane 2). mAb 68.3 only recognized a band of 11/12 kDa on *T. cruzi* ribosome preparation corresponding to the parasite P2 protein and no band on HeLa cells ribosome preparation (Fig. 1B, Panel 2, lane 3). Indeed, the hinge region sequence of *T. cruzi* ribosomal P2 protein represented by peptides 16 and 17 [10] is quite different of its counterpart in mammalian cells [8, 11].

Since mAb 17.2 primarily reacts with the C terminus of TcP2 protein that contains a sequence, AEEEEDD, which is similar to the AESDE anionic amino acid stretch present in the second extra-cellular domain of the β_1-adrenergic receptor, a possible cross-reactivity between membrane proteins of cardiomyocytes expressing this receptor, and this mAb was assessed. While control rabbit anti-mouse antibody revealed nonspecific polypeptides (Fig. 1C, lane 1), protein bands corresponding to the isoforms of the heart receptors [12] were revealed on cardiomyocytes membranes (Fig. 1C, lane 2). The specificity of these bands was demonstrated preincubating mAb 17.2 with the R13 peptide. As shown in Fig. 1C, lane 3, bands completely disappeared after preincubation. Otherwise, neither a band of 60 kDa or 120 kDa was revealed by mAb 68.3 (Fig. 1C, lane 4).

2.2 Localization of P proteins by electron microscopy

To confirm the specificity of mAb 17.2 and to visualize the localization of ribosomal P proteins in the parasite, we performed electron microscopy immunogold staining. Using mAb 17.2 and a polyclonal anti-R13 antibody [10] as a positive control, P proteins were localized in *T. cruzi* (Fig. 2A, B), showing a diffuse cytoplasmic staining. Similar experiments were performed with HeLa cells. In this case the immunogold labeling was predominantly associated with the nucleolus, the site of ribosomal subunit assembly in mammalian cells (Fig. 2C).

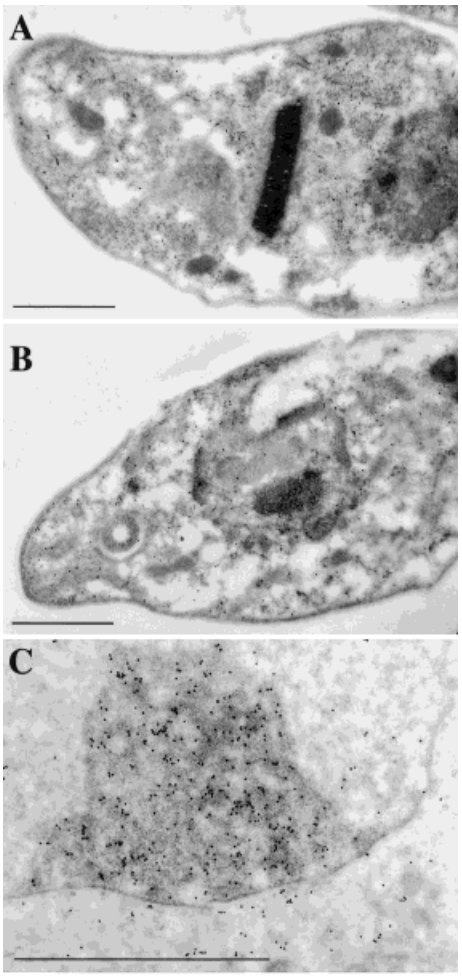

Fig. 2. Localization of ribosomal P proteins by electron microcopy. Immunogold localization of ribosomal P proteins on thin sections of *T. cruzi* epimastigote and of HeLa cells. Both mAb 17.2 (A) and rabbit polyclonal anti-R-13 serum (B) show a diffuse pattern of staining all over the parasite cytoplasm. On HeLa cells a dense staining with mAb 17.2 is observed on nucleolus (C). Bar = 0.5 μm.

2.3 Fine specificity of mAb 17.2 and cross-reactivity

Since the C-terminal peptide is the immunodominant epitope of *T. cruzi* ribosomal P1 and P2 proteins in cChHD and chronic mice [7, 10, 13] and is the epitope recognized by mAb 17.2, alanine mutation scanning was performed to assess the importance of each amino acid in the reactive properties of this peptide. In this case the reactivity of the mAb 17.2 was compared to the polyclonal response of immunized mice. As shown in Fig. 3A, the amino acids essential for recognition were the glutamic acid at position 3, the two aspartic acids at posi-

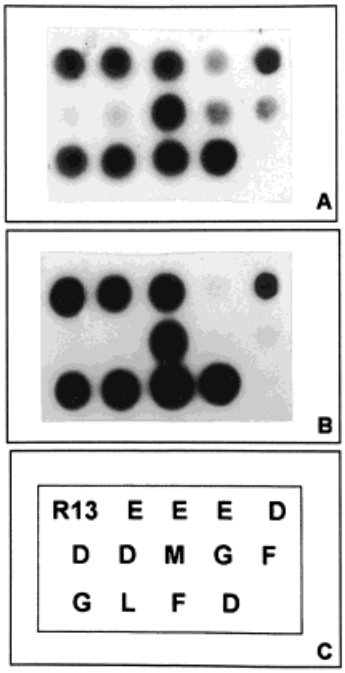

Fig. 3. Comparison of the epitope recognized by mAb 17.2 (A) and an anti-TcP2β polyclonal mouse serum (B) on the R13 peptide or related peptides where each amino acid has been in turn changed into alanine. In (C) a scheme in which each original amino acid of R13 is indicated and located where it has been replaced by alanine. Dot blots show that amino acids 3 (glutamic acid), 5 and 6 (aspartic acids), 8 (glycine) and 9 (phenylalanine) are essential for recognition by mAb 17.2 (A) as well as by the an anti-TcP2β polyclonal mouse serum (B).

tion 5 and 6 and the glycine and phenylalanine at position 8 and 9. The same amino acids were also mapped by the polyclonal mouse anti-P antibody indicating that the mAb reflects the general reactivity of the polyclonal response (Fig. 3B).

Surface plasmon resonance was used to characterize the binding of mAb 17.2 with TcP2β and its corresponding epitope, R13, as well as with its human counterpart, H13, and a peptide representing the C-terminal end of the differing *T. cruzi* P0 protein [8, 11]. Direct binding of mAb 17.2 to TcP2β showed an affinity constant of 3 nM, its K_i for R13 was 400 nM, while that for H13 was 2,000 nM.

These results suggest that, although the main epitope recognized corresponds to the one previously described, the conformational structure of the whole fusion protein is responsible for the high affinity with which the protein is recognized, 3 nM *vs*. 400 nM. It also shows that the change of a Glu in R13, to a Ser in H13, causes a decrease in affinity and that the anti TcP2β response is most specific for the parasite protein. These findings confirm previous results, describing the higher affinity of patient polyclonal anti-P antibodies for R13 [7].

2.4 Functional activity of mAb 17.2

The cross-reactivity of mAb 17.2 with the isoforms of the heart β1-adrenergic receptor led us to examine the functional activity of the antibody. As shown in Fig. 4A, the antibody induced a dose-dependent positive chronotropic effect on neonatal cardiomyocytes in culture, with a maximal value of 30 nM. This effect was abolished by the specific β1-adrenergic antagonist, bisoprolol, as well as by preincubation of the antibody with the peptide H26R corresponding to the second extra-cellular loop of the β1-adrenergic receptor or with the R13 peptide (Fig. 4B).

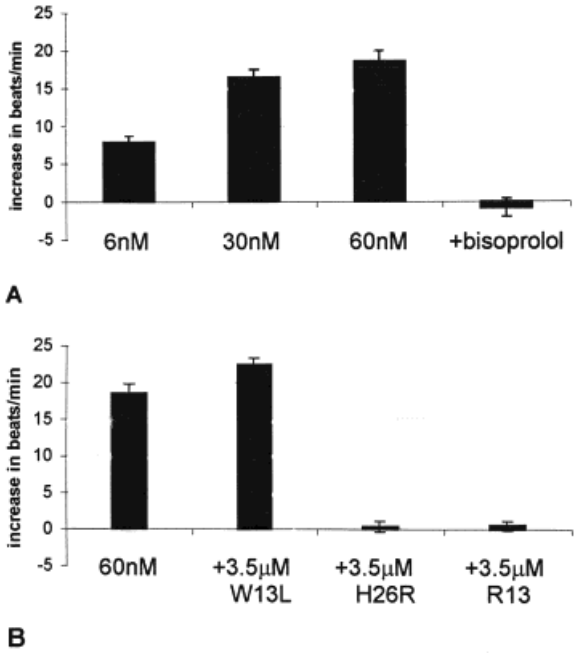

Fig. 4. Positive chronotropic effect of mAb 17.2 on neonatal rat cardiomyocytes. The effect is determined as an increase in beating frequency/min. The abscissa shows the different conditions under which the activity was measured. (A) Dose-dependent response produced by incubating the cardiomyocytes with increasing amounts of mAb 17.2 and reversion of the effect with the β1-adrenergic antagonist bisoprolol 1 μM. (B) Inhibition of the response obtained by preincubating mAb 17.2 with peptides derived from the second extracellular loop of the Huβ$_1$AR (H26R) and from the C-terminal region of TcP2β (R13). By contrast, no inhibition of the response is observed with the peptide derived from the first extracellular loop of the Huβ$_1$AR (W13L).

3 Discussion

Antibodies induced by the *T. cruzi* ribosomal P proteins and cross-reacting with the β-adrenergic and muscarinic receptors [6, 9, 14, 15] could account for abnormalities in the ECG, and even sudden death, since their anti-receptor activities could interfere with the normal heart physiology [6, 7, 9, 10]. In murine experimental *T. cruzi* infection, it was shown that, chronologically, the primary cross-reaction between *T. cruzi* antigens and cardiac receptors was targeted to the β1-adrenergic receptor, since cross-reactive functional anti-β1 receptor antibodies were shown already in the acute phase.

Alanine-mutation scanning of the epitope recognized by the mAb 17.2, confirms that this epitope corresponds to the major anti-P antibody response originated after immunization. The immunochemical and functional properties of the mAb thus reflect the properties of the polyclonal response [10]. Although no binding of the mAb 17.2 to the peptide derived from the human β_1-adrenergic receptor (H26R) could be measured neither by enzyme immunoassay nor by surface plasmon resonance (not shown), a clear functional response was obtained as evaluated on rat cardiomyocytes in our cell culture system. An explanation for this discrepancy may be the fact that only a few cellular receptors must be recognized by the specific ligand to obtain maximal activation of the transduction signal and of the effector cascade, resulting in the chronotropic effect. Otherwise, the conformational epitope on the extracellular domain of the receptor can have a constrained structure more similar to the epitopes recognized on TcP2β. Indeed, this is the case for other antibodies that bind to cardiac receptors [16, 17].

Whatever the mechanisms by which the antibodies against the ribosomal P2 proteins of *T. cruzi* exert their effects, the existence of an mAb against such a protein which is able to activate the cardiac β1-adrenergic receptor confirms that cross-reactivity is the basis of functional antibodies against the β_1-adrenergic receptor. Since autoantibodies with similar properties have been found in idiopathic dilated cardiomyopathy [18], and since induction of such antibodies by active immunization leads to cardiopathic changes in immunized rabbits [19], it can be concluded that at least the electrophysiological pathology of Chagas' cardiomyopathy can be ascribed to such cross-reactive antibodies.

4 Materials and methods

4.1 Peptides

Peptides were synthesized by the Fmoc technique using solid-phase automatic synthesis. Their purity was assessed by HPLC and they were identified by mass spectrometry. Table 1 summarizes the sequence of peptides used in this work.

4.2 mAb production and characterization

BALB/c mice were immunized by i.p. injection of 100 μg MBP-TcP2β [9] emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, USA), and boosted twice with 100 μg MBP-TcP2β emulsified in incomplete Freund's adjuvant (Sigma, St. Louis, USA) every 2 weeks. Splenic B cell fusions were performed according to the standard procedure [20]. Supernatants of fused cells were screened for specific antibody production by an enzyme immunoassay as previously described [10]. Clones were amplified in ascites fluids by i.p. injection into histocompatible mice previously primed with 0.5 ml pristane (Sigma). Ascites fluids were collected and immunoglobulins were precipitated with 40% $(NH_4)_2SO_4$ saturation. The protein concentration was evaluated by measuring the absorbance at 280 nm (1.40 for a solution of 1 mg/ml). All further characterizations were performed with the $(NH_4)_2SO_4$ purified preparations. The fine specificity was assessed according to previously described epitope mapping using a set of 25 biotinylated peptides covering the whole TcP2 sequence as well as isotyping [10].

Table 1. Sequence of peptides

Name	Protein	Sequence number [Ref]	Sequence
R13	TcP2β	107–119 [8]	EEEDDDMGFGLFD
H13	HuP2	102–115 [8]	EESDDDMGFGLFD
H26R	Huβ$_1$AR	197–222 [24]	HWWRAESDEARRCYNDPKCCDFVTNR[a]
W13L	Huβ$_1$AR	121–134 [24]	WGRWEYGSFFCEL[b]

a), b) Sequences of the second extracellular loop (a) and of the first extracellular loop (b) of the Huβ$_1$AR

4.3 Immunoblotting of ribosomal proteins and rabbit heart membranes

Parasite ribosome extracts were obtained from *T. cruzi* epimastigotes CL strain as previously described [10]. Enriched ribosome preparations from HeLa cells were prepared using the same protocol. Membranes from a rabbit heart were prepared using a phosphate buffer (50 mM Na_2HPO_4, 10 mM $MgCl_2$) at pH 7.4, according to the standard procedure.

For enriched ribosome preparations or membrane cardiocyte preparations, SDS-PAGE was performed using 15% or 10% polyacrylamide gels (Bio-Rad, USA), respectively. Polypeptides were electrotransferred to a nitrocellulose membrane and immunodetection was performed either with mAb 17.2, or with an anti-R13 polyclonal monospecific rabbit serum (1:400). A peroxidase-labeled goat anti-mouse IgG (1:2,000) or anti-rabbit IgG (1:1,000) was used as second antibody and the reaction was revealed as described [13].

For membrane cardiomyocytes, strips were incubated with mAb 17.2 or mAb 68.3 (4 μg/ml) or with mAb 17.2 preincubated with 25 μg of the H26R peptide. A peroxidase-labeled goat anti-mouse IgG (1/10,000) was allowed to react with the adsorbed antibodies and visualized using the ECL technique (Amersham, UK).

4.4 Electron microscopy

T. cruzi epimastigotes or HeLa cells were processed for electron microscopy and immunocytochemistry as previously described [21]. mAb 17.2 was used at 1 mg/ml and rabbit polyclonal anti-R-13 antiserum at a 1:100 dilution.

4.5 Epitope mapping and alanine mutation scanning

The fine epitope mapping of the C-terminal region of TcP2β (R13) was performed with the SPOTs®; kit (Genosys Biotechnologies, GB). A set of 14 peptides representing the R13 sequence and its alanine-replacement analogs were synthesized on a membrane as prescribed by the purveyor.

The interactive residues were defined by incubation with a 2.5 μg/ml dilution of mAb 17.2 or with a 1:400 dilution of a TcP2β immunized mouse polyclonal serum for 2 h at room temperature followed by 1-h incubation with peroxidase-conjugated anti-mouse IgG and revealing by the ECL technique (Amersham).

4.6 Surface plasmon resonance

The binding affinities of mAb 17.2 for several peptides and recombinant proteins were determined using a BIACORE 2000 (BIAcore, Uppsala, Sweden) to measure association (k_a) and dissociation (k_d) rate constants. For immobilization, glutathione S-transferase (GST)-TcP2β and GST dissolved in 10 mM acetate buffer pH 4.5 were injected onto a CM5 chip, activated by a standard protocol, at a concentration of 100 μg/ml for 10 min at a flow rate of 5 μl/min. After blocking and washing the surface as described [7], increasing amounts of purified mAb 17.2 were allowed to react with the immobilized proteins for 10 min (5 μl/min) for the determination of kinetic parameters. A 2-min pulse in 3 M KSCN regenerated the surface. Sensorgrams were corrected for nonspecific interaction by subtracting the response on the immobilized GST. Association and dissociation rate constants were calculated separately using a 1:1 Langmuir association model in the BIACORE evaluation software. The equilibrium association constant (K_A) was calculated as k_a/k_d.

Interaction parameters with the different peptides were determined by inhibition experiments. The mAb 17.2 was preincubated at a concentration of 25 nM with increasing amounts of peptides and then allowed to react with the recombinant proteins, immobilized on the chip. Inhibition % was calculated as $(1–k_{0i}/k_0)\times100$ in which k_{0i} and k_0 are the initial reaction velocities in the presence or absence of inhibitor. They reflect the concentration of active molecules remaining in the flow phase. From the dose-dependent inhibition curve, an IC_{50} could be determined and the K_i calculated according to Cheng and Prusoff [22]: $K_i = (1+K_A[A])/IC_{50}$ in which K_i is the inhibition constant, K_A the affinity constant of the mAb, [A] the concentration of the mAb, and IC_{50} the concentration of inhibitor needed for 50% inhibition.

4.7 Functional assay on neonatal rat cardiomyocytes

Rat neonatal cardiomyocytes were prepared from ventricles of 1–2 days Wistar rats by a previously described method [23]. Ten small circular were inspected through the perforation of a metal template. Single beating cells or clusters of synchronously beating cardiomyocytes in each of the ten fields were selected, and the number of contractions counted for 15 s. This procedure was repeated for two to five identically treated culture flasks. The mAb 17.2 was dissolved in the same medium used for the determination of the basal beating rate at concentrations and conditions indicated and incubated with the monolayer for 60 min.

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