Activation of Ca-permeable Cation Channels by Myocarditis-associated Antibody in Guinea Pig Ventricular Myocytes

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Abstract

The pathogenesis of myocarditis and dilated cardiomyopathy is thought to involve autoimmunological processes and myocardial calcium overload. Serum containing antiheart antibodies associated with a murine model of myocarditis increased $[Ca^{2+}]_i$ in guinea pig ventricular myocytes only in the presence of extracellular Ca^{2+}. The antiheart antibody-positive serum activated Ca^{2+}-permeable cation channels that were insensitive to dihydropyridines and membrane stretch. The permeability sequence was $Ba^{2+} > Ca^{2+} > Na^+ \sim K^+$, and the single-channel conductance to Ba^{2+} was 12 pS. The channel was activated by extracellular application of the serum during on-cell recording, which suggests that a soluble intracellular messenger may be involved. The antibody-positive serum did not alter voltage-gated Ca^{2+} currents. We propose that excess Ca entry in myocarditis and dilated cardiomyopathy results from activation of a Ca^{2+}-permeable cationic channel by the autoantibodies. (*J. Clin. Invest.* 1993. 91:1231–1234.) Key words: antiheart antibody • cardiomyopathy • fura-2 • patch-clamp • heart muscle cell

Introduction

Several antiheart autoantibodies have been reported to be associated with the pathogenesis of myocarditis and dilated cardiomyopathy (1–8). Apparently, myocardial calcium overload is responsible for myocardial damage in myocarditis and cardiomyopathy (9). In fact, increased Ca uptake has been observed in the myocardium of cardiomyopathic hamsters (10, 11). Taken together, there is the possibility that circulating antiheart antibodies induce a myocardial calcium rise by increasing Ca entry.

By using the fura-2 and patch-clamp techniques, we found that the serum containing antiheart antibodies associated with a murine model of myocarditis increased the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) through the activation of Ca^{2+}-permeable cation channels in isolated ventricular cells of guinea pig.

Methods

Sera preparation. Sera were collected from inbred male BALB/c mice inoculated with the M variant of the encephalomyocarditis virus, as previously reported (12). In several experiments, the sera were used after inactivating the complement at 56°C for 30 min. The antiheart antibody-positive sera devoid of the Ig fraction were prepared from the positive sera by affinity chromatography using tresyl-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) and affinity-purified goat anti-mouse Ig (Cappel Laboratories, Cochranville, PA). The control antiheart antibody-negative sera were obtained from the age-matched, noninfected mice. Immunofluorescence studies showed that the sera from virus-inoculated myocarditic mice, but not from noninfected mice, contain antibodies that cross-react with proteins on the surface of guinea pig ventricular myocytes (Tominaga, M., and A. Matsumori, unpublished observation).

Cell preparation. Single ventricular cells were isolated from adult Hartley guinea pig hearts using Langendorf's perfusion technique with collagenase (Yakult, Tokyo, Japan), as previously described (13).

Fura-2 experiments. The $[Ca^{2+}]_i$ was monitored by the fura-2 fluorescence ratio, as previously described (14). Myocytes (10^4/ml) were suspended in normal Tyrode's solution containing 10 μM fura-2/acetoxymethyl (Molecular Probes, Inc., Eugene, OR) and 5% bovine serum for 30 min at 36°C. The cells were then washed and stored on ice for at least 1 h before experiments. The fluorescence images at excitation wavelengths of 340 and 380 nm were collected from single ventricular myocytes at room temperature and were digitized by an image processor (Argus 100; Hamamatsu Photonics K.K., Hamamatsu City, Japan). The $[Ca^{2+}]_i$ was estimated from the mean value of the 340:380 nm ratio in a given myocyte by in vivo calibration using the maximum and minimum ratio values obtained in the presence of 1 μM ionomycin and 10 mM Ca^{2+} or 5 mM ethylene glycol bis(β-amino-ethylether)-*N,N,N',N'*-tetraacetic acid (EGTA).

Patch-clamp experiments. Whole-cell and cell-attached single-channel recordings were performed with a patch-clamp amplifier (EPC-7; List, Darmstadt, Germany) at room temperature. The currents were filtered at 3 kHz upon recordings and at 500 Hz on off-line data analyses. Data was sampled at 1 or 2 kHz.

Experimental solutions. Tyrode's solution contained (mM): 143 NaCl, 0.3 NaH_2PO_4, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 5.5 glucose and 5 Hepes-NaOH (pH 7.4). In some experiments, nifedipine (Bayer, Leverkusen, Germany) or Bay K 8644 (a gift from Dr. M. Kameyama, Kagoshima University, Kagoshima, Japan) was added to Tyrode's solution. Low Ca^{2+} Tyrode's solution was made by removing $CaCl_2$ from and adding 10 μM EGTA to normal Tyrode's solution. The free Ca^{2+} concentration was estimated to be ~ 50 nM by taking Ca^{2+} ions (5 μM) contaminating distilled water into consideration. Low Cl^- Tyrode's solution was made by replacing 76.5 mM NaCl with 76.5 mM Na

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1. *Abbreviations used in this paper:* BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; EGTA, ethylene glycol bis(β-amino-ethylether)-*N,N,N',N'*-tetraacetic acid.

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gluconate. An isotonic $CaCl_2$, $BaCl_2$, NaCl or KCl solution contained 110 mM $CaCl_2$, 110 mM $BaCl_2$, 140 mM NaCl or 140 mM KCl, 8 mM Tris, and 12 mM Hepes (pH 7.4). The control pipette solution for whole-cell recordings contained (mM): 110 K-aspartate, 20 KCl, 5 K_2ATP, 5 Na_2-creatinine phosphate, 5 $MgCl_2$, 5 EGTA, 5 Hepes and 21.1 KOH (pH 7.4). Na_2-creatinine phosphate was removed in the experiments to determine the relative permeability to K^+ of Ba^{2+}. In some experiments, 5 mM 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), tetrapotassium salt, was used in place of 5 mM EGTA in the pipette solution. Patch pipettes for single-channel recordings were filled with 110 mM $BaCl_2$ and 10 mM Hepes-CsOH (pH 7.4).

Results

By the fura-2 fluorescence ratio technique, antiheart antibody-positive sera collected from mice inoculated with encephalomyocarditis virus were found to increase $[Ca^{2+}]_i$ reversibly from 53.8±6.9 (SD) to 82.3±6.8 nM ($n = 4$, $P < 0.01$) in single ventricular myocytes of guinea pig (Fig. 1 *A*). In contrast, antiheart antibody-negative sera collected from noninfected mice did not change $[Ca^{2+}]_i$ (Fig. 1 *B*). When the extracellular Ca^{2+} concentration was reduced to about 50 nM, antiheart antibody-positive sera failed to increase the $[Ca^{2+}]_i$ (Fig. 1 *C*).

Whole-cell patch-clamp experiments showed that transient inward currents are induced by puff applications of antiheart antibody-positive sera at negative potentials (Fig. 2 *A*), but not by antiheart antibody-negative sera (Fig. 2 *B*). When EGTA (5 mM) in the pipette solution was replaced with BAPTA (5 mM), essentially identical results were obtained ($n = 3$). These responses had a latency of 5–20 s. The current response diminished after repeated applications of the positive sera. In the current-clamp mode, the positive sera produced a depolarization of 4.2±2.4 (SD) mV from the resting potential of −69.9±3.3 (SD) mV ($n = 8$). This level of depolarization was not sufficient to activate voltage-gated Ca^{2+} channels. Neither nifedipine (5 μM) nor Bay K 8644 (5 μM) affected the serum-induced currents ($n = 4$ and 5, respectively). High voltage-activated Ca^{2+} currents induced by depolarization pulses (0 mV, 300 ms) applied from a holding potential at −40 mV in ventricular myocytes were not affected by antiheart antibody-positive sera (Fig. 2 *A*). This was in contrast to the increase of high voltage-activated Ca^{2+} currents by antibodies against the ADP/ATP carrier (8). The positive sera also did not affect low voltage-activated Ca^{2+} currents (mainly low voltage-activated class) measured by applying depolarizing pulses (−20 mV, 60 ms) from a holding potential at −100 mV ($n = 3$). The positive sera from which the Ig fraction had been removed failed to induce the inward currents (Fig. 2 *C*). Similar current responses were also induced by the positive sera, in which the complement had been heat inactivated ($n = 2$). The positive sera-induced current response showed a reversal potential of ~ 0 mV and the whole-cell current-voltage relation was almost linear in Tyrode's solution (Fig. 2 *D*, filled circles, *Inset a*). Similar results were also obtained in an isotonic NaCl or KCl solution ($n = 6$). Reducing the external Cl^- concentration from 153 to 76.5 mM did not affect the current-voltage relationship ($n = 5$). The positive serum-induced inward currents were also observed in an isotonic $CaCl_2$ (Fig. 2 *D*, *Inset b*) or $BaCl_2$ solution (Fig. 2 *D*, *Inset c*). The reversal potential was +9 and +15 mV in the $CaCl_2$ and $BaCl_2$ solutions (Fig. 2 *D*, *open squares*), respectively. The relative permeabilities of Ca^{2+} and Ba^{2+} to K^+, estimated by the Goldman-Hodgkin-Katz equation assuming bi-ionic conditions (110 mM divalent cations against 161 mM K^+), were 1.2 and 1.9, respectively, at the macroscopic current level.

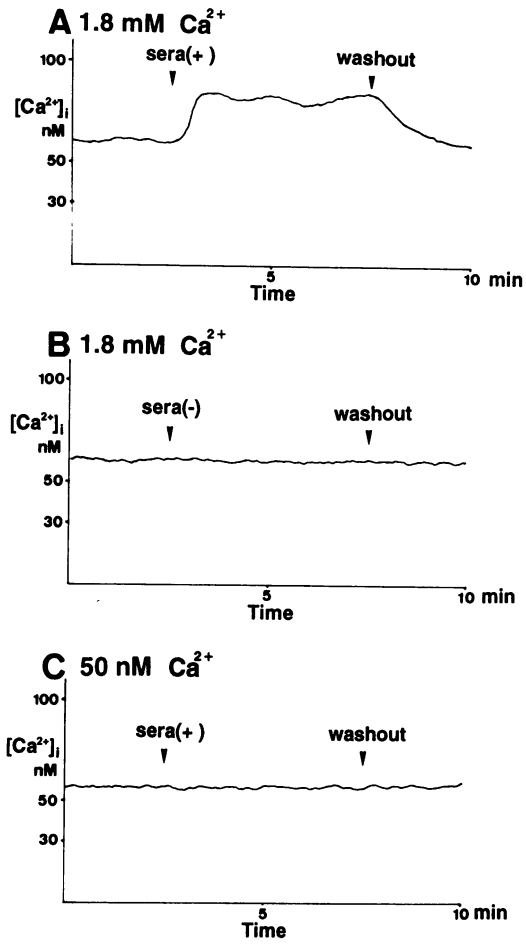

Figure 1. Effects of antiheart antibody-positive (*A*, and *C*) and negative (*B*) sera of mice on $[Ca^{2+}]_i$ in single ventricular cells of guinea pig. (*A*) A $[Ca^{2+}]_i$ increase in response to antiheart antibody-positive sera [*sera(+)*] in a ventricular myocyte in normal Tyrode's solution. (*B*) No $[Ca^{2+}]_i$ response to antiheart antibody-negative sera [*sera(−)*] in normal Tyrode's solution. (*C*) No $[Ca^{2+}]_i$ response to the positive sera in low Ca^{2+} Tyrode's solution containing 10 μM EGTA. At arrowheads, the perfusate was switched from serum-free Tyrode's solution to that containing 1% sera or vice versa. The data of *A*, *B*, and *C* represent four, three, and six similar experiments, respectively.

Under the cell-attached configuration, inward single-channel currents carried by Ba^{2+} were observed at negative membrane potentials in response to the positive sera (Fig. 3), but not to the negative sera ($n = 3$). There is a latency of ~ 15 s and the current lasted for ~ 90 s (Fig. 3 *A*). The single-channel conductance determined from the unitary current-voltage plot was ~ 12 pS (Fig. 3 *B*). The reversal potential estimated by extrapolation (~ +20 mV) agreed with that of the whole-cell current. Single-channel currents (unitary conductance of ~ 16 pS) could be observed with a pipette containing normal Tyrode's solution, and this current-voltage relationship was almost linear with a reversal potential of ~ 0 mV ($n = 9$). Thus, the

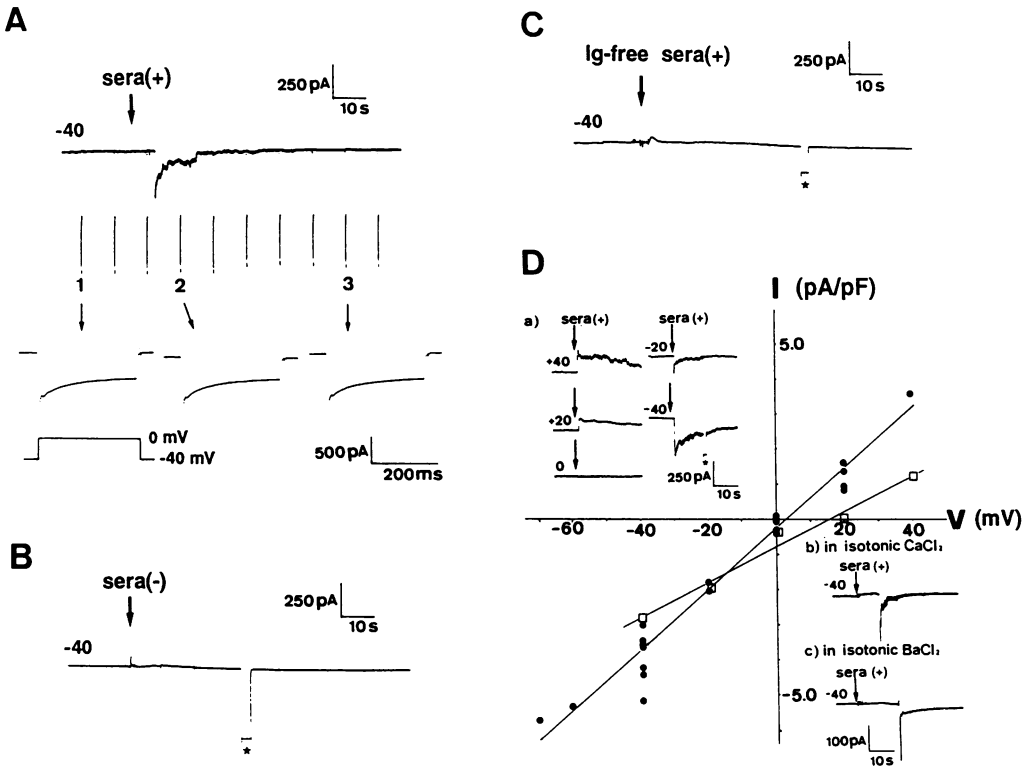

Figure 2. Effects of antiheart antibody-positive (*A* and *D*) and -negative (*B*) sera, as well as the Ig-free–positive sera (*C*) of mice on the whole-cell currents recorded in single ventricular cells of guinea pig. The bathing and pipette solutions were normal Tyrode's solution and the control pipette solution, respectively, unless otherwise indicated. During the current recordings, bath perfusion was interrupted. Thick arrows indicate the time of puff applications (duration ~ 8 s) of ~ 10 μl of Tyrode's solution containing 1% sera. Inward currents are depicted as downward deflection of the trace. Holding potentials were altered from the resting potential (−70 mV measured by current-clamp recordings) and are indicated on the left of individual traces (in millivolts). The voltage-clamp mode was sometimes switched to the current-clamp mode (*). In *A*, high voltage-activated Ca^{2+} currents (*sharp inward deflections*) were also intermittently elicited by applying depolarizing pulses (0 mV, 300 ms) (see expanded traces 1–3). In *D*, the current-voltage relationship was shown for the peak response to the positive sera bathed in normal Tyrode's solution (*filled circles*) and in an isotonic $BaCl_2$ solution (*open squares*). (*Insets*) Representative current traces before and after the application of the positive sera in normal Tyrode's solution (*a*), in the isotonic $CaCl_2$ solution (*b*), and in the isotonic $BaCl_2$ solution (*c*). The pipette solution containing BAPTA was used for the experiments in the isotonic $CaCl_2$ solution. All the traces are representatives of four to seven separate experiments.

cation permeability sequence is $Ba^{2+} > Na^+ \sim K^+$ at the microscopic current level. The antiheart antibody-activated single-channel event was not affected by dihydropyridines (5 μM nifedipine or Bay K 8644: $n = 4$) and membrane stretch induced by suction via patch electrodes ($n = 3$).

Discussion

In the present study, we found that the antiheart antibody associated with myocarditis induces activation of Ca^{2+}-permeable cation channels that are insensitive to dihydropyridines. Recently, Ca^{2+}- and Ba^{2+}-selective background channels were found in rat ventricular cells (15). We confirmed the presence of similar channels in some guinea pig ventricular cells when cell-attached pipette contained isotonic $BaCl_2$. However, the Ca^{2+}- and Ba^{2+}-selective background channel appears to be different from the antiheart antibody–activated cation channel because (*a*) the former (but not the latter) was activated by dihydropyridines; (*b*) the former had a larger channel conductance to Ba^{2+} (~ 17 pS) than the latter (12 pS); (*c*) the reversal potential for the former channel (measured under the same conditions, as in Fig. 3) was more positive (~ +59 mV) than that for the latter channel; and (*d*) the antiheart antibody-positive sera did not alter the former channel activity. The antibody-activated channel is also distinct from the Ca^{2+}-activated nonselective cation channel reported in guinea pig ventricular myocytes (16), since the antiheart antibody-activated nonselective cation current was not abolished by chelation of cytosolic Ca^{2+} with 5 mM BAPTA.

Autoantibodies associated with myasthenia gravis and the Lambert-Eaton myasthenic syndrome are known to act by directly binding to acetylcholine receptor channels (17) and voltage-gated Ca^{2+} channels (18–20), respectively. In contrast, antiheart antibody-positive sera added to the bath could activate the channels in the cell-attached patch membrane to which the sera could not gain access. This and the long latency of the response suggest the involvement of some intracellular messengers in channel activation.

The pathogenesis of myocarditis and cardiomyopathy has been suggested to involve autoimmunity (1–3), as well as calcium overload (9–11). Antibodies against the ADP/ATP carrier have been shown to enhance high voltage-activated Ca^{2+} channels (8). The dihydropyridine-binding sites in heart muscle were reported to increase in the Syrian cardiomyopathic hamster (21, 22), but this remains controversial (23). Here we showed that dihydropyridine-insensitive Ca^{2+}-permeable cation channels, but not voltage-gated Ca^{2+} channels, are activated by antiheart antibodies associated with myocarditis in guinea pig ventricular myocytes. It is likely that excess Ca entry caused by activation of this novel channel by the disease-associated antibodies is involved in the pathogenesis of myocarditis and dilated cardiomyopathy.

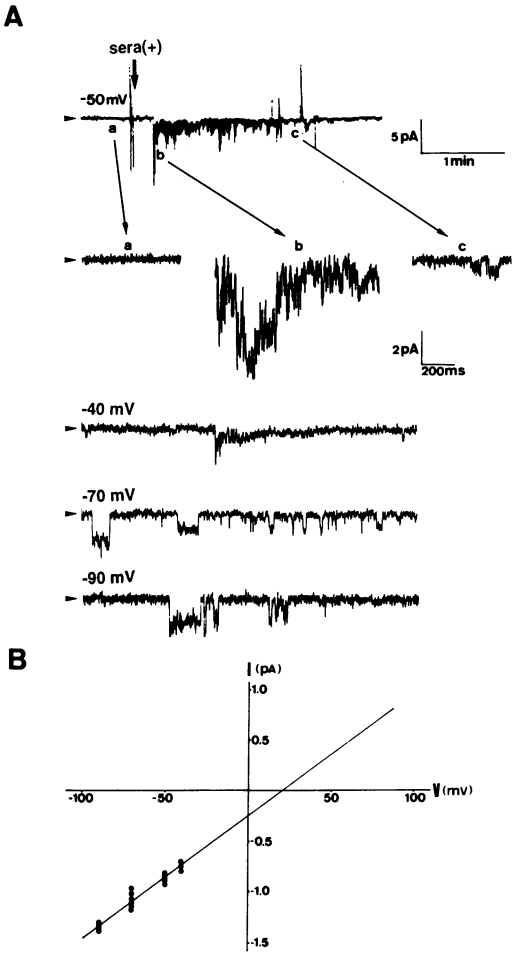

Figure 3. Ba^{2+}-permeable single-channel activities induced by antiheart antibody-positive sera at various holding potentials in cell-attached patches on guinea pig ventricular cells bathed in normal Tyrode's solution. The pipette solution contained 110 mM Ba^{2+}. (*A*) Representative traces of the serum-induced channel activities in four different patches at −40, −50, −70, and −90 mV. Membrane potentials were estimated by assuming that the intracellular potential is equal to −70 mV. A thick arrow indicates the time of puff application (duration ~ 8 s) of the positive sera. The sera were added 40–47 s before recording of the lower three traces. Downward deflections of the current trace represent inward currents. (a–c) Expanded traces of parts of the top trace. The closed level is marked by arrowheads. (*B*) Current-voltage (I-V) relationship. At more positive potentials than −40 mV, isolation of the antiheart antibody-activated channel activities was difficult because the activities were contaminated by other kinds of channels.

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