ANTIBODY-MEDIATED ENHANCEMENT OF CALCIUM PERMEABILITY IN CARDIAC MYOCYTES

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Heart-reactive antibodies have been found in various cardiovascular diseases (1–3). In myocarditis and dilated cardiomyopathy autoantibodies to the ADP/ATP carrier, a protein of the inner mitochondrial membrane, was found (4). The ADP/ATP carrier is a transport system within the membranes of eukaryotic cells enabling direct nucleotide transport between the intra- and extra-mitochondrial compartments (5). Autoantibodies against this carrier appear to cause organ-specific inhibition of nucleotide transport (6-8). In animals immunized with the isolated ADP/ATP carrier, the antibody binding was also found to be organ specific (9).

In this study we investigated the effects of an antibody against the ADP/ATP carrier on isolated adult rat myocytes. Immunobinding and immunofluorescence studies showed that the antibody binds specifically to a cell surface protein. Exposure of the rat myocytes to the antibody resulted in enhanced calcium current. Prolonged exposures caused cell damage and death, which was prevented by addition of organic calcium channel blockers. These studies provide evidence for an interaction of the antibody against the ADP/ATP carrier with the calcium channel and suggest a new antibody-mediated mechanism for cytotoxicity and subsequent cell death.

Materials and Methods

Preparation of Cardiac Myocytes. Cardiac myocytes were isolated using a modified enzymatic procedure (10). Briefly, male Wistar rats, 250–300 g, were killed by a blow to the back of the neck. The chest was opened and the heart was removed and attached to a Langendorff perfusion system (11). The heart was perfused for 15 min with calcium-free "high K" buffer solution (NaCl, 50 mM/l; KCl, 25 mM/l; KH_2PO_4, 1.21 mM/l; Na_2HPO_4, 16 mM/l; $MgSO_4$ 1.21 mM/l; $NaHCO_3$, 25 mM/l; 1% MEM nonessential amino acids; 1% MEM essential amino acids; 1% MEM vitamins [Boehringer Mannheim Biochemicals, Indianapolis, IN]; glutamine, 0.68 mM/l; creatine, 20 mM/l; taurine, 60 mM/l; glucose, 11 mM/l) buffered at pH 7.2–7.4 (10).

The heart was then perfused with another high K buffer containing, in addition, 0.5% pure BSA (Behring Diagnostics, San Diego, CA), 1.0 mg/ml collagenase (Worthington Biochemical Corp., Freehold, NJ), and 1.5 mg/ml hyaluronidase (Sigma Chemical Co., St. Louis, MO). The perfusion rate was controlled between 4 and 8 ml/min. After 45–60 min the heart was removed, minced, and the cells were suspended in a fresh high K buffer containing en-

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zyme mixture for 10 min. After the first 5 min of incubation, calcium was added to give a final concentration of 25 μM Ca^{2+}. The cell suspension was filtered through a 300-μm nylon mesh and, to remove dead cells and fibroblasts, the cell suspension was separated by a BSA gradient centrifugation (10). The final cell pellet was suspended in low K buffer solution (NaCl, 121 mM/l; KCl, 4.85 mM/l; KH_2PO_4, 1.21 mM/l; Na_2HPO_4, 16 mM/l; $MgSO_4$, 1.21 mM/l; 1% MEM nonessential amino acids; 1% MEM essential amino acids; 1% MEM vitamins (Boehringer Mannheim Biochemicals); glutamine, 0.68 mM/l; creatine, 20 mM/l; taurine, 60 mM/l; glucose, 11 mM/l; 0.5% BSA; $CaCl_2$, 0.1 mM/l) buffered at pH 7.2–7.4 for further use.

Cell Counting. Cell morphology was examined by light microscopy and the total number of cells was determined by a hemocytometer (Fuchs-Rosenthal, Wertheim, FRG). The percentage of cells that were rod shaped with clear striations and that excluded 0.4% trypan-blue were measured. Cell yields were calculated by measuring the volume of the final cell suspension.

Analytical Procedures. Protein content was determined by a biuret procedure (12). The ATP content of the cells was determined enzymatically in acid extracts (13, 14).

Antibodies and Solid-phase RIA (SPRIA)[1]. Immunization of rabbits with the ADP/ATP carrier, purification of the antisera and the preimmune sera to IgG fractions, and the indirect micro-SPRIA were carried out as described previously (9, 15). For affinity purification of antibodies to the ADP/ATP carrier, purified ADP/ATP carrier protein was bound to nitrocellulose paper. The free binding sites were blocked by 1% Tx or albumin. After a 12-h incubation with antiserum followed by several washes with a solution ($NaSO_4$, 100 mM/l; morpholinopropanesulfonic acid (MOPS), 10 mM/l; EDTA, 0.05 mM/l; 0.5% Triton-X 100 [Tx]; 0.05% NaN_3) buffered at pH 7.2.

Bound antibodies were released by a 15–30 min incubation with 0.1 M citric acid, pH 2.5. The obtained purified IgG solution was immediately neutralized with saturated Tris buffer, pH 7.4, then extensively dialyzed against PBS, and was concentrated to the original serum volume used for adsorption. The purification procedure was done at 4°C. Chromatographical affinity purification was not attempted because of the instability of the antigen at the column.

Immunofluorescence Studies. Cryosections (4 μm) from rat and rabbit cardiac tissue were obtained with a cryostate (Linde, Koln, FRG) at −22°C. The sections were incubated with 20 μl of affinity-purified IgG to the ADP/ATP carrier for 30 min at room temperature. Cell surface staining of isolated cardiac myocytes was done at 10°C in PBS. Suspended living cells were incubated with affinity-purified antibodies for 30 min. For control experiments, the antibodies were replaced by rabbit preimmune IgG or by antibodies to the ADP/ATP carrier previously adsorbed to the purified antigen (100 μg antigen/1 ml antibody dilution 1:50, 3 h, 0°C). As secondary antibody, goat anti-rabbit IgG conjugated to fluorescein isothiocyanate was used in a dilution of 1:40. This procedure was the same for cryosections and living monocytes. Fluorescence microscopy of labeled tissue and cells was carried out with a microscope (Orthoplan, Wetzlar, FRG) equipped for immunofluorescence. Pictures were taken on a Gilford HPS film.

Radioimmunobinding Assay. To detect bound antibodies, the cells were tested using a radioimmunobinding assay. 2×10^5 myocytes in 400 μl "low K" buffer were incubated with antibodies (IgG fraction) at different concentrations and for different times. The myocytes were then washed with low K buffer and centrifuged (20 *g*; 40 s; three times). ^{125}I-protein A (sp act, 9.8 μCi/μg; NEN) was used to label the bound antibodies (20,000 cpm/2×10^5 cells) for 1 h. After incubation at room temperature the cells were washed with low K buffer once more and recentrifuged (20 *g*; 40 s; four times). The final pellet was measured for radioactivity in a gamma counter (Gammaszint BF 5300; Berthold, München, FRG). The data were then normalized to 10^6 cells for comparative quantification.

Gel Electrophoresis and Immunoblot Analysis. Proteins were separated in a Laemmli buffer system (16) on 5–18% polyacrylamide slab gels. Gels were stained with silver nitrate using a modified technique of Oakley et al. (17). Immunoblotting was performed according to Towbin et al. (18) on nitrocellulose sheets (4°C; overnight; 0.1 A). The nitrocellulose was rinsed with

[1] *Abbreviations used in this paper:* MOPS, morpholinopropanesulfonic acid; SPRIA, solid-phase RIA; TTX, tetrodotoxin; Tw, Tween 20; Tx, Triton-X 100.

PBS containing 1% Tw to reduce unspecific binding, and then incubated with antibody solutions (IgG; 1:400) overnight at 4°C. After washing with PBS/Tween 20 (Tw) the sheets were incubated with peroxidase-conjugated goat anti-rabbit IgG (Miles Laboratories Inc., Naperville, IL) (1:400) and were washed again and analyzed by staining with 4-chloro-naphthol (0.5 mg/ml).

Measurements of Cytotoxicity. In each case 5×10^4 myocytes were incubated in 1 ml low K buffer for 1 h with varying concentrations antibody from 1:25 to 1:6,400; at $(Ca^{2+})_o$ of 1 mM; for varying times up to 180 min with an antibody concentration of 1:100 and $(Ca^{2+})_o$ of 1 mM; for varying times up to 180 min and varying calcium concentrations of 0, 0.1, 0.5, and 1 mM at an antibody concentration of 1:100; and for 1 h after preadsorption of the antibody with its antigen (100 μg antigen/1 ml antibody dilution 1:100; 3 h). After the given times or concentration steps, the cells were counted and examined for their viability, as described in the previous section, Cell Counting.

Whole Cell Electrical Measurements. Isolated rat ventricular myocytes were voltage clamped using the whole cell patch clamp technique (19, 20). Cells were dialyzed with solutions containing high Cs^+ in order to block the potassium currents. The pipet solution contained: CsCl, 100 mM/l; EGTA, 10 mM/l; $CaCl_2$, 1.0 mM/l; Hepes, 20 mM/l; glucose, 10 mM/l; Mg-ATP, 5 mM/l; c-AMP, 0.2 mM/l; buffered at pH 7.3. 10^{-5} M tetrodotoxin (TTX) was used in the bathing solutions to block the fast sodium current. The bathing solution contained: NaCl, 120 mM/l; $CaCl_2$, 1–5 mM/l; KCl, 3 mM/l; glucose, 10 mM/l; Hepes, 10 mM/l; buffered at pH 7.4.

Results

Structure-function Characteristics of the Isolated Cardiac Myocytes. The enzymatic isolation technique used in this study yielded a large number ($4.2 \pm 0.9 \times 10^6$) of healthy cells from adult rat hearts. Using exclusion of trypan blue in combination with rod-shaped morphology as criteria of cell viability, we found 84.1 ± 4.4% of the cells to be viable. Rod-shaped cells had clear cross striations, and were calcium tolerant (Fig. 5a). A very small fraction of cells contracted spontaneously at very low frequencies. The mean protein content of our cells was 5.4 ± 1.8 ng, and the cellular ATP content of 20.5 ± 3.8 nM/mg protein per myocyte was in good agreement with previous studies (14, 21, 22). The cells remained viable for at least 8 h.

Identification and Specificity of the Antibody against the ADP/ATP Carrier. Fig. 1 *a* shows the antibody binding to the antigen, the ADP/ATP carrier. The protein was isolated as described before (5, 9). After coating with antigen, the titer plates were incubated with decreasing amounts of antiserum (affinity-purified IgG) and the bound antibodies were detected with ^{125}I-protein A. The preimmune serum IgG was used as control. Preincubation of the antibodies with different concentrations of antigen and subsequent SPRIA measurement resulted in an inhibition of antibody binding (Fig. 1 *b*), indicating the specificity of the antibody to the ADP/ATP carrier.

Gel electrophoresis of the isolated ADP/ATP carrier (Fig. 2 *a*) and the subsequent immunoblot showed only one band with a molecular mass of 30 kD. To prove whether the antiserum reacted with another protein of the mitochondrial fraction used for isolation of the carrier protein, we blotted mitochondrial membranes on nitrocellulose sheets and incubated them with the anti-ADP/ATP carrier serum. As seen in Fig. 2, lane *d*, the antibody again only reacted with the 30-kD band. After preincubation of the serum with the isolated carrier protein, no antibody binding to the nitrocellulose was detected anymore. Based on these data, one can assume that antibodies raised against the ADP/ATP carrier only react with this mitochondrial protein. Moreover, the specific immune reaction of the antibodies with only one single

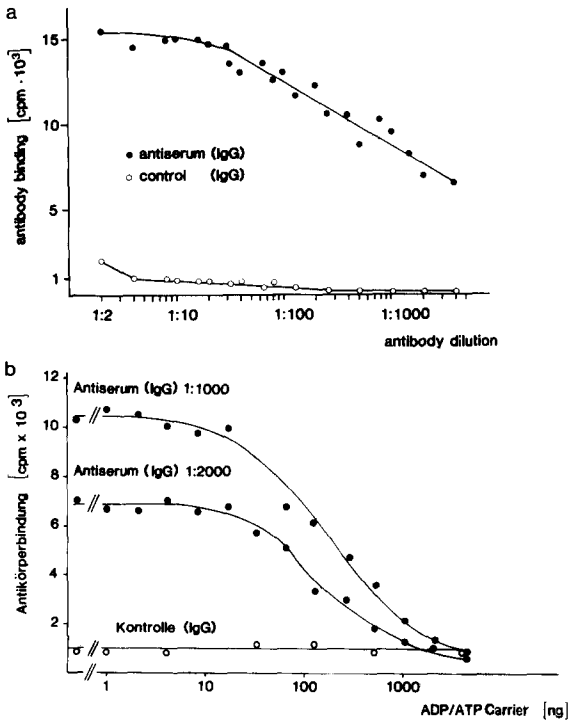

FIGURE 1. Binding of the antibody to its antigen, the ADP/ATP carrier, in SPRIA. (*a*) Concentration-dependent antibody binding to the purified ADP/ATP carrier. (*b*) Inhibition of antibody binding after preincubation with the purified ADP/ATP carrier. Antisera in a dilution of 1:1,000 and 1:2,000 were incubated with increasing amounts of the purified ADP/ATP carrier and then measured for residual binding.

band makes an additional interaction of the antibodies with a minor containment quite unlikely.

The Anti-ADP/ATP Carrier Antibody Binds to Cardiac Tissue and Myocytes. Cryosections of rat, rabbit, and human cardiac tissue incubated with antibodies to the

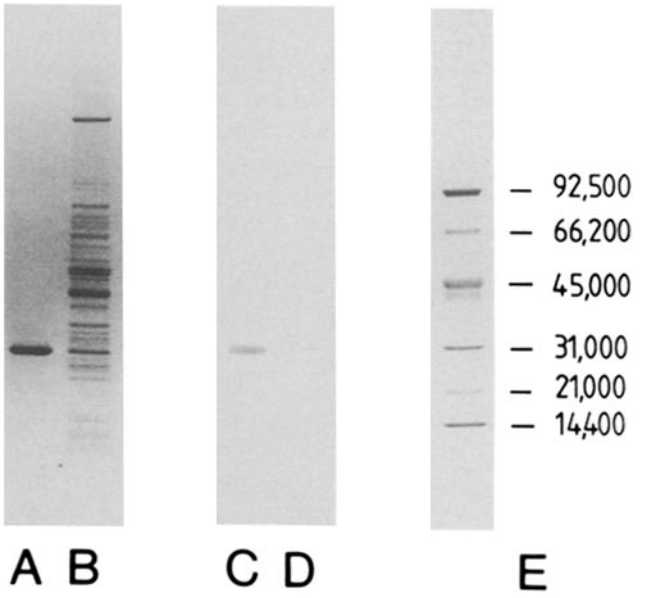

FIGURE 2. Gel electrophoresis (*A* and *B*) and immunoblot (*C* and *D*) of the purified ADP/ATP carrier (*A* and *C*) and total mitochondrial proteins from heart (*B* and *D*); molecular weight standard (*E*). The proteins were separated on SDS polyacrylamide gels. One section of the gels were stained with coomassie blue (*A*, *B*, and *E*), the other section was electrophoretically blotted on a nitrocellulose sheet, incubated with the anti-ADP/ATP carrier antiserum, and then with peroxidase conjugated anti-IgG (*C* and *D*).

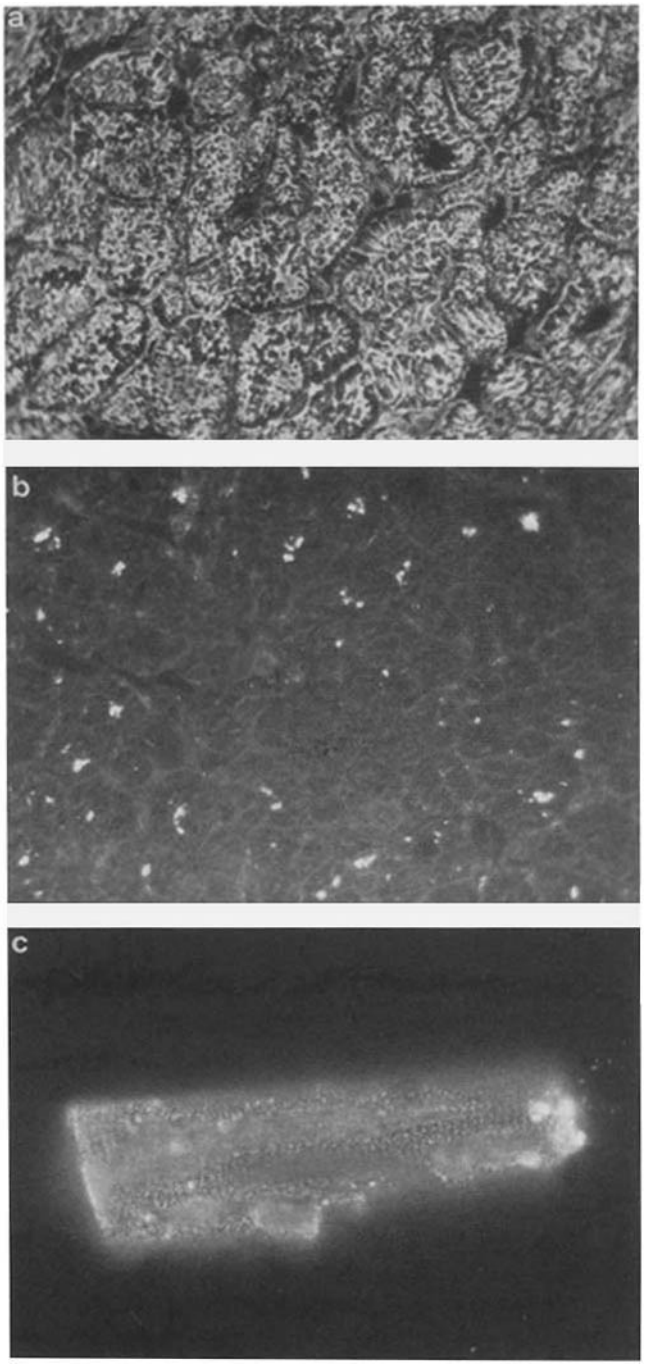

FIGURE 3. Immunofluorescence on cardiac tissue and isolated cardiac myocytes. (*a*) Immunofluorescence staining of mitochondria and cell surface in cross-sections of human papillary muscle with anti-ADP/ATP carrier antibodies. (*b*) Disappearance of cell surface and intracellular reaction after neutralization of antibodies with the purified ADP/ATP carrier antibodies. (*c*) Cell surface staining of isolated adult rat cardiac myocytes with anti-ADP/ATP carrier antibodies.

ADP/ATP carrier (affinity-purified IgG) showed bright immunofluorescence on mitochondrial structures and the cell surface. Fig. 3, *a* and *b* compares the staining of human papillary muscle with antibodies to the ADP/ATP carrier before and after its neutralization with the ADP/ATP carrier. The bright immunofluorescence dis-

appeared from the tissue (Fig. 3 *b*). Preimmune sera (IgG) did not react with any cardiac tissues examined. The antibody (affinity-purified IgG) also stained the surface of cardiac myocytes (Fig. 3 *c*) and could be completely neutralized with the ADP/ATP carrier. These results suggest specificity of the immune reaction and provide evidence that the antibody against ADP/ATP carrier crossreacts immunologically with antigenic determinants on the cell surface. The crossreactivity between the ADP/ATP carrier of the inner mitochondrial membrane and a protein of the cell surface was also demonstrated by a radioimmunobinding assay. Fig. 4 *a* shows that one-half maximum binding of antibodies to the cell surface occurs after 15 min. Preimmune serum IgG (control) did not bind to the surface of myocytes. Antibody dilution from 1:1,000 to 1:6,000 resulted in a reduction of total antibody binding. No significant antibody binding could be detected at dilutions of 1:5,000 (Fig. 4 *b*). The specificity of the antigen-antibody reaction was examined by preadsorption of the antibody to an increasing amount of the isolated ADP/ATP carrier (1–1,500 ng). Fig. 4 *c* shows that increasing the concentration of the ADP/ATP carrier reversed the antibody binding to the cell surface. These results clearly indicate the existence of a cell surface protein that interacts competitively with the antibodies directed against the ADP/ATP carrier.

Antibody-mediated Cytotoxicity. Incubation of isolated cardiac myocytes with an antibody concentration of antibody >1:1,000 resulted in a concentration-dependent cell deterioration and death. Cell deterioration was monitored visually. It was accompanied by oscillatory contractions, bleb formation, cell shortening, granulation, and finally, cell rounding (Fig. 5, *a–c*). Rounded cells took up trypan blue rapidly while rod-shaped cells excluded the dye completely. The ATP content of such cells decreased from 20.5 ± 3.8 to <10 nM/mg protein in a cell suspension incubated for 2 h with antibody dilutions of 1:100.

Using an antibody concentration of 1:100 in cell suspensions containing 1 mM calcium, 20% of the myocytes rounded up after 30 min, 40% after 1 h, and ~80% after 3 h. In contrast, only 10-15% of the myocytes incubated with preimmune serum IgG appeared to be damaged after 3 h (Fig. 6). Decreasing the external calcium concentration decreased the antibody-mediated cytotoxicity. In absence of added calcium with antibody concentrations of 1:100, only 10% of the cells appeared to be damaged after 3 h (Fig. 6). Preadsorption of the antibody with its antigen, the ADP/ATP carrier, markedly reduced cytotoxicity (27% damaged cells after 3 h, not shown).

To examine whether cytotoxicity was mediated by increased calcium current, the effect of calcium channel antagonists on the time-dependent antibody-induced cell damage was tested. Fig. 7 shows that calcium channel blockers markedly reduced the cytotoxic effect of the antibodies. Nifedipine and nitrendipine (10^{-6} M) seemed to be more effective in protecting against cell damage than was verapamil (10^{-6} M). In the absence of antibodies, calcium channel antagonists had no effect on time-dependent cell viability (Fig. 7).

The effects of Antibody on the Calcium Current of Isolated Cardiac Myocytes. The results thus far suggest that the antibody binds specifically to the cell surface of cardiac myocytes and enhances their calcium permeability leading to cell damage. To gain further evidence for a direct interaction of the anti-ADP/ATP carrier antibodies with the calcium channel, we isolated the calcium channel protein complex according to the method of Campbell (30). After SDS gel electrophoresis and transfer of the

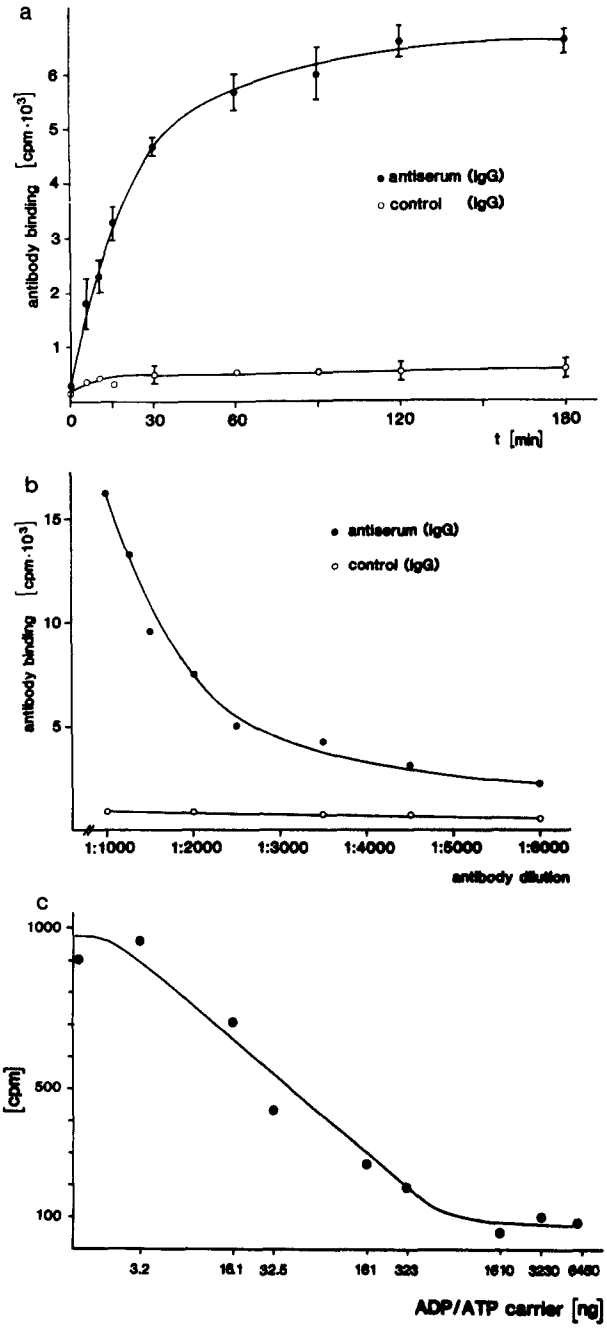

FIGURE 4. Binding of anti-ADP/ATP carrier antibodies to isolated cardiac myocytes measured with radioimmunobinding assay. (*a*) Time-dependent antibody binding. Antibody dilution, 1:2,000; (*b*) concentration-dependent antibody binding. Incubation, 60 min; (*c*) inhibition of antibody binding to isolated cardiac myocytes after preincubation with the purified ADP/ATP carrier. Antiserum in a dilution of 1:2,000 was incubated with increasing amounts of the purified ADP/ATP carrier and then measured for residual binding in radioimmunobinding assay.

protein to nitrocellulose paper, the immunoblot analysis showed that the affinity-purified antibodies against the ADP/ATP carrier protein bind to the different calcium channel subunits (Fig. 8). These data clearly indicate a crossreactivity between the ADP/ATP carrier of the inner mitochondrial membrane and the calcium channel located within the cell surface membrane.

Therefore in another series of experiments, we examined the possible effects of

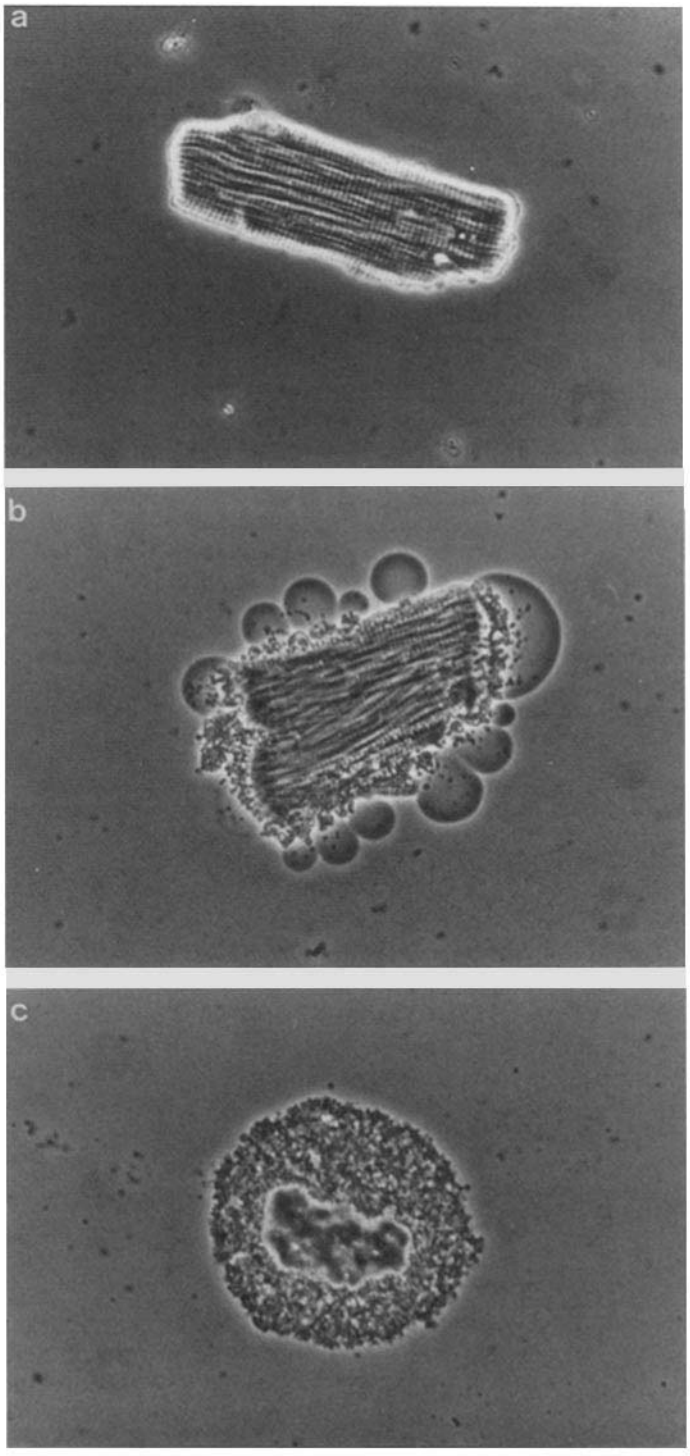

FIGURE 5. Appearance of isolated cardiac myocytes during incubation with anti-ADP/ATP carrier antibodies (dilution 1:100, 1 mM Ca^{2+}). (*a*) Normal, rod-shaped cell; (*b*) after 30 min: cell shortening, granulation, bleb formation; (*c*) after 1 h: final cell death.

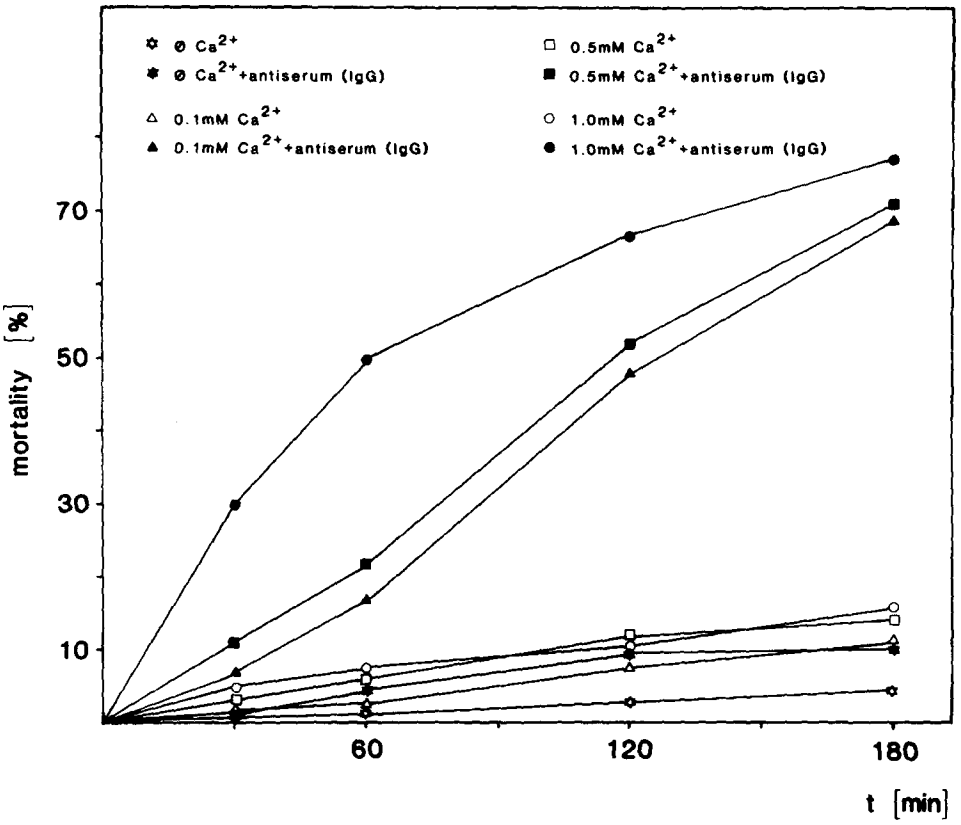

FIGURE 6. Calcium concentration and time-dependent mortality of isolated cardiac myocytes incubated with anti-ADP/ATP carrier antibodies; antibody (IgG) dilution, 1:100.

the antibody on the calcium current in isolated rat myocytes. Myocytes were patch clamped in control tyrodes (1-5 mM Ca^{2+}) and the magnitude of I_{Ca} and its kinetics were examined. Cells were dialyzed with high concentrations of Cs^+ and TEA^+ in order to block the K^+ currents. Intracellular calcium concentration was buffered at $\sim 10^{-8}$ M, using 11 mM EGTA and 1 mM Ca^{2+}. I_{Na} was inactivated and blocked using a combination of TTX (10^{-5} M) and holding potentials of -40 to -50 mV. I_{Ca} was then activated by step depolarization to different potentials from -50 mV. Fig. 9 shows that addition of antibody enhances I_{Ca} rapidly. Peak I_{Ca} increased by ~50-100%. The inactivation of I_{Ca} was also markedly slowed in the presence of the antibody (Fig. 8 *inset*), leading often to activation of large "tail" currents accompanying the termination of the clamp pulse. The effect of antibody on I_{Ca} was slowly reversible on washout of the antibody. Peak I_{Ca} often decreased below control levels, consistent with often-observed "run-down" of I_{Ca}. The enhancement of the I_{Ca} by the antibody was reversibly blocked by Nifedipine (10^{-6} M). β blockers (e.g., propranolol at 10^{-6} M) did not affect the antibody-induced enhancement of I_{Ca}.

The potentiating effect of the antibody on the I_{Ca} was often variable. Fig. 10 il-

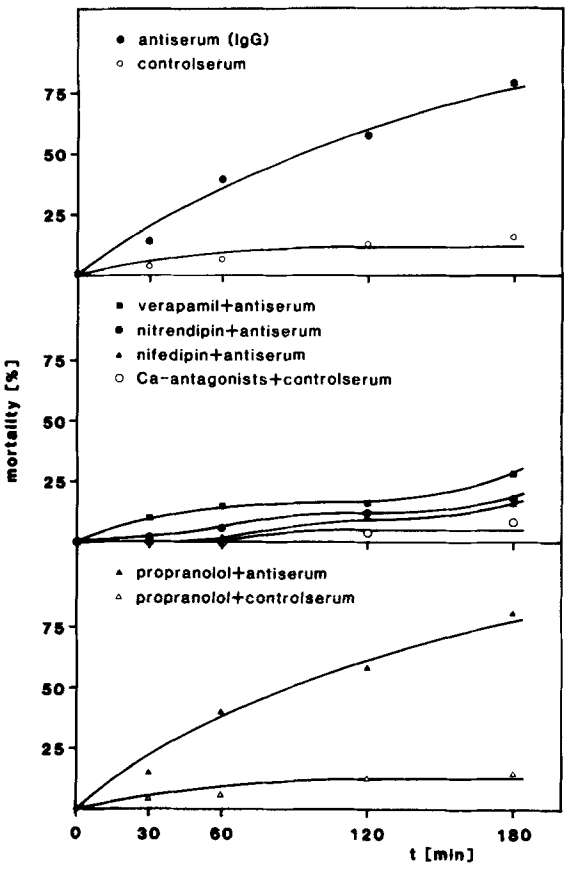

FIGURE 7. The effects of the calcium channel antagonists nitrendipin, nifedipin, and verapamil and the β blocker propranolol on the mortality of isolated cardiac myocytes incubated with anti-ADP/ATP carrier antibodies. 3×10^5 myocytes in 1 ml washing buffer containing 0.5% ESA and 1 mM Ca^{2+} were incubated either with the calcium antagonists or the β blocker for 5 min. Subsequently, to one half of the cells, ADP/ATP carrier antibodies were added in a final concentration of 1:100, and control serum was added to the other half (1:100). After 0, 30, 60, 120, and 180 min, the cells were counted and examined for their variability (see Material and Methods).

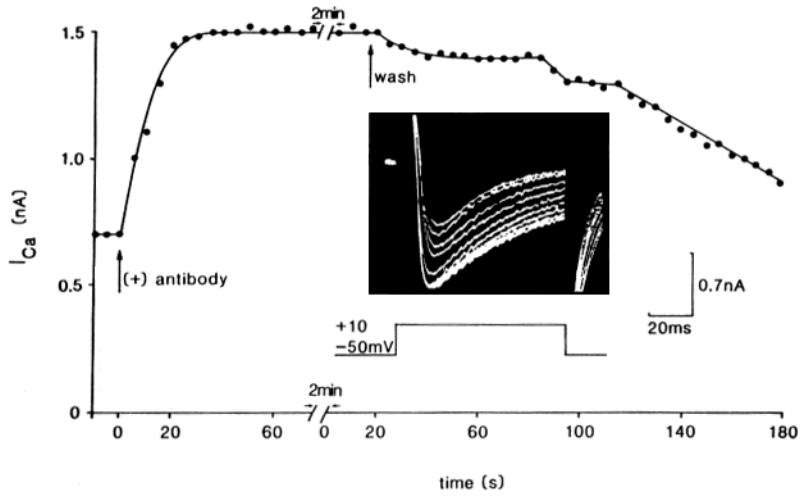

FIGURE 8. Time course of antibody effect on I_{Ca} in a rat ventricular myocyte. (*Inset*) Original superimposed traces of I_{Ca} upon addition of antibody (1:500). Addition of antibody strongly enhanced peak I_{Ca} in this cell. Reversal of the effect was slow. Room temperature, 24°C; $(Ca^{2+})_o$, 2.0 mM; TTX, 10^{-5} M.

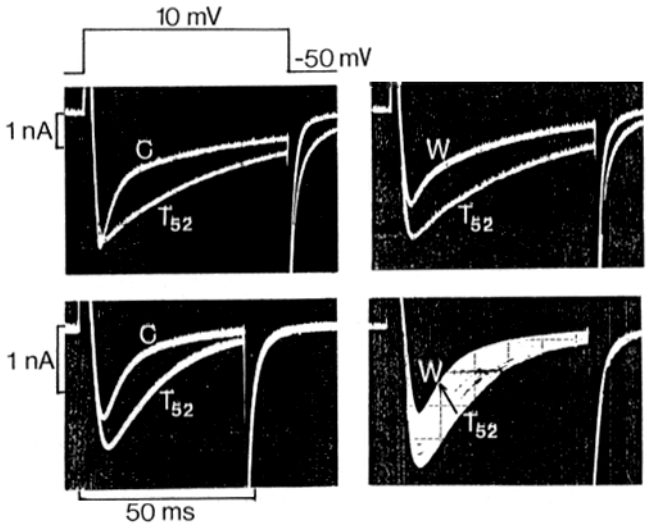

FIGURE 9. Effect of antibody on calcium current in two different rat ventricular cells. Upper and lower panels represent two different cells subjected to antibody concentrations of 1:100. In upper left panel, peak I_{Ca} does not seem to have increased, although the time course of inactivation is markedly slowed (T52-trace). Wash out of the antibody (*W*) (*upper right panel*) reveals larger potentiating effect of antibody on peak I_{Ca}. Most likely because I_{Ca} is known to run down during the time course of experiments. (*Lower left panel*) In another cell where I_{Ca} was more stable, antibody induces potentiating effect on peak I_{Ca} and slows its inactivation. Lower right panel represents complete reversal of antibody effect (*W*). $(Ca^{2+})_o$, 2.0 mM; room temperature, 24°C; Holding potential, −50 mV; TTX, 10^{-5} M.

lustrates the effect of the antibody on peak and the maintained components of I_{Ca} in two different cells. In one cell the antibody appeared to enhance only the maintained component of I_{Ca}, with little or no apparent effect on the peak I_{Ca} (Fig. 10, *upper left panel*), while in the other, both the maintained and the peak I_{Ca} were

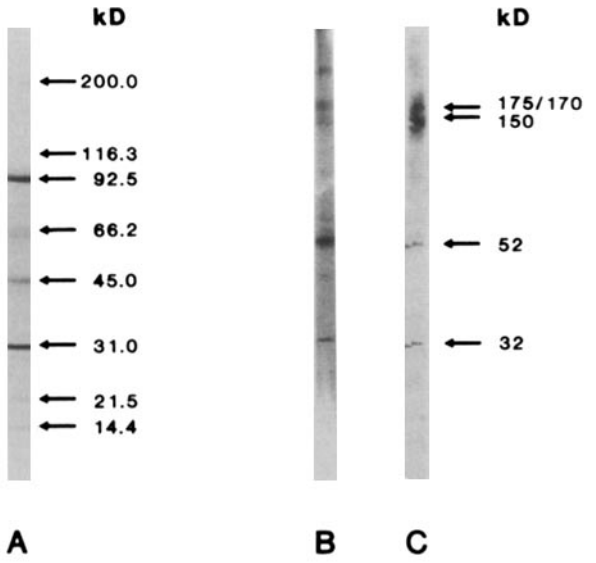

FIGURE 10. Gel electrophoresis and immunoblot of the isolated calcium channel protein (30). The isolated calcium channel protein was separated on SDS polyacrylamide gels. Lane *B* was stained with coomassie blue, lane *C* was incubated after the transfer to nitrocellulose with affinity-purified antibodies (IgG) against the ADP/ATP carrier and stained with peroxidase-conjugated anti-IgG; molecular weight standard (*C*).

enhanced (Fig. 10, *lower left panel*). This effect is most likely due to variable run-down of I_{Ca} in these two cells, since the washout of the antibody in the cell depicted in the upper panels leads to a decrease in peak I_{Ca} significantly below control level. Consistent with this idea was the finding that in the frog ventricular myocytes where the run-down of I_{Ca} is very slow, the antibody consistently and reversibly potentiated peak I_{Ca} (not shown). These results thus support the idea that the antibody crossreacts with a surface membrane protein, most likely the calcium channel in bringing about the above-described effects.

Discussion

The experiments described here provide evidence for a crossreactivity of antibodies against the ADP/ATP carrier of the inner mitochondrial membrane with a protein of cell surface, most likely the calcium channel. The main observations of this report are that this antibody enhances I_{Ca} and the cellular calcium permeability, which may lead to the observed cytotoxicity.

Evidence for crossreactivity of the antibody against the ADP/ATP carrier with a cell surface protein of cardiac myocytes was obtained from several experiments. In immunofluorescence studies, the antibody against the ADP/ATP carrier, not the preimmune serum, stained the cardiac cell membrane. After neutralization of the antibodies with the purified ADP/ATP carrier, no immunofluorescence staining was detected anymore, indicating the specificity of the immunoreaction. These data are in good agreement with those obtained by radioimmunobinding tests, which show a time- and concentration-dependent binding of the antibodies to the cell surface. Once again, neutralization of the antibody binding to the cell surface by preincubation of the antibodies with the isolated ADP/ATP carrier demonstrates the high specificity of this reaction. Together, these results indicate the existence of a crossreaction between the ADP/ATP carrier and a sarcolemmal protein of cardiac myocytes.

Although we can not totally exclude that the "crossreacting antibody" is directed against a minor contaminant in the ADP/ATP carrier preparation, the data shown indicate that the vast majority of the antibodies is directed against the 30-kD protein. Considering the magnitude of crossreaction and the lack of any detectable protein by electrophoresis and immunoblot technique, the immunogenicity of this contaminant moeity and the affinity of the resulting antibody would have to be in an unimaginable range.

Prolonged exposure of cardiac myocytes to high concentrations of antibody exhibited a cytotoxic effect. The antibody-mediated cytotoxicity was thought to be caused directly by the IgG fraction of the serum and was not complement dependent, because cytotoxicity could be induced by affinity-purified IgG in the serum-free media. This observation indicates a new mechanism for an antibody-mediated complement-independent cytotoxicity (23, 24). Cytotoxicity appeared to result from increased cellular permeability to calcium, causing oscillatory contractions and calcium overload (25-27). Consistent with this assertion, cytotoxicity was prevented, in order of effectiveness, by: removal of $(Ca^{2+})_o$, addition of calcium channel blockers, and neutralization of antibody. Thus, the antibody-mediated cytotoxicity may have developed secondary to the enhanced calcium permeability of the myocytes.

Three types of studies suggest that the enhanced calcium permeability most likely is caused by an antibody binding to the calcium channel. (*a*) Immunofluorescence

and binding studies suggest large binding of the antibody to the cell surface. The resulting antibody-induced cell damage could be reversed by nifedipine, nitrendipine, and verapamil, and on withdrawal of $(Ca^{2+})_o$. (*b*) The rapid enhancement of I_{Ca} by the antibody in the isolated myocytes also strongly suggests that the antibody interacts directly with the Ca^{2+} channel modifying its permeability. The β receptor does not appear to mediate this action of the antibody, since neither β blockers nor the presence or absence of c-AMP in the dialyzing patch pipet solution altered the antibody effect on I_{Ca}. These experiments suggest, therefore, that the antibodies against the ADP-ATP carrier interact with a site closely associated with the calcium channel, enhancing I_{Ca} most likely by increasing the mean open time of the channel. (*c*) Supporting evidence for this assertion comes from the finding that the antibody against the ADP/ATP carrier binds directly to the isolated calcium channel protein.

It has been recently suggested that there may be a common structural similarity between various membrane channels (28, 29). A high degree of structural similarity between gap junction polypeptide, sodium channel, and nicotinic acetylcholine receptor has already been described (28). Furthermore, similarities were reported between several voltage-gated channels (K, Na, Ca). The data presented here also show a crossreactivity between two different transport proteins, however, for the first time between a protein of the inner mitochondrial membrane and a cell surface protein. As the crossreacting antibodies influence the function of both proteins, this might indicate a similar mode of function and a common evolutionary origin for both proteins.

Abnormalities in calcium metabolism of myocardium are thought to be involved in pathogenesis of cardiomyopathies (31–33). So far the mechanism underlying disturbed calcium metabolism is not clear. As we can show that antibodies against the ADP/ATP carrier, also found in myocarditis and dilated cardiomyopathy (4, 6, 7, 34), crossreact with the calcium channel, these antibodies could be responsible for the disturbance of calcium homeostasis by enhancing calcium current. This may lead to an an antibody-mediated calcium overload and subsequent cell death (35). Thus, the data presented could be of great clinical relevance in the understanding of the pathogenesis of these suspected autoimmune diseases (36, 37).

Summary

Our study shows that antibodies, specific to the ADP/ATP carrier of the inner mitochondrial membrane, crossreact with the cell surface of cardiac myocytes, where the calcium channel seems to be the antigenic determinant. The antibodies enhanced the calcium current and suppressed its inactivation. Affinity-purified antibodies (IgG) exhibit an acute cytotoxic effect, which required extracellular calcium and was prevented by calcium channel blockers. Our findings suggest that antibody-mediated cytotoxicity results secondary to calcium overload caused by enhanced cellular calcium permeability, requiring no complement-dependent process.

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