

# Heterologous Expression of BI Ca<sup>2+</sup> Channels in Dysgenic Skeletal Muscle

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**ABSTRACT** We have examined the ability of BI (class A) Ca<sup>2+</sup> channels, cloned from rabbit brain, to mediate excitation-contraction (E-C) coupling in skeletal muscle. Expression plasmids carrying cDNA encoding BI channels were microinjected into the nuclei of dysgenic mouse myotubes grown in primary culture. Ionic currents and intramembrane charge movements produced by the BI channels were recorded using the whole-cell patch-clamp technique. Injected myotubes expressed high densities of ionic BI Ca<sup>2+</sup> channel current (average 31 pA/pF) but did not display spontaneous contractions, and only very rarely displayed evoked contractions. The expressed ionic current was pharmacologically distinguished from the endogenous L-type current of dysgenic skeletal muscle ( $I_{\text{dys}}$ ) by its insensitivity to the dihydropyridine antagonist (+)-PN 200-110. Peak BI Ca<sup>2+</sup> currents activated with a time constant ( $\tau_a$ ) of ~2 ms and inactivated with a time constant ( $\tau_h$ ) of ~260 ms (20–23°C). The time constant of inactivation ( $\tau_h$ ) was not increased by substituting Ba<sup>2+</sup> for Ca<sup>2+</sup> as charge carrier, demonstrating that BI channels expressed in dysgenic myotubes do not undergo Ca<sup>2+</sup>-dependent inactivation. The average maximal Ca<sup>2+</sup> conductance ( $G_{\text{max}}$ ) produced by the BI channels was quite large (~534 S/F). In contrast, the average maximal charge movement ( $Q_{\text{max}}$ ) produced in the same myotubes (~2.7 nC/ $\mu$ F) was quite small, being barely larger than  $Q_{\text{max}}$  in control dysgenic myotubes (~2.3 nC/ $\mu$ F). Thus, the ratio  $G_{\text{max}}/Q_{\text{max}}$  for the BI channels was considerably higher than previously found for cardiac or skeletal muscle L-type Ca<sup>2+</sup> channels expressed in the same system, indicating that neuronal BI Ca<sup>2+</sup> channels exhibit a much higher open probability than these L-type Ca<sup>2+</sup> channels.

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## INTRODUCTION

The rabbit brain BI (class A)  $\text{Ca}^{2+}$  channel was the first neuronal  $\text{Ca}^{2+}$  channel to be expressed functionally from its complementary DNA (Mori, Friedrich, Kim, Mikami, Nakai, Ruth, Bosse, Hofmann, Flockerzi, Furuichi, Mikoshiba, Imoto, Tanabe, and Numa, 1991). When heterologously expressed in *Xenopus* oocytes, BI channels mediate an ionic current that is insensitive to dihydropyridine (DHP) antagonists and to  $\omega$ -conotoxin GVIA, but that is partially blocked by crude venom from the funnel-web spider *Agelenopsis aperta* (Mori et al., 1991). More recently, BI channels expressed in oocytes have been shown to be partially suppressed by  $\omega$ -Aga-IVA, a peptide component of *Agelenopsis* venom, and completely blocked by  $\omega$ -conotoxin MVIIC (Sather, Tanabe, Zhang, Mori, Adams, and Tsien, 1993), a peptide predicted by cDNA cloned from the venom gland of *Conus magnus* (Hillyard, Monje, Mintz, Bean, Nadasdi, Ramachandran, Miljanich, Azimi-Zoonooz, McIntosh, Cruz, Imperial, and Olivera, 1992). Thus, the pharmacological sensitivities of expressed BI channels somewhat resemble those of native P-type  $\text{Ca}^{2+}$  channels found in mammalian Purkinje cell neurons (Llinas, Sugimori, Lin, and Cherksey, 1989; Regan, Sah, and Bean, 1991; Mintz, Venema, Swiderek, Lee, Bean, and Adams, 1992). However, quantitative differences in toxin sensitivity and biophysical properties between cloned BI channels and native P-type  $\text{Ca}^{2+}$  channels suggest that these two  $\text{Ca}^{2+}$  channels are not identical (Tsien, Ellinor, and Horne, 1991; Sather et al., 1993; Zhang, Randall, Ellinor, Horne, Sather, Tanabe, Schwarz, and Tsien, 1993; Wheeler, Randall, and Tsien, 1994).

The objectives of the present work were to examine the ability of BI  $\text{Ca}^{2+}$  channels to mediate excitation-contraction (E-C) coupling and to generate the intramembrane charge movement signal that is associated with this process in skeletal muscle (Rios and Pizarro, 1991). We also wanted to measure the ratio of  $\text{Ca}^{2+}$  conductance to intramembrane charge movement produced by gating of the BI channels, so that we could estimate the open probability ( $P_0$ ) of the BI channels for comparison with L-type  $\text{Ca}^{2+}$  channels expressed in the same system. Previously, we have used myotubes prepared from skeletal muscle of mice with muscular dysgenesis as a system for investigating the mechanisms of E-C coupling and for characterizing the biophysical properties of cloned  $\text{Ca}^{2+}$  channels (Tanabe, Mikami, Niidome, Numa, Adams, and Beam, 1993). Dysgenic skeletal muscle is extremely useful for such experiments because it lacks excitation-contraction (E-C) coupling (Powell and Fambrough, 1973) and slowly-activating L-type  $\text{Ca}^{2+}$  current (Beam, Knudson, and Powell, 1986). Furthermore, dysgenic skeletal muscle lacks the high-density intramembrane charge movement that is characteristic of normal skeletal muscle (Adams, Tanabe, Mikami, Numa and Beam, 1990). However, when cDNAs encoding cloned L-type  $\text{Ca}^{2+}$  channels are expressed in dysgenic myotubes, E-C coupling, L-type  $\text{Ca}^{2+}$  currents and intramembrane charge movements are all restored, mirroring the physiological situations in cardiac and skeletal muscle (Tanabe et al., 1993).

L-type  $\text{Ca}^{2+}$  channels appear to exhibit a very low maximum probability of being open ( $P_0$ ) during a depolarizing test pulse. This low  $P_0$  has been revealed by single-channel measurements of native skeletal muscle  $\text{Ca}^{2+}$  channels reconstituted into lipid bilayers (Ma, Mundina-Weilenmann, Hosey, and Rios, 1991; Mejia-Alvarez,

Fill, and Stefani, 1991) and by cell-attached patch clamp recordings from skeletal muscle myotubes (Dirksen, Tanabe, and Beam, 1993) and cardiac ventricular cells (Lew, Hryshko, and Bers, 1991; Ono and Fozzard, 1992). A low  $P_0$  is also reflected in the small ratio of maximum L-type  $\text{Ca}^{2+}$  conductance to intramembrane charge movement ( $G_{\text{max}}/Q_{\text{max}}$ ) found in dysgenic myotubes exogenously expressing the cardiac or skeletal muscle L-type  $\text{Ca}^{2+}$  channels (Adams et al., 1990). We find that BI channels expressed in dysgenic myotubes produce a high-density  $\text{Ca}^{2+}$  conductance but a low density of intramembrane charge movement, suggesting that few BI channels are present in the membrane but that those present exhibit a relatively high  $P_0$ . Significantly, we also find that BI  $\text{Ca}^{2+}$  channels do not restore E-C coupling to dysgenic skeletal muscle. A preliminary report of these results has appeared (Adams, Tanabe, Mori, and Beam, 1994).

#### MATERIALS AND METHODS

Primary cultures of myotubes were prepared from newborn dysgenic mice as previously described (Adams and Beam, 1989). Approximately 7 d after plating, the nuclei of developing myotubes were microinjected (Tanabe, Beam, Powell, and Numa, 1988) with the expression plasmid pKCRCBI-1 or pKCRCBI-2, which carry cDNA encoding the BI-1 or BI-2 isoforms of the BI  $\text{Ca}^{2+}$  channel, respectively (Mori et al., 1991). The expression plasmid pKCRCBI-1, carrying the entire protein-coding sequence of the BI-1  $\text{Ca}^{2+}$  channel, was constructed by inserting the *Hind*III(vector)/*Hind*III(7,932) fragment from pSPCBI-1 (Mori et al., 1991) into the *Hind*III site of pKCRH2 (Mishina, Kurosaki, Tobimatsu, Morimoto, Noda, Yamamoto, Terao, Lindstrom, Takahashi, Kuno, and Numa, 1984). The expression plasmid pKCRCBI-2, carrying the entire protein-coding sequence of the BI-2  $\text{Ca}^{2+}$  channel, was constructed by inserting the *Hind*III(vector)/*Hind*III(7,519) fragment from pSPCBI-2 (Mori et al., 1991) into the *Hind*III site of pKCRH2. 1–3 d after injection with cDNA, myotubes bathed in culture medium were tested for the ability to contract in response to electrical stimulation via a saline/agar-filled extracellular pipette (Tanabe et al., 1988). The culture medium was then replaced by external solution (see below for composition) and ionic  $\text{Ca}^{2+}$  channel currents and intramembrane charge movements were recorded using the whole-cell patch clamp technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) as previously described (Adams and Beam, 1989; Adams et al., 1990). After establishment of the whole-cell configuration, electronic compensation was used to minimize the effective series resistance (usually to  $< 1 \text{ M}\Omega$ ) and the time required to charge the cell capacitance (usually to  $< 0.5 \text{ ms}$ ). The steady holding potential (HP) was  $-80 \text{ mV}$ . Test pulses were delivered at 5-s intervals and each test pulse was immediately preceded by a 1-s prepulse to  $-30 \text{ mV}$  to inactivate endogenous T-type  $\text{Ca}^{2+}$  current (Beam et al., 1986). Linear membrane capacitance and leakage currents were measured for each myotube during 10 voltage steps from  $-80$  to  $-100 \text{ mV}$ ; these control currents (which directly preceded test currents) were averaged, scaled appropriately, and subtracted from test currents.  $\text{Ca}^{2+}$  current densities (expressed in  $\text{pA/pF}$ ) were calculated for each myotube by dividing the leak- and capacitance-corrected test currents by whole-cell linear capacitance. The time constants for activation ( $\tau_a$ ) and inactivation ( $\tau_i$ ) of expressed  $\text{Ca}^{2+}$  currents were derived by fitting single exponential functions to activating or inactivating segments, respectively, of test currents recorded near the peak of the current-voltage ( $I$ - $V$ ) relation. Maximal  $\text{Ca}^{2+}$  conductance ( $G_{\text{max}}$ ) was calculated for individual myotubes by fitting measured values of inward current according to the expression

$$I = G_{\text{max}}(V - V_{\text{rev}})/[1 + \exp\{-(V - V_G)/k_G\}]$$

where  $I$  is the peak BI current activated at test potential  $V$ ,  $V_{\text{rev}}$  is the extrapolated reversal potential,  $V_G$  is the potential for activation of half-maximal conductance, and  $k_G$  is a slope factor.

Intramembrane charge movements were recorded after the addition of 0.5 mM  $\text{Cd}^{2+}$  and 0.1 mM  $\text{La}^{3+}$  to the external solution; this combination of  $\text{Cd}^{2+}$  and  $\text{La}^{3+}$  effectively blocked ionic  $\text{Ca}^{2+}$  currents. To isolate intramembrane charge movements (gating currents) arising from the expressed BI  $\text{Ca}^{2+}$  channels, we employed a voltage protocol modified from Bean and Rios (1989). With this protocol, membrane potential was stepped from the holding potential ( $-80$  mV) to  $-30$  mV for 1 s (the prepulse), then to  $-50$  mV for 20–30 ms (the pedestal), and finally to a variable test potential. As previously demonstrated (Adams et al., 1990), this voltage protocol inactivates endogenous  $\text{Na}^+$  current and T-type  $\text{Ca}^{2+}$  current and immobilizes a component of intramembrane charge movement, presumably that arising from these channels. Importantly, however, this voltage protocol had no effect on ionic  $\text{Ca}^{2+}$  currents produced by expression of the BI channels (see Fig. 4 B). The “immobilization-resistant” intramembrane charge movements recorded with this voltage protocol were corrected for linear leakage and capacitive currents by subtracting an averaged, appropriately scaled control current, obtained during 10 voltage steps between  $-80$  and  $-140$  mV. To prevent amplifier saturation, voltage clamp command pulses were exponentially rounded with a time constant of 50–300  $\mu\text{s}$ . For a given test pulse, the amount of charge that moved outward after the onset of the test pulse ( $Q_{\text{on}}$ ) was obtained by integration.  $Q_{\text{max}}$ , the maximum amount of charge that could be moved, was taken as  $Q_{\text{on}}$  for a test pulse to  $+30$  or  $+40$  mV. Maximum charge movement density (expressed in  $\text{nC}/\mu\text{F}$ ) was calculated by dividing  $Q_{\text{max}}$  for each cell by that cell's linear capacitance.

Patch pipettes were filled with a solution containing (in millimolar) 140 Cs-Aspartate, 10 HEPES, 10  $\text{Cs}_2\text{EGTA}$ , 5  $\text{MgCl}_2$ ; pH 7.4 with CsOH. When immersed in the external solution, filled pipettes had resistances of 1.5–2.1  $\text{M}\Omega$ . The external solution contained (in millimolar) 145 tetraethylammonium (TEA) chloride, 10 HEPES, 10  $\text{CaCl}_2$  (or 10  $\text{BaCl}_2$  where indicated), 0.003 tetrodotoxin; pH 7.4 with TEA-OH. External solution containing 100  $\mu\text{M}$   $\text{CdCl}_2$  was applied via a puffer pipette positioned within 500  $\mu\text{m}$  of the cell. (+)-PN 200–110 (a gift from Drs. A. Lindenmann and E. Rossi of Sandoz Ltd., Basel, Switzerland) was applied by bulk exchange of the external solution. Before examination of the effects of (+)-PN 200–110, control currents were recorded after flushing the dish ( $\sim 1$  ml vol) with 10–15 ml of drug-free solution to control for perfusion effects on  $\text{Ca}^{2+}$  currents. Test currents were subsequently recorded after flushing the dish with 20–30 ml of solution containing 1 or 10  $\mu\text{M}$  (+)-PN 200–110. (+)-PN 200–110 was prepared as a stock (10 mM in 100% ethanol) which was stored in the dark at  $-20^\circ\text{C}$ ; aliquots of this stock were diluted into the external solution immediately before use.

All experiments were performed at room temperature ( $20$ – $23^\circ\text{C}$ ). Numerical values presented in the text are mean  $\pm$  SEM with the number of observations in parentheses.

## RESULTS

Dysgenic myotubes injected with pKCRCBI-1 or pKCRCBI-2 failed to display spontaneous or evoked contractions (see below). However, when we methodically patch clamped every myotube within the injected regions of the culture dishes, we found that  $\sim 10\%$  expressed a high-density  $\text{Ca}^{2+}$  current. Fig. 1 illustrates a representative example of this current, which presumably was mediated by the expressed BI  $\text{Ca}^{2+}$  channels. The current activated rapidly with a time constant ( $\tau_a$ ) of  $2.3 \pm 0.1$  ms ( $n = 38$ ). The time course of inactivation (measured during a 1-s depolarization) was much slower, having a time constant ( $\tau_h$ ) of  $258 \pm 32$  ms ( $n = 6$ ).

With 10 mM Ca<sup>2+</sup> as the charge carrier, the expressed BI Ca<sup>2+</sup> current first appeared in response to test pulses to near -10 mV and peaked in response to test pulses to near +20 mV (Fig. 1B). Because the properties of the expressed macroscopic currents were not different between myotubes injected with pKCRCBI-1 or pKCRCBI-2, results obtained with these two expression plasmids have been combined.

The expressed current was mediated by Ca<sup>2+</sup> channels, as demonstrated by its rapid and reversible block by 100 μM Cd<sup>2+</sup> (Fig. 2A). Furthermore, the BI Ca<sup>2+</sup>

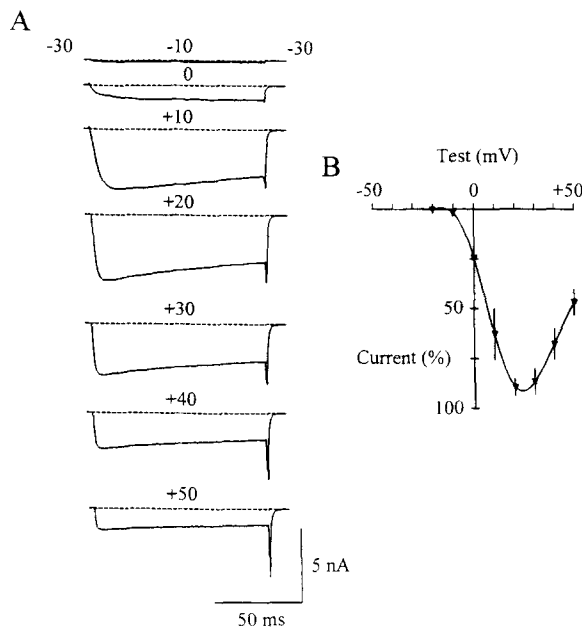


FIGURE 1. Kinetics and voltage dependence of BI Ca<sup>2+</sup> currents expressed in dysgenic myotubes. (A) Representative BI currents. Test pulses to various potentials were immediately preceded by a 1-s prepulse to -30 mV in order to inactivate endogenous T-type Ca<sup>2+</sup> current. Steady holding potential (HP) was -80 mV, and the charge carrier was 10 mM Ca<sup>2+</sup>. Pretest, test, and repolarization potentials are all indicated above the current traces. The peak density of BI current recorded from this myotube was 28 pA/pF (measured at a test potential of +20 mV). The maximum calculated voltage error ( $V_{err}$ ; calculated as the product of peak inward cur-

rent and compensated series resistance) was 4.9 mV. Dysgenic myotube BT93, injected with pKCRCBI-2; linear capacitance ( $C$ ) = 160 pF. (B) Average ( $\pm$ SEM) current-voltage ( $I$ - $V$ ) relationship of BI Ca<sup>2+</sup> currents expressed in six dysgenic myotubes. Current amplitudes in each cell were normalized by the maximum current amplitude recorded from that cell. In these six myotubes, the average density of BI current was  $17.5 \pm 2.2$  pA/pF and the maximum  $V_{err}$  was <5 mV.  $G_{max}$  for these six cells was  $378 \pm 40$  nS/ $\mu$ F; the potential which activated half-maximal BI Ca<sup>2+</sup> conductance ( $V_C$ ) was  $10 \pm 3$  mV ( $n = 6$ ), and the steepness factor ( $k_C$ ) was  $4.2 \pm 0.6$  mV ( $n = 6$ ). Steady HP was -80 mV. All test pulses were immediately preceded by a 1-s prepulse to -30 mV. The charge carrier was 10 mM Ca<sup>2+</sup>.

current was clearly distinguished from the small, endogenous L-type Ca<sup>2+</sup> current ( $I_{dys}$ ) of dysgenic skeletal muscle (Adams and Beam, 1989) by its insensitivity to 1 μM (+)-PN 200-110, a potent dihydropyridine Ca<sup>2+</sup> channel antagonist ( $n = 9$ ; Fig. 2B). A higher concentration (10 μM) of (+)-PN 200-110 also failed to inhibit the expressed BI Ca<sup>2+</sup> current ( $n = 4$ ; not shown). In 8 of 13 myotubes examined, (+)-PN 200-110 appeared to slightly potentiate the expressed BI current; however,

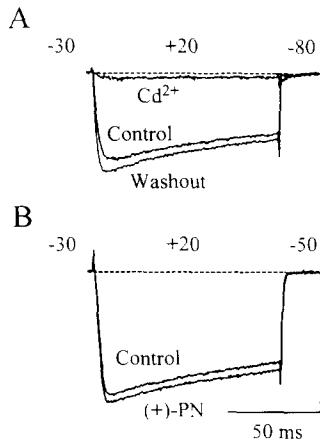


FIGURE 2. Effect of  $\text{Ca}^{2+}$  channel blockers on expressed BI  $\text{Ca}^{2+}$  currents. (A) BI current is reversibly blocked by  $\text{Cd}^{2+}$ . Maximal currents were recorded before (Control), during exposure to  $100 \mu\text{M}$   $\text{Cd}^{2+}$  and after washout (Washout). Cell BV65,  $C = 170 \text{ pF}$ . (B) BI current is not inhibited by  $1 \mu\text{M}$  (+)-PN 200–110. Maximal currents were recorded for a control solution exchange (Control) and during exposure to the drug ([+]-PN). Cell BV95,  $C = 210 \text{ pF}$ . Pretest, test, and repolarization potentials are indicated above the current traces. Vertical calibration is  $2.5 \text{ nA}$  (A) and  $2.0 \text{ nA}$  (B).

this effect could not reliably be distinguished from current “run up” which was typically observed in our experiments.

To identify the mechanisms underlying inactivation of the BI current, we replaced all  $\text{Ca}^{2+}$  in the external solution with equimolar  $\text{Ba}^{2+}$ . For these experiments,  $1 \mu\text{M}$  (+)-PN 200–110 was included in all external solutions to block any endogenous  $I_{\text{dys}}$  that might have been present. As shown in Fig. 3, substituting  $\text{Ba}^{2+}$  for  $\text{Ca}^{2+}$  as charge carrier substantially increased the peak amplitude of the expressed BI current (by  $57 \pm 7\%$ ;  $n = 8$ ) without decreasing the rate of current inactivation. Substituting  $\text{Ba}^{2+}$  for  $\text{Ca}^{2+}$  also shifted the current-voltage relationship to more negative potentials by  $\sim 10 \text{ mV}$  ( $n = 8$ ; not shown).

To examine the ability of the expressed BI  $\text{Ca}^{2+}$  channels to generate intramembrane charge movement, we used a prepulse protocol (see Methods) that effectively isolates “immobilization-resistant” charge movements arising from high-voltage-activated, slowly-inactivating channels (Adams et al., 1990). The upper panel in Fig. 4A illustrates immobilization-resistant charge movement recorded from a BI-expressing dysgenic myotube after the addition of  $\text{Cd}^{2+}$  and  $\text{La}^{3+}$  to block all ionic  $\text{Ca}^{2+}$  currents. The immobilization-resistant charge movement in this cell was little different from that recorded from a typical control dysgenic myotube (Fig. 4A, bottom). In 16 BI-injected myotubes that expressed a peak  $\text{Ca}^{2+}$  current of  $31 \pm 4$

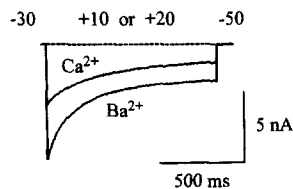


FIGURE 3. BI  $\text{Ca}^{2+}$  channels expressed in dysgenic myotubes do not exhibit  $\text{Ca}^{2+}$ -dependent inactivation. Shown are maximal BI currents recorded with either  $\text{Ca}^{2+}$  or  $\text{Ba}^{2+}$  as charge carrier. The time constants of inactivation ( $\tau_{\text{h}}$ ) for the currents shown were  $278 \text{ ms}$  with  $10 \text{ mM}$   $\text{Ca}^{2+}$  in the external

solution (peak current elicited by a test pulse to  $+20 \text{ mV}$ ) and  $183 \text{ ms}$  with  $10 \text{ mM}$   $\text{Ba}^{2+}$  in the external solution (peak current elicited by a test pulse to  $+10 \text{ mV}$ ). Dysgenic myotube BV95, injected with pKRCBI-2;  $C = 210 \text{ pF}$ . Pretest, test, and repolarization potentials are indicated above the current traces. (+)-PN 200–110 ( $1 \mu\text{M}$ ) was present in the external solutions throughout the experiment, in order to block endogenous  $I_{\text{dys}}$ .

pA/pF ( $G_{\max}$  of  $534 \pm 51 \mu\text{S}/\mu\text{F}$ ), the maximum, immobilization-resistant charge movement ( $Q_{\max}$ ) averaged  $2.77 \pm 0.21 \text{ nC}/\mu\text{F}$ . For comparison,  $Q_{\max}$  in noninjected dysgenic myotubes was  $2.33 \pm 0.24 \text{ nC}/\mu\text{F}$  ( $n = 14$ ). Possibly, some BI channel gating charge might have been immobilized by the prepulse to  $-30 \text{ mV}$  that was a part of the voltage protocol used. However, as shown in Fig. 4 B, the BI Ca<sup>2+</sup> current elicited with the prepulse protocol was indistinguishable from that elicited by a voltage step applied directly from the holding potential. Thus, it appears that expression of BI channels in dysgenic myotubes results in a high density of ionic Ca<sup>2+</sup> channel current but very little immobilization-resistant charge movement.

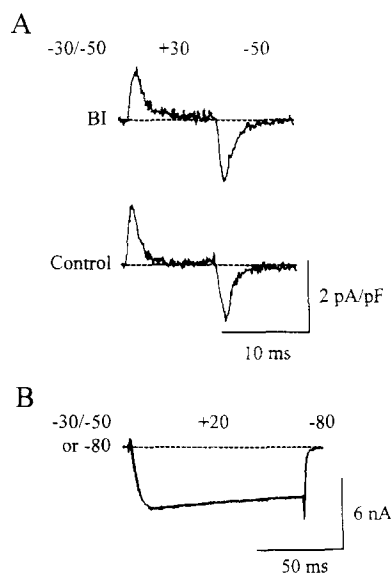


FIGURE 4. Expression of BI Ca<sup>2+</sup> channels produces very little immobilization-resistant intramembrane charge movement. (A) Non-linear charge movement in response to a strong depolarization in a dysgenic myotube expressing BI Ca<sup>2+</sup> channels (BI) and in a noninjected dysgenic myotube (Control). The maximum Ca<sup>2+</sup> current recorded from the myotube expressing BI channels was 38 pA/pF (elicited by a test pulse to +10 mV) and  $Q_{\max}$  in this same myotube was 2.7 nC/ $\mu\text{F}$  (test pulse to +30 mV).  $Q_{\max-dys}$  recorded from the control dysgenic myotube was 2.5 nC/ $\mu\text{F}$  (test pulse to +30 mV). BI: dysgenic myotube BK13, injected with pKCRCBI-1;  $C = 280 \text{ pF}$ . Control: noninjected dysgenic myotube BL71;  $C = 110 \text{ pF}$ . Pretest, test, and repolarization potentials are indicated above the current traces. (B) Effect of the voltage protocol used to measure immobilization-resistant charge movement on ionic Ca<sup>2+</sup> current through the BI channel.

Shown are two superimposed current traces recorded using either the standard voltage protocol (test pulse to +20 mV delivered directly from the steady HP of  $-80 \text{ mV}$ ) or the prepulse protocol (a 1-s step to  $-30 \text{ mV}$  followed by a 25-ms step to  $-50 \text{ mV}$  preceded the test pulse to +20 mV). Cell BV93,  $C = 260 \text{ pF}$ .

Dysgenic myotubes injected with BI channel cDNA were never observed to contract spontaneously, and evoked contractions were extremely rare. Out of more than 300 injected myotubes that were electrically stimulated via an extracellular pipette, only three myotubes displayed evoked contractions. In these three contracting myotubes, whole-cell patch clamp recordings subsequently revealed exceptionally high Ca<sup>2+</sup> current densities ( $> 50 \text{ pA}/\text{pF}$ ). Many other BI-injected myotubes which did not display evoked contractions expressed lower, but still quite significant densities of BI Ca<sup>2+</sup> current (up to  $\sim 40 \text{ pA}/\text{pF}$ ). To identify the type of E-C coupling mediated in these rare instances by the expressed BI channels, we simultaneously recorded Ca<sup>2+</sup> currents and monitored contractions in one of the few BI-expressing myotubes that displayed evoked contractions. Whole-cell Ca<sup>2+</sup> currents were recorded from this cell

using a pipette solution in which the EGTA concentration was reduced from the usual 10 to 0.1 mM, a concentration which does not prevent contraction of normal myotubes or of dysgenic myotubes expressing the cardiac DHP receptor (Garcia, Tanabe, and Beam, 1994). In this particular myotube, a test depolarization to +20 mV elicited a large inward  $\text{Ca}^{2+}$  current and a corresponding contraction. When 100  $\mu\text{M}$   $\text{Cd}^{2+}$  was added to the external solution, the  $\text{Ca}^{2+}$  current was effectively blocked and the contractions were abolished (not shown). Washout of the  $\text{Cd}^{2+}$  completely restored the original density of the  $\text{Ca}^{2+}$  current and also restored the evoked contractions. This experiment indicates that in the rare instances where expression of the BI channel did restore E-C coupling, the coupling was of cardiac-type in that it required the entry of external  $\text{Ca}^{2+}$ .

#### DISCUSSION

The results presented here demonstrate several interesting features of the neuronal BI  $\text{Ca}^{2+}$  channel. When heterologously expressed in dysgenic myotubes, BI channels produce a high-density  $\text{Ca}^{2+}$  conductance which, surprisingly, almost never restores E-C coupling. Furthermore, the large  $\text{Ca}^{2+}$  conductance produced by the expressed BI channels is associated with a very low density of intramembrane charge movement, indicating that the BI channels exhibit a relatively large open probability. These findings are considered in more detail in the following paragraphs.

BI channels have previously been expressed heterologously in *Xenopus* oocytes (Mori et al., 1991; Sather et al., 1993). However, to the best of our knowledge, the present work constitutes the first report of heterologous expression of the neuronal BI  $\text{Ca}^{2+}$  channels in mammalian cells. In the previous studies, Mori et al. (1991) used 40 mM  $\text{Ba}^{2+}$  as charge carrier, whereas Sather et al. (1993) used either 2 or 40 mM  $\text{Ba}^{2+}$  as charge carrier. In contrast, in the present study we used either 10 mM  $\text{Ba}^{2+}$  or 10 mM  $\text{Ca}^{2+}$  as charge carrier because we wished to compare our results for the BI channels with those previously obtained for L-type channels expressed in dysgenic myotubes. Although differences in concentration and ionic species of charge carrier could easily affect the kinetics and voltage dependence of the expressed  $\text{Ca}^{2+}$  channels, it appears that BI channels expressed in dysgenic myotubes behave in a qualitatively similar fashion to BI channels expressed in *Xenopus* oocytes, despite the differences in experimental conditions used for the two systems. In both cell types, the BI currents activate within a few milliseconds, inactivate within a few hundred milliseconds, and are insensitive to DHP antagonists.

Perhaps the most significant result of the present study is that BI channels expressed in dysgenic myotubes produce a large  $\text{Ca}^{2+}$  conductance, but a low density of immobilization-resistant charge movement. If it is assumed that the immobilization-resistant charge movement observed in noninjected dysgenic myotubes is present as a contaminant of the charge movement recorded from BI-injected dysgenic myotubes, then the difference between the average  $Q_{\text{max}}$  of BI-injected myotubes (2.77 nC/ $\mu\text{F}$ ) and noninjected dysgenic myotubes (2.33 nC/ $\mu\text{F}$ ) provides an estimate of the charge movement ( $Q_{\text{max}}$ ) attributable to the expressed BI channels. We and others have previously used the ratio  $G_{\text{max}}/Q_{\text{max}}$  as a measure of the relative ability of expressed DHP receptors to function as L-type  $\text{Ca}^{2+}$  channels (Adams et al., 1990; Beam, Adams, Niidome, Numa, and Tanabe, 1992; Neely, Wei,



Olcese, Birnbaumer, and Stefani, 1993). In an earlier study (Adams et al., 1990), we found that dysgenic myotubes expressing cardiac L-type Ca<sup>2+</sup> channels had a  $G_{\max}$  (488  $\mu\text{S}/\mu\text{F}$ ) very similar to that found in the present study for dysgenic myotubes expressing BI channels (534  $\mu\text{S}/\mu\text{F}$ ; see Results). In contrast, for dysgenic myotubes expressing cardiac L-type channels,  $Q_{\max}'$  was 8.9 nC/ $\mu\text{F}$ , a value much higher than  $\sim 0.44$  nC/ $\mu\text{F}$  for  $Q_{\max}'$  in dysgenic myotubes expressing BI channels. Consequently, the  $G_{\max}/Q_{\max}'$  ratio was much smaller for the cardiac L-type channels (55 nS/pC) than for the neuronal BI channels ( $> 1,000$  nS/pC). If  $G_{\max} = n\gamma P_0$  and  $Q_{\max}' = nq$ , where  $n$  is the number of channels,  $\gamma$  is the single-channel conductance,  $P_0$  is the maximum open probability, and  $q$  is the gating charge of a single channel, then  $G_{\max}/Q_{\max}' = \gamma P_0/q$ . Further, if the ratio  $\gamma/q$  is roughly constant, then  $G_{\max}/Q_{\max}'$  is directly proportional to  $P_0$ . In fact,  $\gamma$  appears to be smaller for BI than for cardiac L-type channels; with 110 mM barium as charge carrier,  $\gamma$  for BI channels expressed in *Xenopus* oocytes is  $\sim 16$  pS, whereas  $\gamma$  for cardiac L-type channels is  $\sim 21$  pS (Mori et al., 1991; Sather et al., 1993). Thus, the higher  $G_{\max}/Q_{\max}'$  ratio found for the BI channels suggests that they exhibit a considerably higher  $P_0$  than cardiac L-type channels.

The actual  $P_0$  for BI channels expressed in dysgenic myotubes can be estimated by the following calculation. If we assume that  $\gamma = 4$  pS (with 10 mM Ca<sup>2+</sup> as charge carrier, estimated using results presented in Gollasch, Hescheler, Quayle, Patlak, and Nelson, 1992), and  $q = 12$  electronic charges ( $e^-$ ; Schoppa, McCormack, Tanouye, and Sigworth, 1992; Zagotta, Hoshi, Dittman, and Aldrich, 1994), then multiplying  $4 \times 10^{-12}$  S/(12  $e^-$ ) by Avogadro's number ( $6.023 \times 10^{23}$   $e^-/\text{mol}$ ) and Faraday's constant (96,487 C/mol) yields a conductance to charge ratio of  $\sim 2.0 \times 10^6$  S/C; note that this value applies only to the special condition where the channels are always open, i.e.,  $P_0 = 1.0$ , and furthermore this value is independent of the number of channels in the membrane. Multiplying  $2.0 \times 10^6$  S/C by the estimated density of gating charge movement attributable to the expressed BI channels (0.44 nC/ $\mu\text{F}$ ) yields a conductance to capacitance ratio of 880 S/F, which is  $\sim 1.65$  times that calculated for BI channels in the present study (534 S/F; see Results). Thus, the  $P_0$  of the expressed BI Ca<sup>2+</sup> channels would have been  $\sim 0.6$  in order to produce the measured conductance to capacitance ratio of 534 S/F; this value of  $P_0$  is considerably higher than  $P_0$  for L-type Ca<sup>2+</sup> channels in cardiac cells (0.03; Lew et al., 1991), but is quite comparable to  $P_0$  for N-type channels in neurons of the frog sympathetic ganglion (0.5; Delcour and Tsien, 1993). It is interesting to speculate that a relatively high  $P_0$  may be a general feature of neuronal, non-L-type Ca<sup>2+</sup> channels, whereas a relatively low  $P_0$  may be a general feature of muscle L-type Ca<sup>2+</sup> channels.

Our present results demonstrate that BI channels expressed in dysgenic myotubes do not exhibit Ca<sup>2+</sup>-dependent inactivation (Fig. 3). This finding is in agreement with that of Sather et al. (1993) for BI channels expressed in cell-attached patches from *Xenopus* oocytes. The absence of Ca<sup>2+</sup>-dependent inactivation may be an intrinsic property of the BI channel protein (the  $\alpha 1$  subunit), or alternatively may reflect the absence in both dysgenic myotubes and *Xenopus* oocytes of ancillary proteins required for Ca<sup>2+</sup>-dependent inactivation. The latter possibility is suggested by previous results (Tanabe, Mikami, Numa, and Beam, 1990) demonstrating that cardiac L-type Ca<sup>2+</sup> channels, which display prominent Ca<sup>2+</sup>-dependent inactivation in their native

cellular environment (cardiac myocytes), do not exhibit prominent  $\text{Ca}^{2+}$ -dependent inactivation when heterologously expressed in dysgenic myotubes.

The dysgenic myotube expression system makes it possible to test the ability of different types of  $\text{Ca}^{2+}$  channels to mediate E-C coupling. We found that myotubes expressing BI  $\text{Ca}^{2+}$  channels almost never showed restored E-C coupling. Thus, only ~1% of dysgenic myotubes injected with BI channel cDNA displayed evoked contractions, whereas E-C coupling was restored in ~17% of dysgenic myotubes injected with cDNA encoding the cardiac L-type  $\text{Ca}^{2+}$  channel (Tanabe et al., 1990). This difference in restored E-C coupling is surprising because the average current densities are comparable in dysgenic myotubes expressing the BI (~30 pA/pF) and cardiac (~28 pA/pF)  $\text{Ca}^{2+}$  channels. It seems noteworthy that heterologous expression of neuronal BIII (N-type)  $\text{Ca}^{2+}$  channels also fails to restore E-C coupling to dysgenic myotubes (Fujita et al., 1993). Why do cardiac and skeletal muscle L-type  $\text{Ca}^{2+}$  channels usually restore E-C coupling in dysgenic myotubes, whereas neuronal BI and BIII  $\text{Ca}^{2+}$  channels do not? One possibility, suggested by the low densities of intramembrane charge movement recorded from myotubes expressing the BI channels, is that so few BI channels are present in the membrane that even if they are directly apposed to the  $\text{Ca}^{2+}$  release channels of the sarcoplasmic reticulum (SR) they cannot activate enough of the release channels to cause contraction. Alternatively, the expressed BI and BIII channels may not become localized sufficiently close to the SR release channels of the sarcoplasmic reticulum (SR), so that even significant entry of  $\text{Ca}^{2+}$  via these neuronal channels is unable to trigger SR  $\text{Ca}^{2+}$  release effectively. Our present results with the BI channel are consistent with the possibility that these channels become targetted to different regions of dysgenic myotubes than exogenously expressed L-type  $\text{Ca}^{2+}$  channels. Future studies will be necessary to determine the subcellular localization of different types of  $\text{Ca}^{2+}$  channels and to elucidate the mechanisms underlying channel localization.

We acknowledge the support of this work by the late Professor Shosaku Numa.

This research was supported by the Ministry of Education, Science and Culture of Japan, the Japanese Foundation of Metabolism and Diseases and by the United States National Institutes of Health (NS 24444 to K. G. Beam). B. A. Adams was supported by a postdoctoral fellowship from the American Heart Association (Colorado Affiliate) during the initial stages of this project.

*Original version received 4 March 1994 and accepted version received 7 June 1994.*

#### REFERENCES

- Adams, B. A., and K. G. Beam. 1989. A novel  $\text{Ca}^{2+}$  current in dysgenic skeletal muscle. *Journal of General Physiology*. 94:429–444.
- Adams, B. A., T. Tanabe, A. Mikami, S. Numa, and K. G. Beam. 1990. Intramembrane charge movement restored in dysgenic skeletal muscle by injection of dihydropyridine receptor cDNAs. *Nature*. 346:569–572.
- Adams, B. A., T. Tanabe, Y. Mori, and K. G. Beam. 1994. Heterologous expression of neuronal BI  $\text{Ca}^{2+}$  channels in dysgenic skeletal muscle. *Biophysical Journal*. 66:A17. (Abstr.)
- Beam, K. G., B. A. Adams, T. Niidome, S. Numa, and T. Tanabe. 1992. Function of a truncated dihydropyridine receptor as both voltage sensor and  $\text{Ca}^{2+}$  channel. *Nature*. 360:169–171.

- Beam, K. G., C. M. Knudson, and J. A. Powell. 1986. A lethal mutation in mice eliminates the slow Ca<sup>2+</sup> current in skeletal muscle cells. *Nature*. 320:168–170.
- Bean, B. P., and E. Rios. 1989. Nonlinear charge movement in mammalian cardiac ventricular cells. *Journal of General Physiology*. 94:65–93.
- Delcour, A. H., and R. W. Tsien. 1993. Altered prevalence of gating modes in neurotransmitter inhibition of N-type Ca<sup>2+</sup> channels. *Science*. 259:980–984.
- Dirksen, R. T., T. Tanabe, and K. Beam. 1993. Single-channel analysis of native and expressed skeletal muscle Ca<sup>2+</sup> channels. *Biophysical Journal*. 64:A6. (Abstr.)
- Fujita, Y., M. Mynlieff, R. T. Dirksen, M.-S. Kim, T. Niidome, J. Nakai, T. Friedrich, N. Iwabe, T. Miyata, T. Furuichi, D. Furutama, K. Mikoshiba, Y. Mori and K. G. Beam. 1993. Primary structure and functional expression of the  $\omega$ -conotoxin-sensitive N-type Ca<sup>2+</sup> channel from rabbit brain. *Neuron*. 10:585–598.
- Garcia, J., T. Tanabe, and K. G. Beam. 1994. Relationship of Ca<sup>2+</sup> transients to Ca<sup>2+</sup> currents and charge movements in myotubes expressing skeletal and cardiac DHP receptors. *Journal of General Physiology*. 103:125–147.
- Gollasch, M., J. Hescheler, J. M. Quayle, J. B. Patlak, and M. T. Nelson. 1992. Single calcium channel currents of arterial smooth muscle at physiological calcium concentrations. *American Journal of Physiology*. 263(*Cell Physiology* 32):C948–C952.
- Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv*. 391:85–100.
- Hillyard, D. R., V. D. Monje, I. M. Mintz, B. P. Bean, L. Nadasdi, J. Ramachandran, G. Miljanich, A. Azimi-Zoonooz, J. M. McIntosh, L. J. Cruz, J. S. Imperial, and B. M. Olivera. 1992. A new *Conus* peptide ligand for mammalian presynaptic Ca<sup>2+</sup> channels. *Neuron*. 9:69–77.
- Lew, W. Y. W., L. V. Hryshko, and D. M. Bers. 1991. Dihydropyridine receptors are primarily functional L-type Ca<sup>2+</sup> channels in rabbit ventricular myocytes. *Circulation Research*. 69:1139–1145.
- Llinas, R., M. Sugimori, J. W. Lin, and B. Cherksey. 1989. Blocking and isolation of a Ca<sup>2+</sup> channel from neurons in mammals and cephalopods utilizing a toxin fraction (FTX) from funnel-web spider poison. *Proceedings of the National Academy of Sciences, USA*. 86:1689–1693.
- Ma, J., C. Mundina-Weilenmann, M. M. Hosey, and E. Rios. 1991. Dihydropyridine-sensitive skeletal muscle Ca channels in polarized planar bilayers. *Biophysical Journal*. 60:890–901.
- Mejia-Alvarez, R., M. Fill, and E. Stefani. 1991. Voltage-dependent inactivation of T-tubular skeletal Ca<sup>2+</sup> channels in planar lipid bilayers. *Journal of General Physiology*. 97:393–412.
- Mintz, I. M., V. J. Venema, K. M. Swiderek, T. D. Lee, B. P. Bean, and M. E. Adams. 1992. P-type Ca<sup>2+</sup> channels blocked by the spider toxin  $\omega$ -Aga-IVA. *Nature*. 355:827–829.
- Mishina, M., T. Kurosaki, T. Tobimatsu, Y. Morimoto, M. Noda, T. Yamamoto, M. Terao, J. Lindstrom, T. Takahashi, M. Kuno, and S. Numa. 1984. Expression of functional acetylcholine receptor from cloned cDNAs. *Nature*. 307:604–608.
- Mori, Y., T. Friedrich, M.-S. Kim, A. Mikami, J. Nakai, P. Ruth, E. Bosse, F. Hofmann, V. Flockerzi, T. Furuichi, K. Mikoshiba, K. Imoto, T. Tanabe, and S. Numa. 1991. Primary structure and functional expression from complementary DNA of a brain Ca<sup>2+</sup> channel. *Nature*. 350:398–402.
- Mori, Y., T. Niidome, Y. Fujita, M. Mynlieff, R. T. Dirksen, K. G. Beam, N. Iwabe, T. Miyata, D. Furutama, T. Furuichi, and K. Mikoshiba. 1993. Molecular diversity of voltage-dependent calcium channel. *Annals of the New York Academy of Sciences*. 707:87–108.
- Neely, A., X. Wei, R. Olcese, L. Birnbaumer, and E. Stefani. 1993. Potentiation by the  $\beta$  subunit of the ratio of the ionic current to the charge movement in the cardiac Ca<sup>2+</sup> channel. *Science*. 262:575–578.

- Ono, K., and H. Fozzard. 1992. Phosphorylation restores activity of L-type  $\text{Ca}^{2+}$  channels after rundown in inside-out patches from rabbit cardiac cells. *Journal of Physiology*. 454:673–688.
- Powell, J. A., and D. M. Fambrough. 1973. Electrical properties of normal and dysgenic mouse skeletal muscle in culture. *Journal of Cell Physiology*. 82:21–38.
- Regan, L. J., D. W. Y. Sah, and B. P. Bean. 1991.  $\text{Ca}^{2+}$  channels in rat central and peripheral neurons: high-threshold current resistant to dihydropyridine blockers and  $\omega$ -conotoxin. *Neuron*. 6:269–280.
- Rios, E., and G. Pizarro. 1991. Voltage sensor of excitation-contraction coupling in skeletal muscle. *Physiological Reviews*. 71:849–908.
- Sather, W. A., T. Tanabe, J.-F. Zhang, Y. Mori, M. E. Adams, and R. W. Tsien. 1993. Distinctive biophysical and pharmacological properties of class A (BI)  $\text{Ca}^{2+}$  channel  $\alpha 1$  subunits. *Neuron*. 11:291–303.
- Schoppa, N. E., K. McCormack, M. A. Tanouye, and F. J. Sigworth. 1992. The size of gating charge in wild-type and mutant *Shaker* potassium channels. *Science*. 255:1712–1715.
- Tanabe, T., A. Mikami, S. Numa, and K. G. Beam. 1990. Cardiac-type excitation-contraction coupling in dysgenic skeletal muscle injected with cardiac dihydropyridine receptor cDNA. *Nature*. 344:451–453.
- Tanabe, T., K. G. Beam, J. A. Powell, and S. Numa. 1988. Restoration of excitation-contraction coupling and slow  $\text{Ca}^{2+}$  current in dysgenic muscle by dihydropyridine receptor complementary DNA. *Nature*. 336:134–139.
- Tanabe, T., A. Mikami, T. Niidome, S. Numa, B. A. Adams, and K. G. Beam. 1993. Structure and function of voltage-dependent  $\text{Ca}^{2+}$  channels from muscle. *Annals of the New York Academy of Sciences*. 707:81–86.
- Tsien, R. W., P. T. Ellinor, and W. A. Horne. 1991. Molecular diversity of voltage-dependent  $\text{Ca}^{2+}$  channels. *Trends in Physiological Sciences*. 12:349–354.
- Wheeler, D. B., A. Randall, and R. W. Tsien. 1994. Roles of N-type and Q-type  $\text{Ca}^{2+}$  Channels in supporting hippocampal synaptic transmission. *Science*. 264:107–111.
- Zagotta, W. N., T. Hoshi, J. Dittman, and R. W. Aldrich. 1994. *Shaker* potassium channel gating II: Transitions in the activation pathway. *Journal of General Physiology*. 103:279–319.
- Zhang, J.-F., A. D. Randall, P. T. Ellinor, W. A. Horne, W. A. Sather, T. Tanabe, T. L. Schwarz, and R. W. Tsien. 1993. Distinctive pharmacology and kinetics of cloned neuronal  $\text{Ca}^{2+}$  channels and their possible counterparts in mammalian CNS neurons. *Neuropharmacology*. 32:1075–1088.