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A Monoclonal Antibody Specifically Modulates Dihydropyridine-Sensitive Calcium Current in BC_3H1 Myocytes

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SUMMARY

The nonfusing muscle cell line BC_3H1 expresses functional Na and Ca channels similar to those found in skeletal muscle (1). We have utilized a monoclonal antibody that cross-reacts with a 45–50-kDa glycoprotein in differentiated BC_3H1 myocytes but not in cardiac or neuronal cells. This antibody, MAb1223, specifically modulates ^{45}Ca influx and slowly activating, dihydropyridine-sensitive Ca currents when added to BC_3H1 myocytes. Low-threshold, dihydropyridine-insensitive Ca currents and Na and K currents are unaffected. MAb1223 action is voltage dependent. At very negative holding potentials, similar to those of the resting cell, MAb1223 increases slow Ca current without significant changes in the voltage dependence of activation. At more positive potentials, at which channel opening probability is reduced by inactivation, exposure to MAb1223 reduces current. Agonist and antagonist dihydropyridines modulate the action of MAb1223 on the slow Ca channel in a manner that suggests that their respective binding sites may interact directly with the channel.

Voltage-gated Ca channels play a central role in the regulation of Ca metabolism in a wide variety of cells. They transduce changes in membrane potential by modulating Ca influx and intracellular effector systems. The structural and functional architecture of Ca channels remains unknown, although there is considerable evidence that the DHPR, associated with the HTC channel, is an oligomeric complex of several polypeptides. Current concepts of the DHPR/Ca channel complex suggest that it contains two high molecular mass (170–190 kDa) α_1 and α_2 components. Several smaller polypeptides are also frequently associated with purified DHPR in variable amounts. These include an intermediate, 50–60 kDa, β component and a 30–40 kDa γ component (2–5). Although functional channels can be reconstituted from membrane preparations containing this complex (6, 7), the roles of individual polypeptide components have not been established.

Ca channels are present throughout early stages of embryonic development. A number of electrophysiological studies have documented the appearance and disappearance of several types of Ca channels in oocytes, fertilized eggs, and early cleavage stage embryos (8–11). In the sea urchin embryo, primary mesenchyme cells differentiate into spicule-forming cells that produce the magnesium calcite skeleton. A monoclonal antibody, MAb1223, which specifically recognizes a 130-kDa glycoprotein on the surface of isolated spicule-forming cells, inhibits uptake of extracellular Ca, and blocks spicule formation (12, 13). This antibody has been shown to recognize an epitope that is part of an anionic *N*-linked carbohydrate chain on the 130-kDa glycoprotein (14). Primary mesenchyme cells have many features in common with excitable cells, such as sensitivity to verapamil and other pharmacological agents known to modify Ca channels (15, 16). Furthermore, MAb1223 can alter binding of DHPs to cultured primary mesenchyme cells.[1] Consequently, MAb1223 is of potential interest as a specific probe for studying Ca channel function in other systems.

The present studies indicate that MAb1223 can specifically modulate the DHP-sensitive HTC current in differentiated BC_3H1 myocytes. MAb1223 modulated ^{45}Ca influx into BC_3H1 myocytes in a manner consistent with its actions on Ca currents in voltage clamp. The epitope recognized in BC_3H1 membranes is contained by a 45–50-kDa glycoprotein. MAb1223 has no effect on the HTC currents in several other excitable cells. There is no effect on low threshold DHP-insensitive Ca currents, TTX-insensitive Na currents, or K currents in the

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[1] M. C. Farach and W. J. Lennarz, unpublished observations.

ABBREVIATIONS: DHPR, dihydropyridine receptor; HTC, high threshold Ca; DHP, dihydropyridine; nmIgG, normal mouse immunoglobulin G; MAb, monoclonal antibody; FBS, fetal bovine serum, HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HBSS, Hank's balanced salt solution; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; TTX, tetrodotoxin.

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BC_3H1 cells. MAb1223 either increases or decreases slow I_{ca}, depending on the membrane holding potential at which it is applied. In addition, it interacts with DHP agonists and antagonists in a manner that suggests that the oligosaccharide epitope is intimately associated with the Ca channel.

Methods

Cell culture. BC_3H1 myocytes (17) were seeded at densities of 3×10^4 cells/cm^2 in 35-mm dishes in medium composed of equal parts Dulbecco's Modified Eagles Medium and Ham's F12 (GIBCO, Grand Island, NY) supplemented with 17 mM HEPES (pH 7.4), 3 mM $NaHCO_3$, 2 mM L-glutamine, 50 μg/ml gentamicin, and 20% heat-inactivated FBS (Hazelton Co., Denver, PA). At a density of approximately 3×10^5 cells/cm^2, differentiation was induced by washing and refeeding the cells with medium in which FBS was reduced to 0.5%. Cells were maintained in this medium for 10 to 21 days to enable maximum differentiation of voltage-dependent ion currents as described previously (1). For biochemical and ion flux experiments, BC_3H1 cultures were grown to the densities indicated below. C2 and PC12 cells were cultured as described elsewhere (18, 19).

MAb 1223. Purified IgG (subclass IgG_1) was isolated on protein A-Sepharose (Sigma Chemical Co., St. Louis, MO) from conditioned media of hybridoma 1223 as described previously (12, 13). Purified nmIgG (PelFreez Co, Rogers, AK) was suspended in the same buffer as MAb1223 by dialysis after reconstitution. nmIgG served as a control in all biochemical and electrophysiological experiments. FAb fragments were prepared from MAb1223 and nmIgG by methods described by Mage (20).

Labeling of BC_3H1 myocytes and identification of immunoreactive peptides. BC_3H1 cells grown to a density of approximately 2×10^6 cells/dish were induced to differentiate by mitogen withdrawal for 7 days. Differentiation medium (DMEM, 0.5% FBS) was removed, the plates were washed twice with HBSS, and the medium was replaced by labeling medium (methionine-free MEM) (Flow Laboratories, McLean, VA) containing 0.5% FBS and [^{35}S]methionine (Amersham, Arlington Heights, IL) at 150 μCi/dish. Labeling proceeded for 4 hr at 35°. The cells were then removed from the culture dish by scraping. A cell pellet was collected by low-speed centrifugation ($1000 \times g$ for 5 min) and washed three times with HBSS. This pellet was solubilized overnight at 4° in a buffer solution consisting of 10 mM Tris (pH 7.4), 2% sodium cholate, and a battery of protease inhibitors (12, 13). The solubilized material was preabsorbed batchwise onto 5 ml of covalently coupled nmIgG-Sepharose 4B beads (Sigma). The nmIgG-Sepharose beads were then collected by centrifugation and the supernatant was transferred to a 1 ml suspension of MAb1223-Sepharose prepared as previously described (13). After overnight incubation, unreacted material in solution was aspirated and the beads were sequentially washed as follows: twice with PBS, twice with PBS containing 0.5 M NaCl, twice with PBS containing 0.05% Tween-20, and finally once with PBS. The washed beads were boiled in SDS-PAGE sample buffer and the eluted material was analyzed by fluorography of SDS-PAGE gels (8.75% running gel/5% stacking gel) (12, 13).

Immunoblotting with MAb1223. Western blots and quantitative dot-blots using MAb1223 and iodinated second antibody were performed essentially as described (12, 13), except that nitrocellulose (Bio-Rad Laboratories, Richmond CA) was used in all assays in place of nylon transfer filters. Blots were dried and analyzed by autoradiography. Exposure times were adjusted to remain within the linear range (from 6 hr to 2 weeks), depending on the cells being studied. Radioactive spots were quantitated by direct scanning of radiograms using a Hoefer GS-300 transmittance-reflectance scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA) equipped with GS-350 data software system.

^{45}Ca uptake assay. The procedure for monitoring ^{45}Ca uptake was a modification of standard methods (21). Assays were performed on BC_3H1 myocytes grown to a density of approximately 3×10^5 cells/35-mm dish and differentiated by nitrogen withdrawal for at least 10 days. Uptake measurements were carried out at room temperature (22°). Culture medium was removed, the cells were washed with normal HBSS, and then incubated for various times with 2 ml of "resting" buffer (containing 132 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM glucose, and 25 mM Tris, pH 7.4) or "stimulating" buffer (5 mM NaCl, 132 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM glucose, and 25 mM Tris, pH 7.4) and ^{45}Ca at 10 μCi/ml. Uptake was terminated by aspirating the labeling solution and washing three times with ice-cold resting buffer. Cell-associated ^{45}Ca was extracted by 2-hr incubation with 0.5 N NaOH and measured by liquid scintillation counting. Protein assays performed on randomly selected plates showed that total protein/dish varied less than than 5%.

Miscellaneous biochemical procedures. Protein determinations were by the method of Lowry *et al.* (22) using bovine serum albumin as a standard. Conditions for SDS-PAGE using reduced cross-linked gels were as described by Lau and Lennarz (23). Protein standards were purchased from Bio-Rad. Prestained standards for Western blots were obtained from Bethesda Research Laboratories (Gaithersburg, MD).

Electrophysiological experiments. Ion currents were recorded using whole cell voltage clamp techniques described in Sakmann and Neher (24) using a LIST EPC-7 voltage clamp system (Medical Systems Corp., Greenvale, NY). Patch pipettes were pulled from thick-wall borosilicate glass (type 7052; Garner Glass Co, Claremont, CA) to a tip diameter of 2 to 4 μm and slightly fire-polished. Tip resistances ranged from 1 to 10 MΩ when filled with internal solutions listed below. Data were digitized stored on a video cassette recorder (JVC model HR-D725U, JVC Corp., Elmwood Park, NJ). Selected experiments were digitized at 25 to 250 μsec/point on a PDP 11/23 microcomputer (Digital Equipment Corp., Marlboro, MA) for further analysis. Only those cells that exhibited a rate of rundown of less than 5% of initial current amplitude during a 10-min control period and stable leakage current levels throughout the experiment were included in these analyses.

Linear components of capacitance and leakage currents were subtracted digitally from net current records using scaled averaged currents elicited by hyperpolarizing voltage steps. Kinetic properties of whole-cell ion currents were evaluated using a linear least-squares (Marquart) algorithm that produced a minimum χ^2 fit to a sum-of-exponentials model.[2]

Electrophysiological solutions. Sodium currents were studied using a modified Tyrode's solution containing (in mM): 130 Na, 5 K, 4 Ca, 143 Cl, 10 HEPES (pH 7.3 with NaOH), and 10 glucose. Calcium currents were studied using barium as the permeant divalent cation, in a solution containing (in mM): 110 *N*-methyl-*d*-glucamine, 20 Ba, 150 glutamate, 10 HEPES (pH 7.3 with CsOH), and 15 glucose. Internal (pipette) solutions contained: 140 *N*-methyl-*d*-glucamine/Cs, 130 glutamate/aspartate, 10 F, 10 EGTA, 10 HEPES (pH 7.3 with CsOH). Potassium currents were studied using the modified Tyrode's as external solution and an internal solution containing (in mM): 140 K, 130 glutamate/aspartate, 10 F, 10 EGTA, and 10 HEPES (pH 7.3 with KOH). Organic (dihydropyridine) Ca^{2+} channel blockers and agonists were stored in absolute ethanol and added to the preparation under reduced illumination.

Results

Immunoreactivity of various excitable cells to MAb1223. An immunoblot survey of MAb1223 reactivity with membrane preparations from a variety of excitable cells (Table 1) detected significant reactivity in BC_3H1 myocytes. Reactivity with differentiated and undifferentiated BC_3H1 cells was detectable at 1/10 and 1/50 (respectively) the levels found in sea urchin embryos; membranes from C2 cells (a fusing mouse muscle

[2] J. M. Caffrey and A. M. Brown, manuscript in preparation.

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TABLE 1

Relative immunoreactivity of MAb1223 with excitable cells from several species

Relative immunoreactivity was determined by quantitative measurement of dot blots as described in the text. In each case, serial (1:1) dilutions of the starting material (30 μg of protein/well) were compared for reactivity with MAb1223. Numbers represent reactivities of preparations expressed relative to that of gastrula stage sea urchin embryos. This was the immunogen used to generate MAb1223 and the reactivity with this preparation was assigned an arbitrary value of 10.

Cell/Source	Relative reactivity
Gastrula stage sea urchin embryos (*S. purpuratus*)	10.0
Differentiated BC3H1 myocytes (embryonic C3H mouse)	1.0
Undifferentiated BC3H1 myoblasts (embryonic C3H mouse)	0.2–0.3
C2 myocytes and myotubes (adult C3H mouse)	NDB[a]
Skeletal muscle transverse tubular membranes (adult rabbit)	NDB
PC12 (pheochromocytoma/neuroendocrine) cells (adult rat)	NDB
Cardiac myocytes (adult guinea pig, neonatal rat)	NDB

[a] No detectable binding.

line), rabbit transverse tubular preparations, adult guinea pig ventricular and atrial muscle, neonatal rat ventricular cells, and neuroendocrine cell lines PC12 and GH3 were unreactive under the conditions of our assay. It is important to note that methods used to generate data in Table 1 cannot distinguish between differences in abundance of antigen and variation in affinity of the antibody. Nonetheless these results are pertinent in relating degree of antibody reactivity in various cells to functional studies (see below).

Detection of a single 45–50-kDa peptide by MAb1223. Fluorograms of SDS-PAGE gels of [^{35}S]methionine-labeled protein from BC_3H1 myocytes specifically bound to MAb1223-conjugated immunoaffinity resin (Fig. 1A) showed a single band with molecular mass between 45 and 50 kDa. Reactivity of MAb1223 with a band of similar molecular mass also was detected in Western transfers of protein from BC_3H1 cells (Fig. 1B). Immunoreactive material was occasionally (Fig. 1A) detectable at the top of the separating gel. Whether this reflects an aggregate of the 45–50-kDa polypeptide or some other as yet uncharacterized component is unknown.

MAb1223 increases ^{45}Ca uptake in differentiated BC_3H1 myocytes. In contrast to inhibitory effects of MAb1223 on ^{45}Ca influx in cultured primary mesenchymal cells of the sea urchin (12), ^{45}Ca uptake by cultures of differentiated BC_3H1 myocytes was increased by approximately 50% in the presence of MAb1223 (Fig. 2). Voltage-dependent ^{45}Ca uptake stimulated by elevation of extracellular K^+ also was doubled in the presence of MAb1223 (not shown). Undifferentiated BC_3H1 myoblasts grown in the presence of 20% FBS, conditions that prevent expression of slow Ca channels (1), are unresponsive to MAb1223 (Fig. 2). K^+-stimulated Ca uptake in undifferentiated myoblasts was not significantly different from resting uptake and was likewise unaffected by MAb1223 (not shown). FAb fragments of MAb1223 also stimulated ^{45}Ca influx into differentiated myocytes. Addition of nonspecific IgG or FAb fragments had no effect on ^{45}Ca uptake.

MAb1223 specifically increases slow Ca current in BC_3H1 myocytes. Correspondent to the reactivity of Mab1223 with BC_3H1 myocyte membranes and its effects on

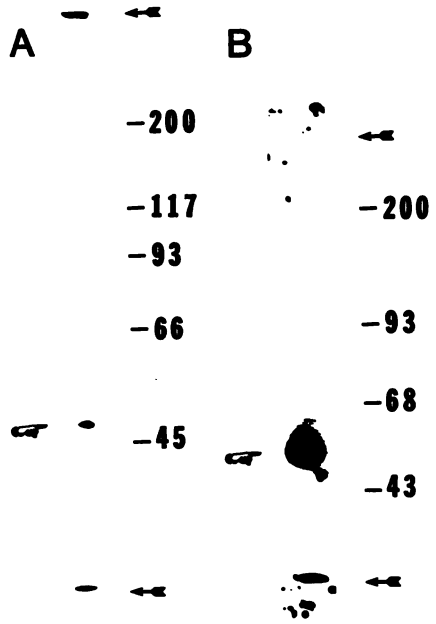

Fig. 1. MAb1223 recognizes a single 45–50-kDa polypeptide in differentiated BC_3H1 myocytes. The protein recognized by MAb1223 in BC_3H1 cells was identified in extracts of [^{35}S]methionine-labeled myocytes (A) and in Western transfer blots (B) as described in the text. The fluorogram shown in A was obtained from a 4-day exposure. The autoradiograph shown in B shows the results of 2-week exposure at −70° of an SDS-PAGE gel loaded with 100 μg/lane of total cellular protein. The positions of molecular weight standards are indicated to the *right* of the lanes; the tops of the gels and the dye front are indicated by *arrows* at the *right*. Values reported for apparent molecular weights of the protein standards are those specified by the manufacturer. The position of detected radiolabel is highlighted at the *left* of the lanes.

^{45}Ca influx, inward Ba^{2+} currents measured under voltage-clamp conditions were found to be modulated by Mab1223 when added to the extracellular solution. This produced an increase in the slow, high-threshold current that has been shown to be similar to the dihydropyridine-sensitive T-tubular calcium current in skeletal muscle[3] (1). The time constants for activation, inactivation (current decay), and deactivation (tail currents) were not significantly changed during this increase ($n = 11$, Fig. 3A). Likewise, the voltage threshold for activation and the membrane potential at which current was maximal were largely unchanged by Mab1223 ($n = 8$, Fig. 3B) in contrast with action of DHP agonists such as BAY k8644 (25).

The threshold concentration for detectable increase in slow Ca current by Mab1223 was approximately 100 ng/ml, with a maximum effect at 10 μg/ml. The maximum amount of increase measured at a test potential of +10 mV was 68 ± 27% ($n = 11$). Antibody effects appeared poorly reversible over attempted washout of 10 to 20 min duration but interpretation of these small effects were complicated by irreversible rundown of slow current (cf. 25); thus experiments were designed assuming that binding and functional effects were effectively irreversible. FAb fragments of MAb1223 also increased slow current ($n = 4$). Control additions of nonspecific mouse IgG had no detectable effect on any of these membrane properties at concentrations up to 100-fold those used to observe MAb1223 effects.

The 'slow' Ca currents in myocytes and myotubes of the

[3] J. M. Caffrey, M. D. Schneider, and A. M. Brown. Two skeletal muscle-like calcium channels in BC_3H1 myocytes: macroscopic and single channel properties. Submitted.

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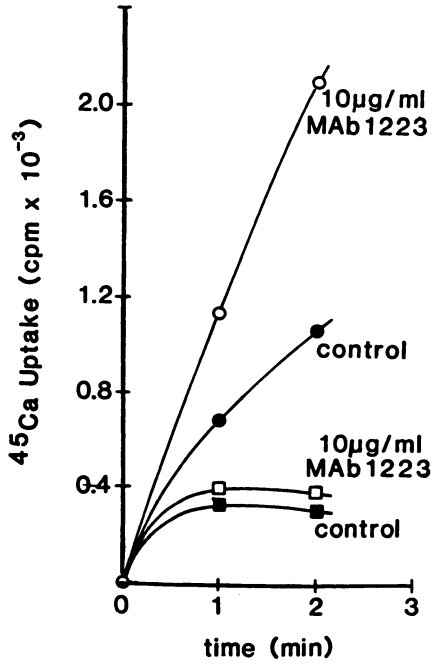

Fig. 2. ^{45}Ca uptake into differentiated BC_3H1 myocytes is stimulated by MAb1223 whereas uptake into undifferentiated BC_3H1 myoblasts is unaffected. *Closed* and *open* symbols show resting uptake in the absence and presence of MAb1223, respectively. *Circles* and *squares* describe resting uptake in differentiated myocytes and undifferentiated myoblasts, respectively. Ca uptake was assayed over a 2-min time course after addition of labeling medium as described in the text. In these experiments, MAb1223 was added to a final concentration of 10 µg/ml. Each *point* represents the average uptake in three separate 35-mm culture dishes. Variation in cell number and protein content was determined to be less than 5%. Variation in uptake at all points was determined to be less than 20% over four separate experiments, consistent with observed variability in the extent of differentiation (*vis-a-vis* expression of Ca current) in BC_3H1 cultures of different passage numbers (unpublished observation).

fusing muscle cell line C2, derived from the same strain of mouse as the BC_3H1 line (18), were unresponsive to Mab1223 (n = 4, Fig. 4A). This was consistent with the lack of immunoreactivity to Mab1223 of membranes prepared from C2 cell cultures or from transverse tubular membranes of adult skeletal muscle. Myocytes isolated from neonatal rat ventricle, adult guinea pig ventricle, and the neuroendocrine cell line PC12 (n = 4, Fig. 4B) showed no responsiveness to Mab1223 under virtually identical recording conditions.

The specificity of Mab1223 action for calcium channels was evaluated on other voltage-gated current types in BC_3H1 myocytes. Both a transient, TTX-resistant Na current and rapidly activating, slowly inactivating, DHP-insensitive ('fast') Ca current are expressed concurrently with slow currents during differentiation induced by mitogen withdrawal (1). Na current was isolated using normal Tyrode's solution with full (140 mM) Na and low (2 mM) Ca. Fast Ca current was isolated (as Ba current) by depolarization of holding potential to −40mV, which preferentially inactivates slow current[3] (1). As shown in Fig. 5, A and B, Mab1223 had no effect on either fast current or Na current at 10 times the concentration maximally effective on slow current (n = 5, or 4, respectively).

Under ionic conditions designed to study outward potassium currents, addition of MAb1223 produced increases in K current in 44% (4/9) of the cells tested from differentiated myocyte cultures (Fig. 5C). This is similar to the incidence of BC_3H1 myocytes that fail to differentiate under these culture conditions.[1] Although Ca currents are not usually (directly) detectable under these ionic conditions, such cells also lacked transient Na current, which is coordinately expressed with fast and slow Ca currents (1). Specific effects of MAb1223 on K channels (in the absence of Ca influx) can be more clearly defined in undifferentiated myoblasts that do not express voltage-gated Ca channels (1). As illustrated in Fig. 5D, addition of MAb1223 had no effect (n = 0/7) on K current undifferentiated myoblasts, suggesting that the effects seen on differentiated myocytes was due to Ca influx-dependent modulation of K channels.

MAb1223 modulates steady state inactivation properties and DHP Action on Slow Ca Currents: To test further the association between the MAb1223 binding site and the slow Ca channel, the interaction of MAb1223 with representitives of the two functional classes of DHPs, drugs postulated to act specifically on calcium channels, was studied. These compounds have been shown elsewhere[3] to act only on slow current; fast current is unaffected by micromolar levels of either agonist or antagonist DHPs. This current contributes between 5 and 10% of the net Ba current signal under control conditions at test potentials used in these experiments.[3]

Slow current elicited from a holding potential of −80 mV could be half maximally blocked using 100 nM nitrendipine, a concentration approximately 200 times greater than the K_D for binding to membrane preparations (26). Under these conditions, addition of MAb1223 had no effect at 10 times the concentration that would produce a maximal response in control cells not treated with nitrendipine (n = 5; Fig. 6). However, when slow inward current was increased by an intermediate concentration (100 nM) of Bay k8644, the subsequent addition of MAb1223 elicited a reduction of current toward control levels (n = 6, Fig. 7). This reduction occurred promptly (within 1–3 min) after addition of MAb1223 and reached a stable level within 5 min. Increases in current could be recovered in these circumstances by elevation of either Bay k8644 or MAb1223 (Fig. 7, B and D, respectively).

Many substances, including DHPs, that interact with ion channels appear to exhibit voltage-dependent modulation of their affinity or potency, depending on the functional state of the channel (27–30). In BC_3H1 myocytes, slow current is inactivated by changes in holding potential between −80 and −40 mV, a more negative potential range than observed for other HTC currents[3] (1). Current evoked from a holding potential of −60 mV, at which the channels were held inactivated by more than 60% for 1 to 3 min (to verify stability of current), was reduced by addition of MAb1223 (n = 8, Fig. 8). This occured in cells whose slow current was specifically selected for a low rate of rundown during a control recording period. As with previous experiments, onset of the effect occurred promptly upon addition of MAb1223 and reached a steady level after several minutes. Thus, upon changing holding potential over a range that inactivates or reduces availability of slow current, the action of MAb1223 changed from a stimulatory to an inhibitory one.

Discussion

MAb1223 binding is strongly correlated to DHPR and the slow Ca channel in BC_3H1 myocytes. The oligosac-

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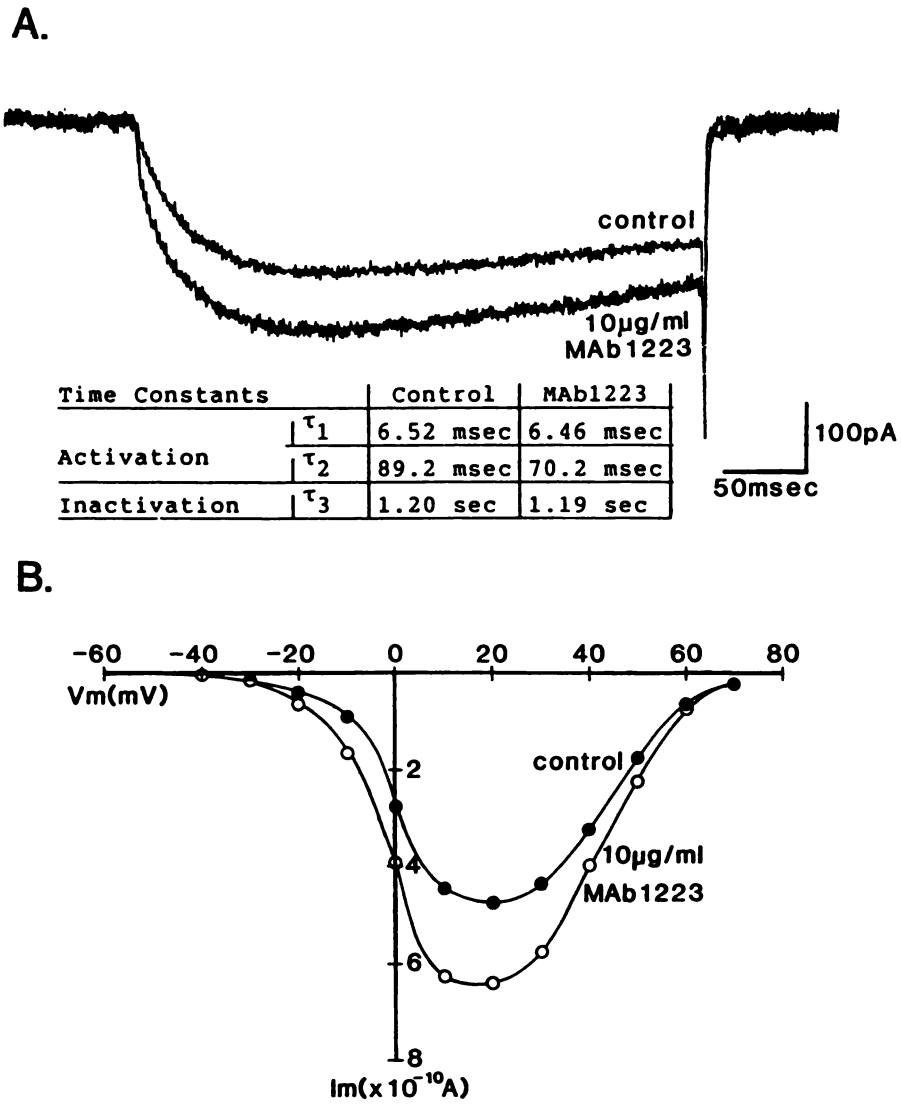

Time Constants		Control	MAb1223
Activation	τ_1	6.52 msec	6.46 msec
	τ_2	89.2 msec	70.2 msec
Inactivation	τ_3	1.20 sec	1.19 sec

Fig. 3. MAb1223 increases slow Ca current in differentiated BC_3H1 myocytes. Inward current through Ca channels was isolated by using Ba as the permeant divalent cation and by substitution of the impermeant monovalent cation NMDG for Na and K. A, Net membrane currents before and 5 min after addition of MAb1223 to the external solution to a final concentration of 10 μg/ml. Holding potential, −80 mV; test potential, +10 mV. B, The amplitude of peak inward Ba current before and after addition of 10 μg/ml MAb1223 at several test potentials.

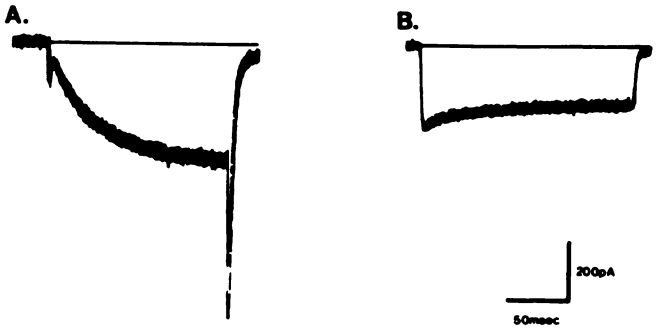

Fig. 4. MAb1223 does not affect high-threshold, DHP-sensitive Ca currents in other excitable cells. A, Inward Ba currents recorded in C2 myocytes differentiated for 5 days in reduced serum (see Methods). Net membrane currents (which include a transient low-threshold component not present in BC_3H1 cells) are shown before and 8 min after addition of MAb1223 to a final concentration of 100 μg/ml. Holding potential, −90 mV. Test potential, 0 mV. B, Membrane currents obtained from PC12 cells before and 8 min after addition of MAb1223 to 100 μg/ml. Holding potential, −80 mV; test potential, +10 mV.

charide epitope recognized by MAb1223 appears to be intimately associated with the slow Ca channel in BC_3H1 myocytes, based on several functional tests. (i) Its action was selective for the slow, DHP-sensitive (HTC) Ca channel with no cross-reactivity with other voltage-gated currents in BC_3H1 myocytes. The stimulation of K currents that was sometimes observed in cells from differentiated myocyte cultures appears to be due to modulation of K channel activation after voltage-dependent Ca influx.[1] (ii) MAb1223 modulated the activity of the HTC in a voltage-dependent manner; at very negative holding potentials, its action is to increase current without changes in activation kinetics or voltage dependence. At more positive holding potentials, at which HTC are partially inactivated, addition of MAb1223 decreases current. (iii) MAb1223 also interacts with dihydropyridine modulation of HTC function. Prior addition of nitrendipine is sufficient to block MAb1223 increase in current. Addition of MAb1223 to HTC modified by the stimulatory DHP BAY k8644 caused a reduction of current, which could be reversed by additional increase in concentration of BAY k8644 or MAb1223.

The dependence of MAb1223 action on holding potential has some similarities to that of DHPs. In cardiac muscle, it has

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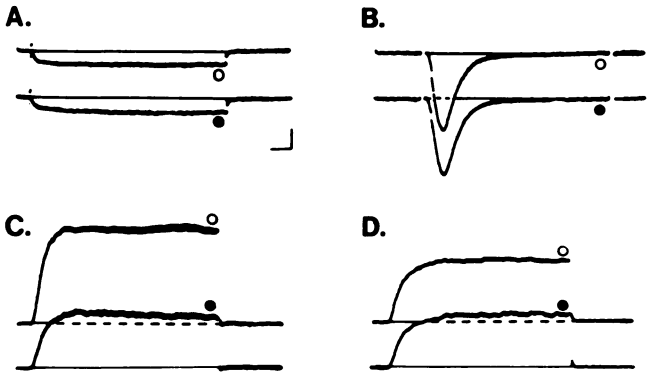

Fig. 5. MAb1223 does not affect other ion channels in BC_3H1 cells. In each panel, the *lower* of each pair of traces is control current (●); the *upper* is in the presence of the indicated amount of MAb1223 (○). A, Rapidly activating, DHP-insensitive (fast) Ba currents in differentiated BC_3H1 myocytes before and 10 min after addition of MAb1223 to 30 μg/ml. Slow Ca current was eliminated using depolarized holding potentials as described in the text. Vertical calibration, 100 pA; horizontal calibration, 50 msec; holding potential, −40 mV; test potential, 0 mV. B, Transient Na current before and 8 min after addition of MAb1223 to a concentration of 100 μg/ml. Vertical calibration, 100 pA; horizontal calibration, 5 msec; holding potential, −90 mV; test potential, −30 mV. C, An example of outward K currents from a culture of differentiated myocytes increased by addition of 10 μg/ml MAb1223 after 5 min. D, The failure of 100 μg/ml MAb1223 to affect K currents in undifferentiated myoblasts. Vertical calibration, 200 pA; horizontal calibration, 50 msec; holding potential, −90 mV; test potential, +10 mV.

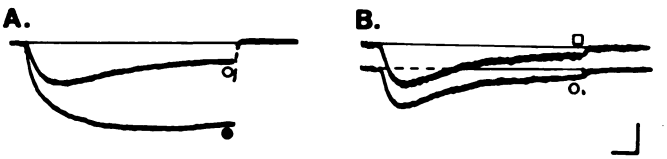

Fig. 6. Barium current in BC_3H1 myocytes is insensitive to MAb1223 in the presence of nitrendipine. In A, slow inward current was partially blocked by addition of 100 nM nitrendipine. B, traces (separated for clarity) before and 10 min after addition of 30 μg/ml MAb1223 in the continued presence of nitrendipine. ●, control; ○, nitrendipine; □, nitrendipine plus MAb1223. Vertical calibration: 200 pA. Horizontal calibration: 50 msec. Holding potential: −80 mV. Test potential: +20 mV.

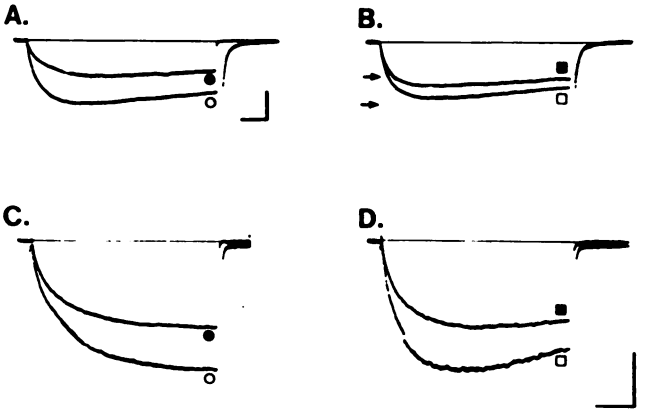

Fig. 7. The stimulatory action of BAY k8644 on slow inward current is reversed by addition of MAb1223. A illustrates inward current in control (●) and after increase by addition of 30 nM BAY k8644 (○). B shows that in the continued presence of 30 nM BAY k8644 addition of 30 μg/ml MAb1223 reduced inward current to control levels (■). Increase of inward current could be recovered by further elevation of BAY k8644 concentration (60 nM; □). *Arrows* delimit current amplitudes from control and 30 nM BAY k8644, shown in A, to clarify the scale of MAb1223 effects. C illustrates the increase of slow current in another cell by 300 nM Bay k8644 (control, ●; BAY k8644, ○). D shows that Bay k8644-enhanced current is reduced toward control levels by addition of 10 μg/ml MAb1223 (■) but further increase can be obtained by elevation of MAb1223 concentration (100 μg/ml, □). Vertical calibration: 200 pA in A and B, 100 pA in C and D. Horizontal calibration: 50 msec in A and B; 30 msec in C and D. Holding potential: −80 mV. Test potential: +10 mV in A and B; 0 mV in C and D.

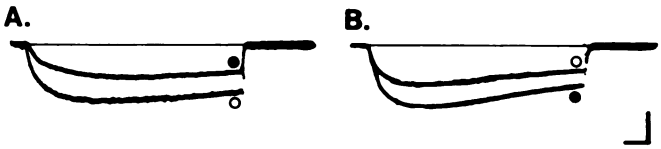

Fig. 8. The modulation of slow Ba current in BC_3H1 myocytes is dependent on holding potential. A shows current before (●) and after (○) addition of 10 μg/ml MAb1223 at a holding potential of −80 mV. B illustrates with data from another cell, that addition of 10 μg/ml MAb1223 at a holding potential of −60 mV, at which slow currents are partially inactivated, leads to a reduction of current (control, ●; MAb1223, ○). Vertical calibration: 200 pA in A, 100 pA in B. Horizontal calibration: 50 msec. Test potential: +10 mV.

been reported that nitrendipine and BAY k8644 can be either stimulatory or inhibitory, depending upon concentration and the holding potential at which they are applied (28–31). Although such bimodal effects of nitrendipine and BAY k8644 on BC_3H1 slow Ca currents are not observed,[1,3] the present results show that similar effects can be obtained by MAb1223 binding to an extracellularly accessible site.

Because MAb1223 does not significantly shift activation voltage dependence of slow Ca current and is inhibitory at partially depolarized resting potentials, the stimulation of both resting and K-stimulated ^{45}Ca influx by MAb1223 seems unusual. These data might be reconciled by observations that spontaneous electrical and contractile activity are frequently observed in developing muscle cultures (cf. 32) and some evidence of this has been noted in differentiated BC_3H1 myocytes (17).

MAb1223 modifies the effects of BAY k8644 in such a manner as to suggest allosteric interaction, causing displacement for BAY k8644 from a stimulatory site on the slow Ca channel. Other interpretations are also possible. Although these experiments were designed to optimize the stimulatory effects of both BAY k8644 and MAb1223, their interaction might have unmasked inhibitory effects of BAY k8644 (cf. 31) that are normally unobservable under these conditions. However, allosteric displacement of MAb1223 by BAY k8644 seems unlikely given the poor reversibility of MAb1223 (and other antibodies) under control conditions.

Interaction with inhibitory DHPs is more complex but has a similar interpretation. Ca currents partially blocked by nitrendipine are unresponsive to MAb1223, even at 10 times the nominal maximum concentration. This is consistent with the observation that MAb1223 has no effect on binding of [^{3}H] PN200-110 to membrane preparations from differentiated BC_3H1 myocytes.[4] DHPs are thought to exert at least part of their inhibitory action by high affinity binding to and stabilization of the inactivated state of the Ca channel (27). However, if nitrendipine partially stabilized the channel in the inactivated state, one might expect MAb1223 to decrease current further; because MAb1223 reduces current at holding potentials at which the channel is partially inactivated. This is not observed nor does MAb1223 increase current in the presence of

[4] D. Rampe and A. M. Brown, unpublished observations.

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nitrendipine at holding potentials at which where it would be stimulatory in control conditions.

One potential explanation for divergent MAb1223 effects on nitrendipine- and BAY k8644-modified slow Ca channels can be found by noting that the K_D of nitrendipine for skeletal muscle-type DHPR is significantly greater than that of BAY k8644 (0.5 versus 25 nM, respectively; Ref. 33). Assuming a molecular mass of 150 kDa for IgG (20), an ED_{50} in the range of 5 μg/ml for MAb1223 increase of slow current (when added at a holding potential of −90 mV) suggests a binding affinity of approximately 30 nM. This is closer to that of BAY k8644 than nitrendipine; thus, interactions between MAb1223 and BAY k8644 might be expected to be more readily detectable than with nitrendipine (or PN200-100). This might also be interpreted to mean that stimulatory and inhibitory DHP binding sites are separate entities (28) and that MAb1223 interacts with the former but not the latter.

These stimulatory effects of MAb1223 are in marked contrast to those associated with the β-adrenergic receptor and cyclic AMP-dependent protein kinase system, whose activation increases HTC channel opening probability and macroscopic current. The actions of β agonists or protein kinase A are not dependent on holding potential (34, 35), nor do they inhibit DHP interaction with the Ca channel (36). Moreover, our experiments were conducted with internal solutions formulated without Mg or exogenous nucleotides and were preceded by a control period of at least 10 min wherein endogenous regulatory components would be significantly reduced by dialysis. Therefore, it is unlikely for these effects of MAb1223 to represent modulation of an intracellular second messenger system.

It is also important to note that the effects of intact MAb1223 could be reproduced by its FAb fragments. This eliminates the possibility that the effects observed arise merely from cross-linking and/or aggregation of epitope-containing molecules on the cell surface.

MAb1223 Action on the slow Ca channel are distinct from those of a MAb directed against DHPR. The functional effects of MAb1223 are distinct from those of another MAb, MAb1A, raised against the 170–190-kDa α_1 component of skeletal muscle DHPR. MAb1A immunoprecipitates proteins of 220, 200, 65, and 35 kDa from adult skeletal muscle T-tubular membranes, which copurify with the DHPR complex (37), and cross-reacts with a reduction-insensitive 210-kDa protein in BC_3H1 membranes (38). This antibody reduces slow Ca current in BC_3H1 myocytes (38) in contrast to the increase produced by MAb1223. MAb1A also modulates kinetic properties of the TTX-insensitive Na current found in BC_3H1 myocytes, but not those of the DHP-insensitive fast Ca current or of the outwardly rectifying K current. By contrast, MAb1223 has no effect on Na currents, as well as failing to modify other ion currents. Preliminary studies comparing the binding of MAb1223 with that of MAb1A in BC_3H1 cells suggest that their respective antigens are not cross-reactive. These two respective polypeptides appear to be of equivalent (low) abundance in these cells, as judged by the time course for appearance of signal in Western blots. If the DHP binding site(s) reside within the 170–190-kDa subunit as reported (see below), then our data suggest that the polypeptides containing these distinct epitopes must interact to modulate slow Ca current and its DHP sensitivity.

MAb1223 recognizes a 45–50-kDa glycoprotein in BC_3H1 myocytes. The precise relationship of the MAb1223-binding protein with the slow Ca channel is still unclear. The interaction of MAb1223 with DHP modulation of HTC function suggests direct interaction with DHPR. The 45–50-kDa protein identified by MAb1223 is unlikely to contain the DHP binding site, because photoaffinity labeling of skeletal muscle DHPR results in specific incorporation of label into the 170–190-kDa nonreducing α_1 subunit (39, 40). This does not, however, rule out the possibility of allosteric interactions of the α_1 subunit with other components that might affect DHP binding and function. A protein of similar molecular weight (50–60-kDa) copurifies in variable amounts with DHPR from skeletal muscle (2–5) but its functional role has not been clarified in reconstitution experiments (cf. 6, 7).

A similar situation appears to exist with regard to the functional role of the β subunit(s) of Na channels. These 33- and 36-kDa proteins copurify in varying amounts with the saxitoxin receptor from brain and muscle (41, 42). Functional reconstitution studies and expression of cDNAs for these proteins in oocytes (43) have suggested that these components are not crucial in the formation of voltage-gated channels. However, recovery of complete sensitivity to neurotoxins in reconstituted and expressed channels does appear to require the presence of β subunits (44).

Although nothing is presently known regarding the expression of individual components of DHPR and/or Ca channels, it is interesting that the MAb1223 epitope is detectable by Western blot analysis in undifferentiated myoblasts and that its levels increase by a factor of 5 during differentiation. Experiments are underway to determine whether the increased levels of MAb1223 binding sites represent increased synthesis or mobilization of intracellular antigen to the cell surface. The appearance of functional HTC and DHP binding in BC_3H1 myocytes is strongly regulated during differentiation induced by mitogen withdrawal (1, 45). Specific [^{3}H]PN200-110 binding and functional Ca channels are undetectable before days 3–5 of differentiation. ^{45}Ca uptake in myoblasts is unaffected by changes in membrane potential induced by changes in extracellular K^+ and by MAb1223. Likewise, the activation of outward K^+ currents, which can be influenced by Ca influx, are unaffected by MAb1223 or changes in extracellular Ca in undifferentiated myoblasts. This is consistent with the selectivity of MAb1223 action on HTC and absence of functional channels in myoblasts but does not conclusively demonstrate lack of surface binding sites for MAb1223 in myoblasts. Labeling of MAb1223 bound to intact BC_3H1 cells with fluorescent second antibodies produced weak punctate signals only on differentiated myocytes (not shown) but the signal levels were too low to address the question of whether surface sites are present (at extremely low levels) on undifferentiated myoblasts.

Future directions. Because BC_3H1 cells and C2 are both myogenic cell lines derived from the C3H mouse strain, they might be expected to share most of their membrane antigenic determinants. An exception to this would be components specifically modified during the more extensive course of differentiation undertaken in C2 cells, which, unlike BC_3H1 myocytes, fuse to form myotubes and evolve a more elaborate sarcotubular membrane system. Differences in the steady state voltage dependence of slow current inactivation between

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BC_3H1 and C2 myocytes have been observed,[5] although activation kinetics and voltage dependence in the two preparations are indistinguishable. It is tempting to speculate that the MAb1223 epitope reflects a developmentally regulated post-translational modification of DHPR/Ca channels that appears during embryonic development and disappears from adult forms of muscle cells. In this context, it is also interesting to note that DHP binding affinity in chick skeletal muscle has been observed to undergo a discontinuous transition at hatching from a high affinity embryonic form (K_d = 0.1 nM, [^{3}H] nitrendipine) to a lower affinity one (K_d = 0.5 nM) identical to adult skeletal muscle (46). The molecular mechanisms underlying this phenomenon have not been investigated, but these observations establish a precedent for developmental modifications of DHPR and/or Ca channel complexes. Precedent for changes in carbohydrate composition of integral membrane proteins during development can found in analyses of the polysialic acid chains of the cell surface adhesion molecule neural cell adhesion molecule in skeletal muscle, which disappear during postnatal development (47, 48). Present studies in our laboratory are directed toward the assembly and expression of Ca channel subunits by BC_3H1 cells, including the oligosaccharide chain recognized by MAb1223.

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