Schistosome Calcium Channel $\beta$ Subunits

UNUSUAL MODULATORY EFFECTS AND POTENTIAL ROLE IN THE ACTION OF THE ANTI SCHISTOSOMAL DRUG PRAZIQUANTEL

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Andrea A. Kohn‡, Peter A. V. Anderson§.§, Jessica M. Roberts-Misterley§, and Robert M. Greenberg¶¶
From the Whitney Laboratory and the Departments of Neuroscience and Physiology, University of Florida, St. Augustine, Florida 32080

Schistosomes are parasitic flatworms that cause schistosomiasis, a major tropical disease. The current drug of choice against schistosomiasis is praziquantel (PZQ), which has minimal side effects and is potent against all schistosome species. The mode of action of PZQ is unknown, though the drug clearly affects Ca$^{2+}$ homeostasis in worms, and there is indirect evidence for interaction of PZQ with schistosome voltage-gated Ca$^{2+}$ channels. We have cloned and expressed two Ca$^{2+}$ channel $\beta$ subunits, one from Schistosoma mansoni and one from Schistosoma japonicum. These two subunits (SmCa$\beta_1$A and SjCa$\beta_1$) have structural motifs that differ from those found in other known $\beta$ subunits. Surprisingly, coexpression of either SmCa$\beta_1$A or SjCa$\beta_1$ with a cnidarian (CvCa$1$) or mammalian (Ca$2.3$) Ca$^{2+}$ channel $\alpha_1$ subunit results in a striking reduction in current amplitude. In the case of Ca$2.3$, this current reduction can be partially reversed by addition of 100 nM PZQ, which results in a significant increase in current amplitude. Thus, these unusual schistosome $\beta$ subunits can confer PZQ sensitivity to an otherwise PZQ-insensitive mammalian Ca$^{2+}$ channel, indicating that a possible target for PZQ action is the interaction between $\beta$ subunits and pore-forming $\alpha_1$ subunits in schistosomes.

Praziquantel (PZQ) is the drug of choice against schistosomiasis, a debilitating disease caused by parasitic flatworms of the genus Schistosoma. The mode of action of PZQ is not known, although initial effects of the drug include a rapid influx of calcium (Ca$^{2+}$) into the worm and a Ca$^{2+}$-dependent muscle contraction and paralysis (reviewed in Refs. 1–3). Voltage-gated Ca$^{2+}$ channels represent a possible site of action for this drug, but this hypothesis has not been tested directly because of the inability to date to record robust Ca$^{2+}$ currents from schistosomes.

Voltage-gated Ca$^{2+}$ channels are heteromultimeric membrane protein complexes consisting of a pore-forming, voltage-sensing $\alpha_1$ subunit that is modulated by $\beta, \alpha_\delta$, and other auxiliary subunits (reviewed in Ref. 4). Previously we have cloned three high voltage-activated Ca$^{2+}$ channel $\alpha_1$ subunit cDNAs from Schistosoma mansoni (5). Here, we report the cloning and expression of two Ca$^{2+}$ channel $\beta$ subunits from Schistosoma japonicum and S. mansoni. These schistosome $\beta$ subunits (SmCa$\beta_1$A and SjCa$\beta_1$) have novel structural features and unusual modulatory effects on $\alpha_1$ subunits. Furthermore, coexpression of either of these two $\beta$ subunits with mammalian Ca$2.3$ ($\alpha_{1K}$) confers PZQ sensitivity to this otherwise PZQ-insensitive $\alpha_1$ subunit.

EXPERIMENTAL PROCEDURES

Isolation of $\beta$ Subunit cDNAs from Schistosomes—The initial cDNA fragment was amplified by PCR with degenerate primers using either a cDNA pool (RT-PCR) or a cDNA library as template. For RT-PCR, total RNA was extracted from fresh mixed-sex adult S. japonicum by homogenization of the tissue in Trizol reagent (Life Technologies, Inc.). RNA was precipitated in isopropanol and resuspended in diethylpyrocarbonate-treated water. cDNA was synthesized from 10 $\mu$g of total RNA using 1 pmol of an oligo(dT) adapter primer and 200 units of SuperScript II reverse transcriptase (Life Technologies, Inc.). Incubation was for 2 h at 42 °C. Degenerate primers for PCR were designed against highly conserved regions from other Ca$^{2+}$ channel $\beta$ subunits. The sense primer (AAYAYAGGTTGGAT) was based on the amino acid sequence NNDWVI. The antisense primer (GCTYTGTGTGTATCRTC) was based on the amino acid sequence DMMQK.

PCR consisted of 1 $\mu$l of template in a reaction containing 50 pmol of each degenerate primer and $\lambda$ DNA (PharGene) as the enzyme. 30 cycles of (1 min, 52, 1 min) were used. A single, ~500-bp band was gel-purified (QIAquick; Qiagen) and cloned into pCR4-TOPO TA cloning vector (Invitrogen). Plasmids were sequenced on an ABI 310 Genetic Analyzer using the BigDye Terminator Cycle Sequencing Kit (ABI). Both the RT and library generated identical clones containing a 584-bp insert encoding sequence similar to known $\beta$ subunits. Both 5’ and 3’ ends of the cDNAs were obtained from the cDNA library by PCR, using a combination of a degenerate primer and a gene-specific primer. Only one of the two libraries contained the entire coding sequence, determined by PCR from a different cDNA pool was used to amplify a full-length copy of the coding sequence, providing a consensus sequence named SjCa$\beta_1$. SmCa$\beta_1$A was cloned using similar methods but using cDNA pools and libraries made from S. mansoni RNA.

Expression in Xenopus Oocytes—RNA was transcribed from cDNA clones containing full-length coding regions of SjCa$\beta_1$, SmCa$\beta_1$A, and other subunits, using the T7 mMessage mMachine in vitro transcription kit (Ambion). The amount of purified, transcribed RNA was estimated by gel analysis using 10% SDS-PAGE. RNA was injected into oocytes by microinjection using a pipette tip. Oocytes were injected with 40 nl of 100 mM BAPTA dissolved in 10 mM HEPES (pH 7.4) to block passive Ca$^{2+}$ entry into the oocyte. Oocytes were incubated in modified Barth’s solution containing 1.8 mM CaCl$_2$, supplemented with 2.5 mM sodium pyruvate, 100 units/ml penicillin, 100 $\mu$g/ml streptomycin, and 5% horse serum at 17 °C for 3–7 days. In some cases, other amounts of RNA were also tested. Because levels of channel expression vary over time, recordings on any given day were from oocytes in all treatment groups. Oocytes were injected with 40 nl of 100 mM BAPTA dissolved in 10 mM HEPES at least 1 h prior to recordings to eliminate potential artifacts from the endogenous Ca$^{2+}$-activated chloride current in oocytes. The bath solution consisted of 40 mM Sr(OH)$_2$, 40 mM N-methylglycine, 10 mM glucose, and 10 mM HEPES, pH 7.4. Sr$^{2+}$ was used as the charge carrier, because both $\alpha_1$ subunits used (CvCa1, and Ca2.3) are most
permeable to that ion (6, 7). Similar results were obtained using Ba$^{2+}$/H$^{+}$ as the charge carrier. Currents were recorded using a Warner Instruments two-electrode voltage clamp and 3 M KCl-filled borosilicate microelectrodes with impedances of 0.1–1 megohms. Oocytes were clamped at −90 mV. Data were recorded and analyzed with pClamp6 and pClamp8 software (Axon Instruments), and leakage currents were subtracted on-line. All experiments were conducted at room temperature.

Praziquantel (Sigma) was dissolved as a 10$^{-2}$ M stock solution in Me$_2$SO and diluted in bath solution to the final concentration. Effects of PZQ were measured within seconds following application of the drug. Me$_2$SO alone had no effect at the dilutions used.

RESULTS

Cloning of Ca$^{2+}$ Channel β Subunit cDNAs from Schistosomes—We used degenerate oligonucleotide primers and PCR to amplify a 564-bp portion of a cDNA from $S$. japonicum with similarity to other known Ca$^{2+}$ channel β subunits. The 2319-bp full-length coding region of the sequence was subsequently obtained and named $Sj$Ca$\beta$. Northern blots probed with a portion of this sequence reveal several bands, ranging in size from 2.4 to 4.4 kilobases (data not shown). The cDNA for $Sj$Ca$\beta$ codes for a predicted protein of 84 kDa, which, as with other invertebrate subunits, does not cluster with any of the mammalian subtypes. We also cloned a similar (88% identical) subunit cDNA from $S$. mansoni ($Sm$Ca$\beta$A). $Sm$Ca$\beta$A codes for a slightly larger protein of 87 kDa. As is found for other schistosome sequences, coding regions for both of these sequences are very A/T-rich (62%). Although the sequences of $Sj$Ca$\beta$ and $Sm$Ca$\beta$A are more similar to other known β subunits than to any other proteins, they both have unusual structural features in comparison to other β subunits (see Fig. 1). For example, both of these schistosome subunits are larger than other subunits, containing extended sequence in the C-terminal region. Both sequences also show differences within the β-interaction domain (BID).

FIG. 1. $Sj$Ca$\beta$ and $Sm$Ca$\beta$A sequences. A, alignment of the two schistosome β subunits ($Sj$Ca$\beta$ and $Sm$Ca$\beta$A) with the β2a subunit from rat (Ca$\beta$2a; accession number M80545). Amino acid sequences were aligned with ClustalW. Identities are shaded black. The BID is shown. Note the extended C-terminal sequence in the two schistosome β subunits. B, alignment of regions corresponding to the BID consensus sequence (8, 9) from several Ca$^{2+}$ channel β subunits. Two serine residues that represent potential phosphorylation sites found in nearly every known BID are not conserved in the two schistosome β subunits (black shading). The only other known BID that does not contain both these serine residues is found in a putative β subunit sequence in the Caenorhabditis elegans genome. The serine residue shown to reduce increases in current amplitude when mutated to arginine (8) is denoted by an asterisk. In $Sj$Ca$\beta$, the final residue in this region is a serine that is also a potential protein kinase C phosphorylation site (box). Accession numbers are as follows: rabbit β1a, M25817; rat β1b, X61394; human β1c, M76560; human β2a, AAD33730; rabbit β2b, X64298; rat β3, M88751; rat β4, L02315; Drosophila,AAF21096; C. elegans-1, AAB53056; C. elegans-2 (partial sequence), AAK21500; jellyfish (Cyanea capillata), AAB87751.
Fig. 2. Modulatory effects of schistosome β subunits on Ca\(_{2.3}\) and CyCa\(_{1.1}\). A, SjCa\(_{\beta}\) reduces current amplitude of the Ca\(_{2.3}\) α subunit. Typical current traces were recorded from Xenopus oocytes injected with Ca\(_{2.3}\) RNA alone or with Ca\(_{2.3}\) plus SjCa\(_{\beta}\) RNAs. B, SjCa\(_{\beta}\) and SmCa\(_{\beta}\)A reduce peak current amplitude of Ca\(_{2.3}\). Coexpression of either SjCa\(_{\beta}\) (n = 13) or SmCa\(_{\beta}\)A (n = 18) results in a significant reduction in peak current compared with Ca\(_{2.3}\) alone (n = 11). Coexpression with the mammalian β2a subunit results in a slight increase in current (n = 7). Asterisks designate significant differences from Ca\(_{2.3}\) alone (p < 0.05, two-tailed t-test). Error bars represent S.E. C, SjCa\(_{\beta}\) and SmCa\(_{\beta}\)A also reduce peak current amplitude of CyCa\(_{1}\). Coexpression of either SjCa\(_{\beta}\) (n = 14) or SmCa\(_{\beta}\)A (n = 12) results in a significant reduction in peak current compared with CyCa\(_{1}\) alone (n = 14). CyCa\(_{\beta}\) (n = 11) increases peak current. Asterisks designate significant differences from CyCa\(_{1}\) alone (p < 0.05, two-tailed t test). D, SjCa\(_{\beta}\) and SmCa\(_{\beta}\)A shift the current/voltage relationship of Ca\(_{2.3}\) in a hyperpolarizing direction. Current/voltage relationships were recorded in oocytes expressing Ca\(_{2.3}\) alone (n = 10) or Ca\(_{2.3}\) plus Ca\(_{\beta}\)2a (n = 5), CyCa\(_{\beta}\) (n = 3), SjCa\(_{\beta}\) (n = 10), or SmCa\(_{\beta}\)A (n = 13) and normalized to peak current values. Peak current measurements were determined by 250-ms voltage steps from a holding potential of -90 mV. The curves represent fits of the Boltzmann function \(I_{\text{norm}} = \frac{I_{\text{max}}}{1 + \exp(V - V_{1/2})/k}\), where the normalized conductance \(g_{\text{norm}} = 0.021, 0.024, 0.018, 0.018, \) and 0.021; the reversal potential \(V_{\text{rev}} = 69.7, 56.6, 66.6, 56.8, \) and 53.6 mV; the slope factor \(k = 5.5, 6.1, 4.8, 3.3,\) and 5.0; and the half-activation potential \(V_{1/2} = +4.6, -3.2, -6.0, -12.4,\) and -9.8 mV for Ca\(_{2.3}\) alone or with Ca\(_{\beta}\)2a, CyCa\(_{\beta}\), SjCa\(_{\beta}\), or SmCa\(_{\beta}\)A, respectively.

Fig. 3. Ca\(_{2.3}\) exhibits sensitivity to PZQ when coexpressed with schistosome β subunits. The ratio of the peak current in the presence and absence of 100 nM PZQ is shown for different α/β combinations (x axis). If PZQ has no effect, then the ratio will equal one. Results are shown for Ca\(_{2.3}\) alone (n = 6), Ca\(_{2.3}\) plus a jellyfish β subunit (CyCa\(_{\beta}\), n = 8), a jellyfish L-type α subunit (CyCa\(_{1}\) plus SjCa\(_{\beta}\)), Ca\(_{2.3}\) plus SjCa\(_{\beta}\) (n = 7), Ca\(_{2.3}\) plus SjCa\(_{\beta}\) plus SmCa\(_{\beta}\)A (n = 12), and Ca\(_{2.3}\) plus SmCa\(_{\beta}\)A (n = 13). Asterisks indicate significant differences from the first column (p < 0.005, two-tailed t test).

Ca\(_{2.3}\) Exhibits Sensitivity to PZQ When Coexpressed with SjCa\(_{\beta}\) or SmCa\(_{\beta}\)A—Currents were measured in oocytes expressing different combinations of α1 and β subunits in the presence or absence of 100 nM PZQ (Fig. 3). Oocytes expressing Ca\(_{2.3}\) alone or CyCa\(_{1}\) alone (not shown) showed no increase in current in the presence of 100 nM PZQ. Similarly, Ca\(_{2.3}\) showed no sensitivity to PZQ when coexpressed with a jellyfish β subunit (CyCa\(_{\beta}\)) or with a mammalian β subunit (Ca\(_{\beta}\)2a; not shown). However, oocytes coexpressing mammalian Ca\(_{2.3}\) with either SjCa\(_{\beta}\) or SmCa\(_{\beta}\)A showed nearly a doubling of current amplitude in the presence of 100 nM PZQ. In contrast to Ca\(_{2.3}\), the jellyfish CyCa\(_{1}\) α1 subunit remained insensitive to PZQ when coexpressed with either SjCa\(_{\beta}\) or SmCa\(_{\beta}\)A.

The primary site of β subunit interaction with the α1 subunit (see Ref. 8 and Fig. 1B). Two conserved serine residues that represent potential phosphorylation sites in the BID are changed to other residues in both SjCa\(_{\beta}\) and SmCa\(_{\beta}\)A. Interestingly, for SjCa\(_{\beta}\) the final residue of the BID consensus sequence is a serine, creating a potential protein kinase C phosphorylation site not found in other β subunits.

SjCa\(_{\beta}\) and SmCa\(_{\beta}\)A Decrease Current Amplitude—Each of the schistosome β subunits was coexpressed with either human Ca\(_{2.3}\) or jellyfish CyCa\(_{1}\) α1 subunits. For both α1 subunits, coexpression of either of these schistosome β subunits results in a dramatic reduction in current amplitude (Fig. 2, A–C). SmCa\(_{\beta}\)A appears to be somewhat more potent at reducing peak currents than SjCa\(_{\beta}\), with up to 10–20-fold reductions in current amplitude. Similar levels of current reduction were found when expression included a mammalian α2−β subunit (data not shown). Coexpression of CyCa\(_{1}\) or Ca\(_{2.3}\)α1 subunits with β subunits from other organisms showed expected large (CyCa\(_{\beta}\)) or minor (β2a) increases in current amplitude (Fig. 2, B and C).

Other than their unusual effects on current amplitude, both SjCa\(_{\beta}\) and SmCa\(_{\beta}\)A modulate α1 subunits as other β subunits do. For example, these β subunits shift the current/voltage relationship of expressed Ca\(_{2.3}\) (see Fig. 2D) and CyCa\(_{1}\) (not shown) in a hyperpolarizing direction and have effects on inactivation rates and voltage dependence of inactivation similar to those found for other β subunits.2

Manuscript in preparation.
In this report, we describe the cloning and functional expression of Ca\(^{2+}\) channel \(\beta\) subunit cDNAs from two schistosome species. Both of these \(\beta\) subunits have unusual structural features compared with other known \(\beta\) subunits. Furthermore, in contrast to other known \(\beta\) subunits, coexpression of these schistosome \(\beta\) subunits with two different \(\alpha_1\) subunits results in a dramatic decrease in current amplitude. Finally, coexpression of the PZQ-insensitive Ca,2,3 with either of these schistosome \(\beta\) subunits results in an expressed current that is sensitive to PZQ, indicating that these \(\beta\) subunits, in combination with particular \(\alpha_1\) subunits, may be important molecular targets of PZQ action.

The two schistosome \(\beta\) subunits we describe here clearly modulate \(\alpha_1\) subunits in an unusual manner. Instead of increasing current amplitude as other \(\beta\) subunits do, these \(\beta\) subunits dramatically decrease amplitude of currents expressed in *Xenopus* oocytes. An explanation for this unusual effect may reside within the BID. Both of these schistosome \(\beta\) subunits lack two conserved serines within their BIDs. These serines are consensus protein kinase C phosphorylation sites. Mutation of one of these serines (Fig. 1B, *) to arginine in the \(\beta_{1b}\) subunit results in a reduced enhancement of current amplitude with no detectable effect on \(\alpha_1/\beta_1\) binding (8). Perhaps the loss of both serines in these schistosome \(\beta\) subunits results in an even more dramatic effect on current amplitude. Other modulatory effects of these \(\beta\) subunits on \(\alpha_1\) subunits are similar to those found for other \(\beta\) subunits, indicating that the reduction in current is likely not the result of some nonspecific interaction.

Perhaps most interestingly, both of these schistosome \(\beta\) subunits appear to confer PZQ sensitivity to an otherwise insensitive mammalian channel. Coexpression of either of these \(\beta\) subunits with human Ca,2.3 results in a significant increase in voltage-gated current amplitude in the presence of the drug. Besides potentially providing insight into the mode of PZQ action, this result also indicates that the reduction in current amplitude induced by schistosome \(\beta\) subunits cannot be explained entirely by a defect in channel assembly. Clearly, channels appear to be present in the membrane but are either not gating currents or are gating reduced currents. In this cross-species heteromer, PZQ appears to partially reverse this inhibitory effect.

These schistosome \(\beta\) subunits are likely competing with the endogenous \(\beta\) subunit found in *Xenopus* oocytes (14) for binding to expressed \(\alpha_1\) subunits. Indeed, we have preliminary data from coexpression experiments (not shown) indicating that these schistosome \(\beta\) subunits compete with other expressed \(\beta\) subunits in a concentration-dependent manner to modulate \(\alpha_1\) subunits.

Interestingly, PZQ-sensitive currents do not result when the jellyfish L-type \(\alpha_1\) subunit (CyCa,1) is coexpressed with SjCa,\(\beta_1\) or SmCa,\(\beta_\alpha\). There are at least two possible explanations for this difference. First, it may reflect some subtle distinction in the interaction of L-type (CyCa,1) and non-L-type (Ca,2.3) \(\alpha_1\) subunits with these schistosome \(\beta\) subunits or with PZQ. Verterbrate non-L-type Ca,2.1 and Ca,2.3 \(\alpha_1\) subunits have been shown to contain a secondary \(\beta\) subunit interaction domain at the C terminus (14, 15). Second, it is possible that the difference reflects structural motifs that are common to Ca,2.3 and, presumably, schistosome \(\alpha_1\) subunits but not found in CyCa,1. These differences would be independent of whether the channel is L-type or non-L-type. For example, schistosome and other flatworm L-type \(\alpha_1\) subunits have a non-charged residue at a conserved negatively charged site in the Domain I pore region (5). Ca,2.3, but not CyCa,1, also has a non-charged residue at that site. These and other hypotheses are currently being tested.

The results presented here suggest a model for PZQ action that is consistent with the observed effects of PZQ on Ca\(^{2+}\) homeostasis in schistosomes. These unusual schistosome \(\beta\) subunits presumably inhibit currents through those schistosome \(\alpha_1\) subunits with which they are associated. We speculate that PZQ may disrupt this \(\alpha_1/\beta_1\) interaction in these channels, thereby either allowing more channels to open or allowing more current to flow through individual channels. As a consequence, normal Ca\(^{2+}\) homeostasis is disrupted. We are currently testing the effects of PZQ directly on schistosome Ca\(^{2+}\) channels expressed in a heterologous system. Eventually, recording of currents through single channels will refine this model, and coexpression of different combinations of schistosome \(\alpha_1\) and \(\beta\) subunits will be used to test it more rigorously.

Interestingly, we have cloned a third schistosome \(\beta\) subunit (SmCa,\(\beta_\alpha\)) that more closely resembles other \(\beta\) subunits in its structure. We would expect that these two (or more?) types of schistosome \(\beta\) subunits may have characteristic patterns of expression in the worm and may have distinct modulatory effects on the different schistosome \(\alpha_1\) subunits.

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