The N Terminus of a Schistosome β Subunit Regulates Inactivation and Current Density of a Ca\(_{v}\)2 Channel

Vicenta Salvador-Recatalà\(^1\) and Robert M. Greenberg

From the Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

The β subunit of high voltage-activated Ca\(^{2+}\) (Ca\(_{v}\)) channels targets the pore-forming α\(_1\) subunit to the plasma membrane and tunes the biophysical phenotype of the Ca\(_{v}\) channel complex. We used a combination of molecular biology and whole-cell patch clamp to investigate the functional role of a long N-terminal polyacidic motif (NPAM) in a Ca\(_{v}\)β subunit of the human parasite Schistosoma mansoni (β\(_{Sm}\)), a motif that does not occur in other known Ca\(_{v}\)β subunits. When expressed in human embryonic kidney cells stably expressing Ca\(_{v}\)2.3, β\(_{Sm}\) accelerates Ca\(^{2+}\)/calmodulin-independent inactivation of Ca\(_{v}\)2.3. Deleting the first 44 amino acids of β\(_{Sm}\), a region that includes NPAM, significantly slows the predominant time constant of inactivation (\(\tau_{fast}\)) under conditions that prevent Ca\(^{2+}\)/CaM-dependent inactivation (β\(_{Sm}\); \(\tau_{fast} = 66\) ms; β\(_{Sm}A2–44\); \(\tau_{fast} = 111\) ms, \(p < 0.01\)). Interestingly, deleting the amino acids that are N-terminal to NPAM (2–24 or 2–17) results in faster inactivation than with an intact N terminus (\(\tau_{fast} = 42\) ms with β\(_{Sm}A2–17\); \(\tau_{fast} = 40\) ms with β\(_{Sm}A2–24\); \(p < 0.01\)). This suggests that NPAM is the structural determinant for accelerating Ca\(^{2+}\) inactivation. We also created three chimeric subunits that contain the first 44 amino acids of β\(_{Sm}\) attached to mammalian β\(_{1a}\), β\(_{2a}\), and β\(_{3}\) subunits. For any given mammalian β subunit, inactivation was faster if it contained the N terminus of β\(_{Sm}\) than if it did not. Co-expression of the mammalian α\(_1\)β\(_{1}\) subunit resulted in doubling of the inactivation rate, but the effects of NPAM persisted. Thus, it appears that the schistosome Ca\(_{v}\) channel complex has acquired a new function that likely contributes to reducing the amount of Ca\(^{2+}\) that enters the cells in vivo. This feature is of potential interest as a target for new antihelminthics.

The cytoplasmic β subunit of high voltage-activated Ca\(^{2+}\) (Ca\(_{v}\)) channels targets the pore-forming α\(_1\) subunit to the plasma membrane and affects its biophysical phenotype (1, 2). An important function of Ca\(_{v}\)β subunits is to modulate the inactivation rate of Ca\(_{v}\) channels. Inactivation of Ca\(_{v}\) channels is a life-sustaining property that maintains tight control of intracellular levels of Ca\(^{2+}\) (3). For instance, in neurons, the spatial-temporal dynamics of Ca\(^{2+}\) microdomains, which determine qualitative and quantitative aspects of neurotransmission, depend on the biophysical properties of the Ca\(_{v}\) channels, such as conductance and inactivation rate as well as the nature of the intracellular buffers (for review, see Ref. 4).

Invertebrate Ca\(_{v}\) channels are less well characterized than mammalian Ca\(_{v}\) channels. However, Ca\(_{v}\) channels are validated targets widely exploited in pharmacotherapy as well as by naturally occurring toxins (5). Understanding the idiosyncrasies of invertebrate Ca\(_{v}\) channels could be useful for design of appropriate therapeutic strategies against invertebrate pathogens such as schistosomes. Schistosomes are parasitic flatworms that are the causative agents of schistosomiasis, a tropical disease that affects an estimated 200 million people worldwide. The current drug of choice against schistosomiasis is praziquantel, which affects Ca\(^{2+}\) homeostasis in adult worms (6). Adults of Schistosoma mansoni express Ca\(_{v}\)2 and Ca\(_{v}\)1 orthologues as well as two β subunit subtypes (7, 8). The modus operandi of the schistosome Ca\(_{v}\) channels remains largely unknown primarily due to technical challenges in expressing α\(_1\) subunits and to fast rundown of Ca\(^{2+}\) currents in native preparations (9, 10). It is expected that these channels would express better in helminthic clonal cell lines as these may have some specific factors that are needed for functional expression of these α\(_1\) subunits; to our knowledge these cells lines are not yet available. In contrast, the schistosome cytoplasmic β subunits express robustly in mammalian cells (11) and in Xenopus oocytes (8). The less conventional of the two β subunits has unusual structural and functional properties and appears to be involved in the action of praziquantel (8). In contrast, less data are available for the more conventional schistosome β subunit, which does not appear to play a role in praziquantel action. This subunit, referred to as β\(_{Sm}\) heretofore, resembles mammalian β subunits in that it significantly increases current density, modulates steady-state inactivation, and displaces the peak of the voltage-current relationship to the hyperpolarized direction. However, unlike other β subunits, β\(_{Sm}\) has a long N-terminal poly acidic motif (abbreviated as NPAM herein) of 15 aspartate and glutamate residues that mediates rapid rundown of Ca\(_{v}\)2.3 currents (11). Although NPAM appears to represent the main difference between schistosome and mammalian β subunits, one has to consider that even very minor differences between ion channel subunits can have major physiological and pharmacological consequences. Indeed, subtle changes are currently exploited in a variety of selective drugs and toxins. For example, pyrethroids, which are commonly used as insecticides, target the voltage-gated Na channels (Na\(_{v}\)) of insects but not those of mammals despite the extensive structural and functional simi-

\(^{*}\) This work was supported, in whole or in part, by National Institutes of Health Grants AI 4052, AI 73660, and AI 082390.

\(^{1}\) The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

\(^{2}\) To whom correspondence should be addressed: 226 Rosenthal Bldg., Dept. of Pathobiology, University of Pennsylvania, 3800 Spruce St, Philadelphia, PA 19104. Tel.: 215-898-5655; E-mail: vicenta@vet.upenn.edu.
larities between the Na\textsubscript{v} channels of insects and mammals. Indeed, a single amino acid is responsible for the sensitivity of insect Na\textsubscript{v} channels to pyrethroid insecticides (12, 13). Similarly, the capability of the other schistosome (variant) \(\beta\) subunit to confer praziquantel sensitivity to an otherwise non-susceptible \(\alpha\)1 subunit can be abolished by mutating a single amino acid residue (14). In another case the sensitivity of the GABA receptor to ethanol requires a string of 8 amino acids in one of the \(\gamma\) subunits (15). By analogy with this latter study, it is reasonable to suppose that the 15-amino acid-long polyacidic motif of the schistosome \(\beta\textsubscript{sm}\) subunit could serve as a drug target itself or might influence the action of drugs on schistosome Ca\textsubscript{v} channels. Nevertheless, the rationale for our study goes beyond identifying a pharmaceutical target against schistosomes and is geared to obtaining a better understanding of an important player in the physiology of the neuromuscular system of these invertebrates, i.e. Ca\textsubscript{v} channels. These insights will almost certainly be an advantage in the design and/or identification of effective pharmacophores.

Because acidic motifs that occur in channel and non-channel proteins bind Ca\textsuperscript{2+} ions to modulate protein function (16, 17), we hypothesized that the polyacidic motif in \(\beta\textsubscript{sm}\) would bind Ca\textsuperscript{2+} to interfere with Ca\textsuperscript{2+}/calmodulin-dependent inactivation. To this end we recorded Ca\textsubscript{v,2,3} currents in conditions that allow Ca\textsuperscript{2+}/calmodulin-mediated inactivation and in conditions that prevent it. Surprisingly, we found that the effects of NPAM\textsuperscript{2} on Ca\textsubscript{v} inactivation appear to be Ca\textsuperscript{2+}/calmodulin-independent. Here we show a detailed characterization of the role of NPAM in modulating the inactivation properties of Ca\textsubscript{v,2,3} currents and provide a discussion of how the biophysical modulation of Ca\textsubscript{v} channels by NPAM-containing \(\beta\) subunits, which are found so far only in parasitic trematodes, may contribute to the parasitic mode of life of these species.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tissue culture dishes were purchased from Corning, Dulbecco’s modified Eagle’s media (DMEM) was purchased from Invitrogen, and poly-L-lysine, ATP, and the calcium phosphate transfection kit were purchased from Sigma. Restriction enzymes were purchased from New England Bio-labs and oligonucleotide primers were from MWG biotech.

**Cell Culture and Transfection**—Human embryonic kidney (HEK) cells stably transfected with human Ca\textsubscript{v,2,3d} were cultured in DMEM supplemented with l-glutamine, glucose, and 10% fetal bovine serum in a humidified atmosphere (95%) with 5% CO\textsubscript{2} at 37 °C. Cells were used for up to 25 passages and were split every 2–4 days. For electrophysiological recordings, cells were seeded in Petri dishes coated with poly-L-lysine, and transfection of \(\beta\) subunits and, in some cases the \(\alpha\textsubscript{\delta}-1\) subunit (accession number AF286488.1), was performed with calcium phosphate on cells seeded on 60-mm Petri dishes at a confluence of 50–60% using 10 \(\mu\)g of the construct per dish. Using standard methods, we cloned all \(\beta\) subunits into the pXOOM vector (18), which contains the gene for green fluorescent protein (GFP) as a marker for transfection.

**Construction of \(\beta\) Chimeras**—Chimeric \(\beta\) subunits were constructed by splicing the N-terminal region of the schistosome \(\beta\textsubscript{sm}\) (corresponding to amino acids 1–47) to the nearly full-length coding regions of the three mammalian \(\beta\) subunits. The respective portions were amplified from plasmid templates by PCR using GoTaq DNA Polymerase Green Master Mix (Promega) and primers containing appropriate restriction sites. For \(\beta\textsubscript{1b}\) and \(\beta\textsubscript{1}, \beta\textsubscript{2}\) chimeras, the forward primer for amplification of \(\beta\textsubscript{sm}\) (5'-GGGGATCCATGCAATGTTGTCAGGATATTCA-3') was designed to correspond to the first 8 amino acids of the sequence and contained a BamHI site at its 5' end. The \(\beta\textsubscript{sm}\) reverse primer was designed against amino acids 55–61 and contained a BglII site at its 5' end (5'-GGGAGATCTTTTA-TAATCATTCTCATCCTT-3'). For the \(\beta\textsubscript{2a}\) chimeras, the \(\beta\textsubscript{sm}\) forward primer (5'-GGGGATCCATGCAATGTTGTCAGGATATTCA-3') was designed to maintain the palmitoylated cysteine residues found in \(\beta\textsubscript{2a}\) at positions 3 and 4, thought to be important for the effects of this subunit on inactivation (19). The portions of the different mammalian \(\beta\) subunits were amplified using subunit-specific primers with a BglII restriction site at the 5' end of the forward primers and a NotI restriction site at the 5' end of the reverse primers. For \(\beta\textsubscript{1b}\) (accession number X61394; amino acids 7–597), the forward primer was 5'-GGGAGATCTATGCGGCGGCTACCCA-3'; for \(\beta\textsubscript{2a}\) (accession number M80545; amino acids 9–604), the forward primer was 5'-GGGAGATCTTTTATATATCATCCTTAATCCTT-3'; and for \(\beta\textsubscript{2}, \beta\textsubscript{3}\) (accession number M88751; amino acids 5–484), the forward primer was 5'-GGGAGATCTTAGTGCCCGGGTTTGTGAGGAC-3'. The reverse primers were: 5'-GCGCGGCCGCCTACAGGATGACGCCTTGA-3' (\(\beta\textsubscript{1b}\)); 5'-GCGCGGCCGCCCTACATGCGGATGATATACAT-3' (\(\beta\textsubscript{3}\)); 5'-GGCGGCCGCTCACTGAATCGTACCTT-3'. These reverse primers were all designed against the final amino acids and stop codon of the open reading frames. All fragments were amplified using standard PCR conditions (annealing temperature = 50 °C). After cleanup of the reactions over Qiaquik columns (Qiagen), the eluted products were digested with BglII, re-purified, and ligated. A small portion of this ligation mix was used as the template for another amplification of the full-length, chimeric construct using \(\beta\textsubscript{sm}\) forward and mammalian \(\beta\) subunit reverse primers. This PCR product was gel-purified, digested with BamHII and NotI, and ligated into the BamHII/NotI-digested pXOOM, and this ligation was used to chemically transform Top 10 (Invitrogen) competent cells. All clones were sequenced, and only those without PCR (or other) errors were used for subsequent experiments. The strategy we used changes amino acid 47 of \(\beta\textsubscript{sm}\) from Glu to Arg.

**Confocal Microscopy of Enhanced Green Fluorescent Protein** (EGFP)-tagged \(\beta\textsubscript{sm}\) and \(\beta\textsubscript{sm}\textsubscript{Δ2–44} \) Subunits—\(\beta\textsubscript{sm}\) and \(\beta\textsubscript{sm}\textsubscript{Δ2–44}\) were amplified by PCR using a reverse primer that removed the stop codon at the end of the open reading frame. Amplification with this primer, therefore, allowed for insertion of the \(\beta\) subunit coding region into the pcDNA3-EGFP plasmid (Addgene #13031) such that the \(\beta\) subunits would be tagged with EGFP at
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the C terminus. HEK cells stably expressing Ca2.3d were transfected with these EGFP-tagged β subunit constructs as previously described. For confocal microscopy experiments, cells were plated onto poly-L-lysine-coated glass-bottomed 35-mm tissue culture dishes (MatTek). Confocal images of the cellular distributions of the EGFP-tagged βSm and its N-deletion mutant βSmΔ2–44 were acquired 24 h after transfection with a spinning disc confocal microscope (Leica) using a 100× oil-immersion objective. Images were acquired using an argon laser (excitation, 488 nm; emission, BP emission filter).

Protein Extraction and Western Blots—Membrane proteins from EGFP-βSm– or EGFP-βSmΔ2–44–transfected HEK cells were extracted using the ProteoJET Membrane Protein Extraction kit (Fermentas) according to the manufacturer's instructions. Briefly, cells were washed and permeabilized and then transferred to microcentrifuge tubes. After centrifugation at 10,000 × gmax, membrane protein extraction buffer was added to the supernatant and incubated for 30 min at 4 °C with shaking, then the membrane protein extract was centrifuged at 10,000 × gmax for 15 min at 4 °C to remove debris, and the supernatant was saved at −70 °C for further analysis. Protein concentrations were determined using the Bradford Assay (Fermentas). Two micrograms of protein were separated on a 4–12% Bis-Tris Nupage gel (Invitrogen) and transferred to a nitrocellulose membrane (Invitrogen). After blocking with TBS, Tween (TBST) plus 5% nonfat dry milk, the blot was incubated in a 1:1000 dilution of anti-GFP rabbit polyclonal (Pierce) according to the manufacturer's instructions. For Western Blots, the blots were incubated in goat anti-rabbit IgG (1:15,000; Jackson ImmunoResearch), washed, and visualized with Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific Pierce) according to the manufacturer's instructions.

Electrophysiology—Whole-cell patch clamp recordings were obtained 24–48 h after transfection of the β subunits using an Axopatch 200B (Molecular Devices). Cell capacitances were 10–15 picofarads. Series resistance was compensated by 70%. Voltage pulses from −20 mV to +70 mV were delivered in 5-mV intervals every 5 s from a holding potential of −80 mV. Data were acquired at sampling intervals of 50 μs and filtered at 5 kHz during acquisition. The bath solution contained 10 mM CaCl2 or BaCl2, 160 mM triethylammonium (TEA) chloride, 10 mM HEPES, and 0.1 mM EGTA, pH (TEA-OH) 7.4. Patch pipettes were pulled from borosilicate glass and fire-polished before each experiment and had resistances between 1 and 1.5 megohms. Membrane seals were obtained by applying negative pressure. All experiments were performed at room temperature (22 °C). The pipette solution contained 110 mM cesium methane sulfonate, 10 mM HEPES, 0.5 mM EGTA, and 5 mM Mg2+/ATP, pH (CsOH) 7.3. In experiments designed to prevent Ca2+/calmodulin-dependent inactivation, EGTA was replaced with BAPTA (5 mM). In some control experiments, no chelators were added to the pipette solution. Current-voltage relationships were obtained as a first step to ensure that the cell produced viable currents. Maximum amplitude currents were used to estimate the time constants of the fast and slow components of macroscopic inactivation. To estimate the kinetics of macroscopic inactivation, the decaying phases of maximal Ca2+ currents evoked with depolarizing pulses from a holding potential of −80 mV were fitted to a double exponential equation of the form \( y = y_0 + A_1 \exp(-x/\tau_1) + A_2 \exp(-x/\tau_2) \), where \( \tau_1 \) and \( \tau_2 \) are the time constants, \( A_1 \) and \( A_2 \) are the amplitudes, and \( y_0 \) is the offset. Some β subunits induce faster inactivation than others. When inactivation was particularly slow, such as when β2a is coexpressed, longer pulses of at least 1 s were used. For consistency, the same portion of the pulse length is shown for all cases. To estimate the kinetics of macroscopic activation, the activating phases of maximal Ca2+ currents evoked with depolarizing pulses from a holding potential of −80 mV were fitted to a single exponential equation of the form \( y = y_0 + A \exp(-x/\tau) \). The voltage dependence of steady-state inactivation was determined by plotting the normalized peak current evoked by a depolarizing pulse to elicit the maximum current as a function of the voltage of a preceding 2-s pre-pulse test (between −120 and +20 mV). Currents were normalized with respect to the current elicited from −120 mV. Steady-state inactivation curves were fitted by a Boltzmann function of the form \( F(V) = I_{\text{max}}/[1 + \exp(V_{0.5} - V)/K] \), where \( I_{\text{max}} \) is the maximal current, \( V \) is the pre-pulse voltage, \( K \) is the slope factor, and \( V_{0.5} \) is the voltage at which inactivation is half-maximal. Data were acquired with an Axopatch 200 B amplifier and Clampex 2.0 software and analyzed with Clampfit 2.0 software (Molecular Devices). Inactivation kinetics analyses were performed with IGOR PRO (WaveMetrics, Lake Oswego, OR). To determine the significance of differences in data, Student’s t tests were performed. In the cases where the variances between the two groups being compared were significantly different, the Welch correction was applied.

RESULTS

We previously described an unusual, highly acidic domain (NPAM) near the N terminus of the “conventional” Ca2.3 channels with wild type, deleted, or modified β subunits containing the schistosome NPAM domain and studied how they modulate inactivation of Ca2.3 channels in conditions that allow or suppress the Ca2+/calmodulin-dependent inactivation process. Fig. 1 shows a diagram of the experimental setup. The N Terminus of β Sm Accelerates Inactivation of Ca2+ Currents through Ca2.3 when Ca2+ Is Chelated with 5 mM Internal BAPTA—In this set of experiments 5 mM BAPTA was included in the pipette solution to prevent Ca2+/calmodulin-dependent inactivation in Ca2.3 channels, so that inactivation occurs via voltage-dependent inactivation (VDI) only (20, 21). The subunits tested included native βSm; βSmΔ2–44, with the N-terminal 44 amino acids deleted (βSmΔ2–44), and chimeric mammalian β subunits with the N-terminal region of βSm attached to the N terminus. The inactivating portion of the Ca2+ currents was optimally fitted to a bi-exponential function. In these experiments, co-expression of β subunits containing the N terminus of βSm robustly accelerated inactivation kinetics of Ca2+ currents through Ca2.3 channels (Fig. 2A). Both fast and slow components of inactivation were accelerated for all subunits, except for βSmΔ1b, which accelerated only the fast component of inactivation with respect to β1b (Fig. 2, B and C).
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The N Terminus of βsm Does Not Significantly Modulate Ca2+ Currents through CaV2.3 When Ca2+ Is Chelated with 0.5 mM Internal EGTA—In these experiments 0.5 mM EGTA was included in the pipette solution, a condition that is expected to allow for accumulation of the intracellular Ca2+ necessary for Ca2+/calmodulin-dependent inactivation of CaV2.3 (20, 21). In most of these experiments, the addition or deletion of the N terminus of βsm to a β subunit did not significantly alter the rate of inactivation of CaV2.3 currents (Fig. 3A). An exception is βsm-β2a, which accelerated both fast and slow kinetic components of inactivation with respect to β2a (Fig. 3, B and C). Data obtained from whole-cell patch clamp experiments in which no chelators had been added to the patch solution (supplemental Fig. S1) were similar to those obtained with 0.5 mM EGTA in the patch pipette (Fig. 3).

The N Terminus of βsm Accelerates Inactivation of Ba2+ Currents through CaV2.3—Taken together, the data shown in the previous section do not support our initial hypothesis that the acidic motif in the N terminus of βsm modulates the inactivation process mediated by Ca2+/calmodulin. Instead, our data strongly point to the likelihood that the βsm N terminus accelerates the VDI process. To further confirm this finding, we tested the action of the N terminus of βsm on Ba2+ currents, as Ba2+ does not bind calmodulin (22), and therefore, using Ba2+ as the charge carrier is an alternative approach to suppress Ca2+/calmodulin-dependent inactivation. As shown in Fig. 4, A and B, Ba2+ currents also inactivated significantly faster with coexpression of NPAM-containing β subunits than with coexpression of β subunits that did not contain NPAM. When NPAM was attached to βsm, β1b, or β3, it accelerated only the predominant fast component of inactivation (Fig. 4, B and C). When attached to βzm, it modulated both fast and slow components of inactivation as the combination Ca2+out/BAPTAin was used (compare with Fig. 3). To further assess the role of NPAM on inactivation kinetics of CaV2.3, we plotted the fast and slow time constants of Ba2+ current inactivation as a function of voltage. Fig. 5, left and center panels, shows that inactivation kinetics were weakly dependent on voltage. The addition or deletion of NPAM did not alter the voltage sensitivity of inactivation kinetics. However, the relative weights of the two components of inactivation were modulated by NPAM (Fig. 5, right panels). Specifically, the relative weight of the fast component was significantly augmented (concomitantly with a decrease of the relative weight of the slow inactivating component) in all cases when the coexpressed β subunit contained NPAM. In the...
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FIGURE 3. The N terminus of a schistosome β subunit (β_{Sm}) plays no significant role in modulating inactivation kinetics of Ca_{2,3} Ca^{2+} currents chelated with 0.5 mM internal EGTA. A, show whole-cell Ca_{2,3} currents elicited by a depolarizing pulse to maximum peak current from a holding potential of −80 mV in the presence of 0.5 mM EGTA with the following coexpressed β subunits: β_{Sm}; β_{SmΔ2–44}; β_{1b}; the chimera β_{Sm}; β_{2a}; the chimera β_{Sm}; β_{2a}β_{2b}β_{3} or the chimera β_{Sm}; β_{3}. Current amplitudes are normalized to highlight the differences in inactivation kinetics for each pair of β subunits (with and without NPAM). Currents produced when NPAM-containing subunits were coexpressed are shown in red. A, show average values of the fast time constant (τ_{fast}) of macroscopic inactivation for Ca_{2,3} channels coexpressed with wild type and modified β subunits. B, show average values of the slow time constant (τ_{slow}) of macroscopic inactivation for Ca_{2,3} channels coexpressed with wild type and modified β subunits. Fast and slow r values were derived by two-exponential fits of the inactivating portions of Ca^{2+} currents. Bars represent mean ± S.E. Statistical significance of differences between the means was obtained from unpaired Student’s t tests. n = 3–9.

Whole-cell Ba^{2+} currents were then recorded in the presence of either of these β_{Sm} versions. To our surprise, Ba^{2+} currents inactivated faster with coexpression of either β_{SmΔ2–17} or β_{SmΔ2–24} than with coexpression of the full β_{Sm} (Fig. 7B). Both fast and slow components of inactivation were significantly accelerated by deleting the amino acids that precede NPAM (Fig. 7, C and D). This suggests that the region that precedes NPAM somehow suppresses NPAM-induced acceleration of Ca_{2,3} inactivation.

The N Terminus of β_{Sm} Accelerates Activation of Ca_{2,3}—Although we focused on the inactivation kinetics, we could not fail to observe that currents that inactivated faster due to coexpression of NPAM-containing β subunits also activated faster. Fitting the activating portion of the inward currents to single exponential functions yielded time constants of activation (τ_{act}) for all channel combinations. In all cases, τ_{act} had lower values (faster activation) in the presence of NPAM-bearing β subunits than in the presence of the corresponding β subunits without NPAM. In the case of β_{2a} and β_{3}, this difference was statistically significant (Table 1).

The N Terminus of β_{Sm} Decreases the Amplitude of Whole-cell Currents through Ca_{2,3}—Although we focused on the inactivation kinetics, we could not fail to observe that currents that inactivated faster due to coexpression of NPAM-containing β subunits also activated faster. Fitting the activating portion of the inward currents to single exponential functions yielded time constants of activation (τ_{act}) for all channel combinations. In all cases, τ_{act} had lower values (faster activation) in the presence of NPAM-bearing β subunits than in the presence of the corresponding β subunits without NPAM. In the case of β_{2a} and β_{3}, this difference was statistically significant (Table 1).
activation portion of each individual I-V relationship was well fitted by a Boltzmann function. The presence of any β subunit displaces the peak of the I-V to the left, which is well represented by the midpoints of activation given by this Boltzmann fit. However, midpoints of activation are not significantly altered by NPAM (Fig. 8E). I-V relationships obtained from patch clamp experiments where the cell interior was dialyzed with a chelator-free solution show that there are no appreciable differences between cells expressing NPAM-containing β subunits and cells expressing NPAM-less β subunits, except for β2a, which decreases average current amplitude if it contains the schistosome N terminus (supplemental Fig. S2).

To test whether the decrease of whole-cell current density by NPAM-bearing β subunits was due to reduced expression, we generated EGFP-tagged βSm and EGFP-tagged βSm2–44 subunits and compared their subcellular distribution in HEK cells. Fig. 8F shows that there are no detectable differences between the cellular distributions of these two β subunits. Both localize to the perinuclear membrane, to a localized region of the cytoplasm, presumably the endoplasmic reticulum and/or the Golgi apparatus, and to the plasma membrane. Supplemental Fig. S3 shows a Western blot of both subunits using a primary antibody against EGFP.

The N Terminus of βSm Does Not Modulate Steady-state Inactivation of Cav2.3—Because some structures of Cav channels appear to have dual roles in determining inactivation kinetics and inactivation gating (24), we wanted to know whether the N terminus of βSm would also affect steady-state inactivation. However, we found no significant differences between midpoints of steady-state inactivation between the NPAM and non-NPAM versions for any given β subunit (Fig. 9).

**DISCUSSION**

Here we report the role of a novel polyacidic motif of 15 aspartate and glutamate residues in a Cav channel β subunit from the human parasite S. mansoni (βSm). This motif is located at position 29–44 in the N terminus and is preceded by a smaller acidic motif, at position 18–24.
subunit that lacks the first 44 amino acids of \( \beta_{\text{Sm}} \) accelerates the decay of Ca\(^{2+} \) currents through Cav2.3. We also created chimeric mammalian \( \beta \) subunits that contained the N terminus of \( \beta_{\text{Sm}} \). These chimeric \( \beta_{\text{Sm}}-\beta_2 \) subunits also accelerated inactivation of Cav2.3, compared with their wild type counterparts. Surprisingly, when the amino acids that precede the long polyacidic motif were deleted from \( \beta_{\text{Sm}} \), inactivation of Cav2.3 currents occurred even faster than with an intact N terminus. The simplest explanation is that the amino acids that precede the polyacidic motif (NPAM) antagonize the NPAM function, perhaps by physically preventing NPAM from reaching its target. One can also hypothesize that the schistosome \( \alpha_1 \) subunit has an interaction site for these initial amino acids, which is not present in the mammalian \( \alpha_1 \) subunit. Taken together, the data presented here show that the N terminus of \( \beta_{\text{Sm}} \) contains structural determinant(s) with a role in accelerating inactivation, with the long polyacidic motif as a likely candidate.

Our data showing the accelerating effect of \( \alpha,\beta-1 \) on Cav2.3-\( \beta \) complexes is consistent overall with previous work. Interestingly, whereas Yasuda et al. (25), working with the mammalian cell line HEK ts-201, found that \( \alpha,\beta-1 \) does not accelerate the currents produced by Cav2.3-\( \beta_{2a} \) complexes, Qin et al. (23), using oocytes as the expression system, found that \( \alpha,\beta \) accelerates inactivation of Cav2.3-\( \beta_{2a} \) complexes. We found that all Cav2.3-\( \beta \) complexes were accelerated by \( \alpha,\beta-1 \), but this effect was significantly less pronounced in the case of Cav2.3-\( \beta_{2a} \). More specifically, the time constant of the fast inactivating component in the presence of \( \beta_{1b}, \beta_{\text{Sm}}, \) and \( \beta_2 \) was reduced by 50%, essentially doubling the rate of inactivation. When \( \alpha,\beta-1 \) was coexpressed with Cav2.3-\( \beta_{2a} \), the time constant of the fast inactivating component was reduced by about 30% (compare Figs. 3B and 5B). Thus, because we observe a lesser effect of \( \alpha,\beta-1 \) on inactivation rate in the case of \( \beta_{2a} \), our data are consistent with the findings of Qin et al. (23) but do not completely agree with those of Yasuda et al. (25). Perhaps the causes for this difference lie in the slightly different materials and conditions used, such as the different cell lines, HEK ts-201 (25) and HEK AD 298 here. Given that \( \beta_{2a} \) interacts with the plasma membrane, one might expect that \( \beta_{2a} \) behaves differently in different expression systems. It is also possible that \( \beta_{2a} \) modulation is affected by whether Cav2.3 is stably expressed (here) or whether it is transiently expressed (25).

Whereas recent work (26, 27) has shown that the longer the N terminus of \( \beta_{1a} \), the greater its accelerating effect on a Cav1 channel, our data (Fig. 6) show that the N terminus of \( \beta_{\text{Sm}} \) does not follow this rule, as shortening the N terminus by deleting the first 27 amino acids (which precede the polyacidic motif)
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that presence of the $\beta_{\text{sm}}$ N terminus results in reduced current density (Fig. 8). Clearly, the function of the N terminus of $\beta_{\text{sm}}$, and NPAM in particular, is quite different from that of the mammalian $\beta$ subunits.

**A Possible Mechanism(s) Used by the N Termminus of $\beta_{\text{sm}}$ to Accelerate Inactivation of Ca$_{\text{2.3}}$**—The fact that acceleration of Ca$_{\text{2.3}}$ inactivation mediated by the N terminus of $\beta_{\text{sm}}$ occurred only when Ca$^{2+}$ was used as the charge carrier, and 5 mBAPTA was added to the pipette solution. Solid lines represent Boltzmann fits to the data, $n = 3–6$.

VDI—The fact that currents consistently activated faster with coexpression of NPAM-containing $\beta$ subunits than with coexpression of NPAM-lacking $\beta$ subunits, would favor a model in which this motif modulates the voltage-dependent mechanism of inactivation. It is generally accepted that voltage dependence of inactivation occurs because the regions of the channel involved in this mechanism are coupled with the parts of the ion channel that sense a change in transmembrane voltage and move to produce VDI (28). This view of VDI largely derives from a model proposed by Aldrich et al. (29, 30) in which a slowing in macroscopic inactivation is the result of a delay in channel opening at the single channel level. The ration-

 accelerates inactivation of Ca$_{\text{2.3}}$. If the length of the N terminus were the only factor that determined the degree of the accelerating effect of $\beta_{\text{sm}}$ on Ca$_{\text{2.3}}$, then deleting 27 amino acids, a significant length by comparison with the experiments performed by Herzig and collaborators (26, 27), should have resulted in slowing of inactivation compared with that seen with wild type $\beta_{\text{sm}}$. Instead, we observed increased acceleration of inactivation. These authors also found that the N terminus of $\beta_{1a}$ does not play a role in determining current density of an L-type channel, a result in stark contrast to our data showing
N Terminus of β Accelerates Non-L-type Channel Inactivation

TABLE 2
Amino acid sequence of N-terminal polyacidic clusters in β subunits of four parasitic (S. mansoni, Schistosoma japonicum, Taenia solium, and Clonorchis sinensis) and two free-living platyhelminths (Schmidtea mediterranea and Dugesia japonica)

| Species                  | Accession no. | Sequence                                  | Position |
|--------------------------|---------------|-------------------------------------------|----------|
| S. mansoni               | AY023599      | EEYDDEEYCARDDDDDDEEDDDDDEDDYKEE            | 17–24, 30–44 |
| S. japonicum             | CA82734       | EEYDDEEYCARDDDDDDEEDDDDDEDDYKEE            | 17–24, 30–44 |
| T. solium                | AU624029      | DGEDDDDED                                  | 34–41    |
| C. sinensis              | AR267713      | EEDEEDEDEEDEEEVEDEEGEGEGEDDEEEDEEE        | 23–60    |
| S. mediterranea          | EG348863      | ELSEEEKE                                   | 17–25*   |
| D. japonica              | FJ485940      | ELSEEEKE                                   | 17–25    |

* Putative position, by alignment with the corresponding sequence in D. japonica.

The channel. This model is based on β2α2 subunits, whose palmitylooyot motif in the N terminus interacts with the plasma membrane. However, it is not known how the N terminus of other β subunits is positioned (38). The current models of the three-dimensional structure of the β subunits (for review, see Ref. 39) focus on core regions of β subunits and their interaction with the I–II loop of the α1 subunit. Nevertheless, drawing from the model for β2α2 (37), we hypothesize that the addition of the polyacidic motif disrupts the interaction of the palmitylooyot motif with the plasma membrane, thus preventing interaction of the N terminus with the plasma membrane.

Physiological Significance—Although it is fair to presuppose that the conditions in supplemental Fig. S2 (no internal chelators) or the addition of mild chelators (0.5 mM EGTA) are reasonable approximations of normal physiological conditions, by analogy with the mammalian system, it is not clear that they are physiological in the case of schistosome cells. Therefore, the fact that NPAM has little role in modulating Cav activity under these low buffering conditions in vitro does not necessarily indicate that NPAM does not modulate Cav channels in the native conditions. In fact, there are data indirectly suggesting that schistosomes have strong intracellular Ca²⁺ buffering mechanisms. For instance, adult schistosomes reside in blood vessels and feed on blood, which contains significant amounts of Ca²⁺; indeed, Shaw and Erasmus (40) propose the existence of special or additional cellular mechanisms to prevent excess Ca²⁺ influx. Perhaps β Sm represents one of these mechanisms, working to reduce Ca²⁺ influx by increasing the rate of Cav channel inactivation (and decreasing current density). The second, “variant” schistosome β subunit also appears to dampen Ca²⁺ currents (8). Additionally, it is known that schistosomes express a variety of Ca²⁺-buffering proteins. In parasites, these proteins appear to have an important role in adapting the parasite to the various environments they encounter in their complex life cycles and are also likely involved in secretion of proteins with roles in neutralizing host attack (41). The function of these buffering agents may not be restricted to host defense, but they may also serve to counteract deleterious increases in intracellular Ca²⁺. According to our data, the polyacidic motif of β Sm would be functionally relevant in this buffered environment, further contributing to minimize an intracellular increase in Ca²⁺.

Modulation of channel kinetics without a concomitant modulation of state-state inactivation, although uncommon, may be relevant physiologically. For instance, consider the relatively slow inactivating rate of the cardiac L-type Ca₉ channels that leads to accumulation of Ca²⁺, which in turn determines the plateau of the cardiac action potential, making possible the con-
traction of the heart. Mutations that minimally affect the inactivation rate of these channels are likely to have a significant impact on cardiac physiology even if they do not affect the voltage dependence of inactivation. In fact, data in support of this idea have been published and show that a splice variant of β2S with a relatively short N terminus, is expressed in the heart and that these two variants differ in their inactivation kinetics but not in steady-state inactivation (42). The magnitude of the difference in inactivation kinetics is comparable with that observed in our study and is likely to have significant effects on cardiac physiology. By analogy, it seems likely that changes in inactivation kinetics of schistosome Cav channels will have an impact on important physiological processes of these organisms.

Non L-type Cav2 channels play important roles in synaptic transmission (43–46), secretion (47, 48), and long term potentiation (49). One would expect that both Cav2 channels of S. mansoni (6) are also relevant to the correct functioning of the nervous system of the parasite. However, it is possible that the environment of adult S. mansoni in the human circulatory system does not demand as many Ca2+−dependent responses as the lifestyle of free-living flatworms. Interestingly, none of the Cav2 subunits of the free-living flatworms whose genome is available contain NPAM-type motifs, whereas all Cav2 subunits from parasitic trematodes examined to date contain polyacidic motifs of 15 amino acids or longer (Table 2). Thus, the polyacidic motif in the N terminus of the only conventional β subunit in S. mansoni (7) could represent a new gene function that appeared after the evolution of the parasitic lifestyle. In their native context, β2S subunits may decrease the total amount of Ca2+ that enters through Cav2 channels not only by accelerating inactivation and by decreasing current density (this work) but also by promoting Ca2+ current run down (11). The putative Ca2+−binding properties of NPAM suggest possible additional roles in the schistosome neuromuscular system, including perhaps as a transcription factor.

Conclusion—In summary, we have identified a structural determinant of Cav2 channel inactivation, a polyacidic motif in the N terminus of a Cav2 subunit, that differs greatly from those previously known. We have discussed several mechanisms by which this long polyacidic motif may induce fast kinetics of inactivation; (i) by modifying voltage−dependent inactivation, (ii) by preventing interaction of α1 and G-proteins, and (iii) by means of its putative Ca2+−binding function. This study highlights a unique feature of the schistosome Cav2 channel complex that is potentially amenable to pharmacological manipulation.

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Acknowledgments—We thank Dr. Toni Schneider for the HEK cell line stably expressing human Cav2.3, Dr. Diane Lipscombe for the β3 and α6-1 subunits, and Dr. Annette Dolphin for the β14 subunit. We thank William Morgan and Dr. Ravi Kasinathan for help with Western blots and Dr. Lingli Zhang for advice on confocal imaging. We also appreciate the critical comments provided by Dr. Richard Horn, Dr. Ji-Fang Zhang, and Dr. Cristina Veláquez. Schistosome nucleic acids and proteins used in these experiments were generated from material supplied by the NIAID Schistosomiasis Resource Center at the Bio medical Research Institute (Rockville MD) through NIAID, National Institutes of Health Contract N01-AI-30026.
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