

# Leaky sodium channels from voltage sensor mutations in periodic paralysis, but not paramyotonia



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## ABSTRACT

**Background:** Hypokalemic periodic paralysis (HypoPP) is associated with mutations in either the  $Ca_v1.1$  calcium channel or the  $Na_v1.4$  sodium channel. Some  $Na_v1.4$  HypoPP mutations have been shown to cause an anomalous inward current that may contribute to the attacks of paralysis. Herein, we test whether disease-associated  $Na_v1.4$  mutations in previously untested homologous regions of the channel also give rise to the anomalous current.

**Methods:** The functional properties of mutant  $Na_v1.4$  channels were studied with voltage-clamp techniques in an oocyte expression system.

**Results:** The HypoPP mutation  $Na_v1.4$ -R1132Q conducts an anomalous gating pore current, but the homologous R1448C mutation in paramyotonia congenita does not.

**Conclusions:** Gating pore currents arising from missense mutations at arginine residues in the voltage sensor domains of  $Na_v1.4$  are a common feature of HypoPP mutant channels and contribute to the attacks of paralysis. *Neurology*® 2011;76:1635-1641

## GLOSSARY

**HypoPP** = hypokalemic periodic paralysis; **PMC** = paramyotonia congenita.

Hypokalemic periodic paralysis (HypoPP) is a channelopathy of skeletal muscle for which the causative mutation may be either in *CACNA1S*<sup>1,2</sup> encoding the  $Ca_v1.1$  L-type calcium channel (~60% of families) or in *SCN4A*<sup>3,4</sup> encoding the  $Na_v1.4$  sodium channel (~20% of families). Fourteen of the 15 identified HypoPP mutations are missense substitutions at arginine residues in the voltage-sensor domains of either  $Ca_v1.1$  or  $Na_v1.4$  (figure 1). This remarkable convergence suggests there may be a common functional defect produced by these voltage-sensor mutations that would explain why a mutation in either channel causes HypoPP.<sup>5</sup>

Expression studies of HypoPP mutant sodium channels have revealed an anomalous “gating pore” current that is active at the resting potential.<sup>6–8</sup> This small current (<1% of the peak  $Na^+$  current during an action potential) may cause the aberrant depolarization during attacks of weakness.<sup>9,10</sup> Prior studies have demonstrated that all 5 missense mutations at the arginines of the domain II voltage sensor in  $Na_v1.4$  support a gating pore current.<sup>6–8</sup> An arginine to glutamine mutation in the domain III voltage sensor (R1132Q) was recently reported in HypoPP.<sup>11</sup> We now show that R1132Q creates a gating pore current, thereby increasing the number of HypoPP  $Na_v1.4$  mutants with this anomalous current to 6 of all 6 tested, and also extending the affected sites to the domain III voltage sensor. Moreover, we show that a homologous arginine to cysteine mutation in the domain IV voltage sensor (R1448C) associated with paramyotonia congenita (PMC) does not produce a gating pore current.

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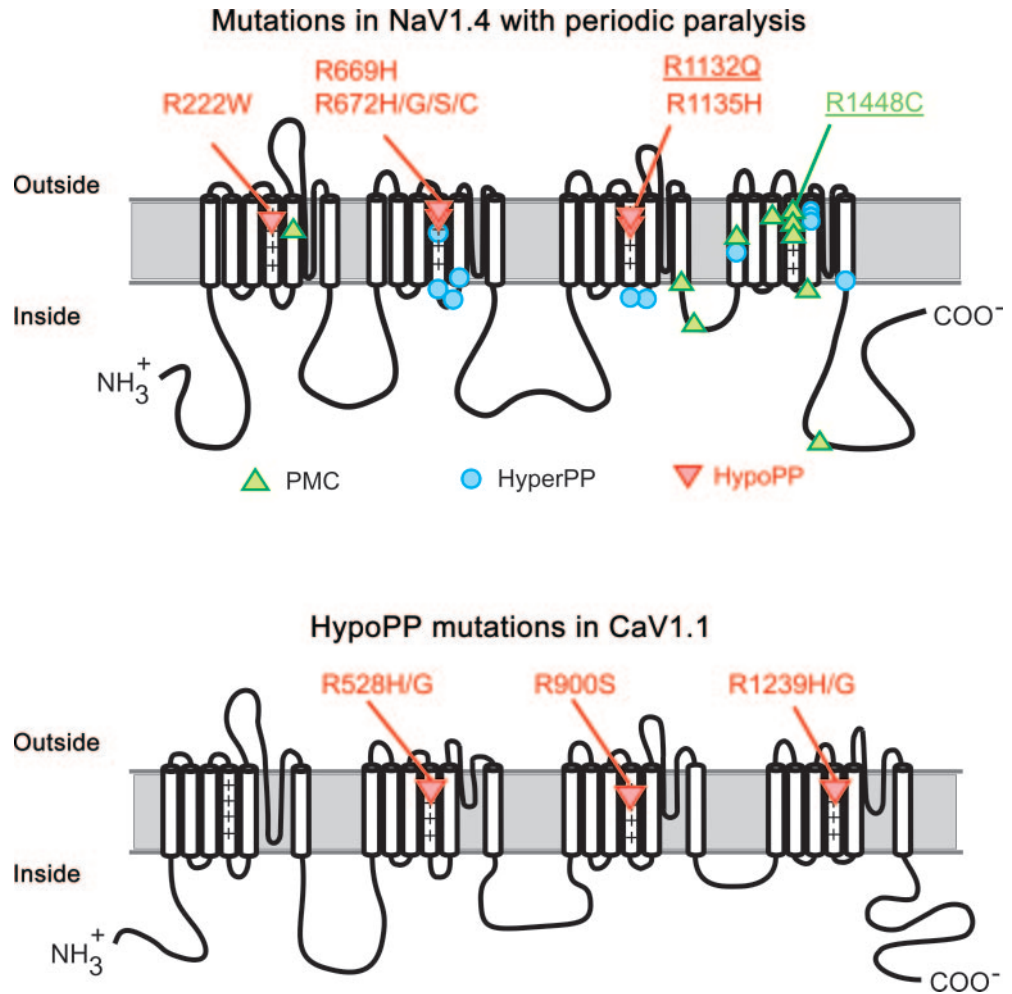
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**Figure 1** Mutations of the sodium channel (Na<sub>v</sub>1.4) or calcium channel (Ca<sub>v</sub>1.1) associated with periodic paralysis



Diagrams show the membrane folding topology and location of missense mutations associated with periodic paralysis. Three allelic disorders with periodic paralysis occur in Na<sub>v</sub>1.4 (paramyotonia congenita [PMC], hyperkalemic periodic paralysis [HyperPP], hypokalemic periodic paralysis [HypoPP]). HypoPP mutations (red triangles) occur at arginine residues (R) in the voltage-sensor domains of Na<sub>v</sub>1.4 or Ca<sub>v</sub>1.1. The Na<sub>v</sub>1.4 mutations investigated in this study are underlined.

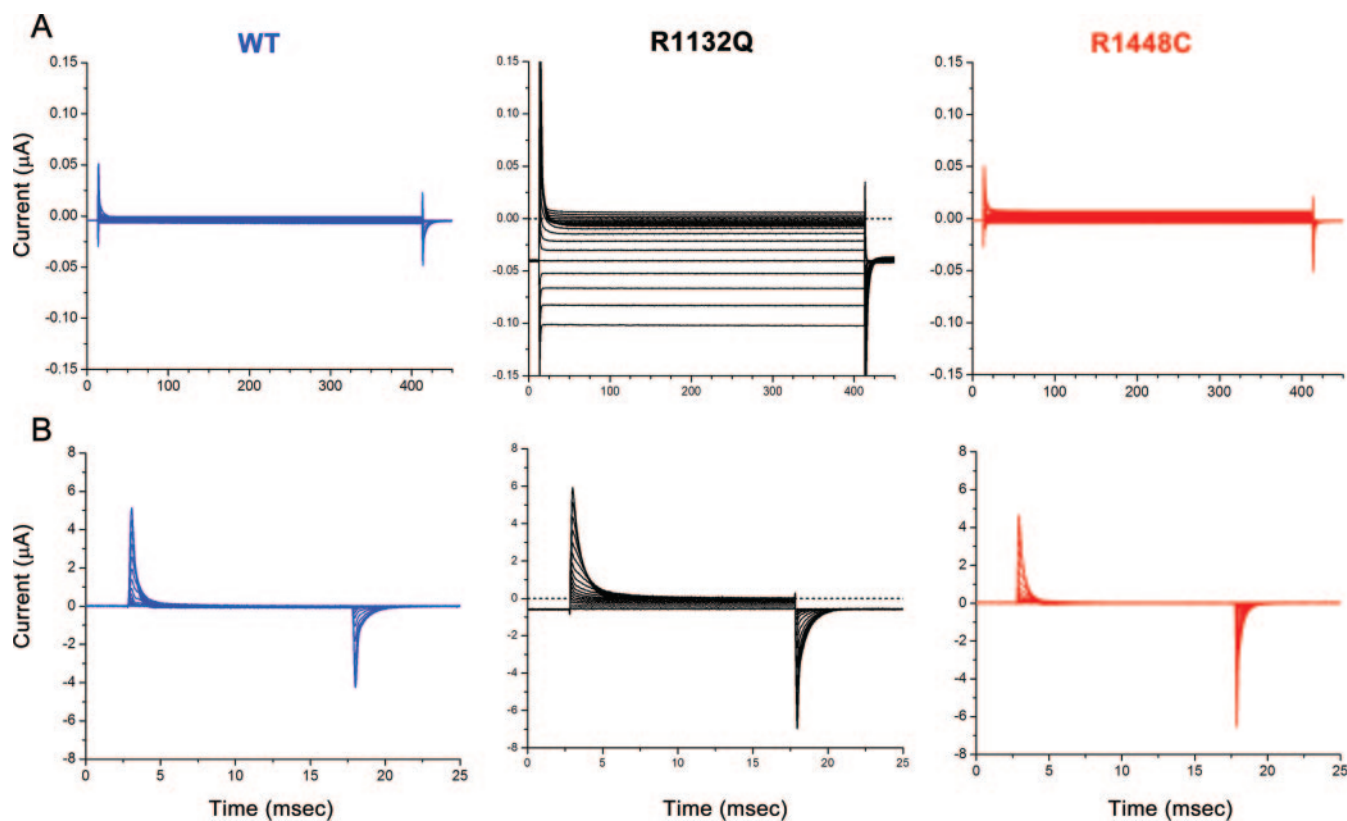
**METHODS Construction of Na<sub>v</sub>1.4 mutants.** The rat adult skeletal muscle sodium channel  $\alpha$  subunit cDNA (rNa<sub>v</sub>1.4) was subcloned into the vector pGEMHE, which is optimized for high-level protein expression in *Xenopus* oocytes.<sup>12</sup> The R1125Q missense mutation of rNa<sub>v</sub>1.4 (ortholog of the human HypoPP mutation R1132Q) was introduced using the QuikChange Mutagenesis kit (Stratagene, LA Jolla, CA) and confirmed by sequencing the entire cDNA. The rNa<sub>v</sub>1.4-R666G mutant in pGEMHE (ortholog of human HypoPP mutation R672G) was generated previously in our laboratory.<sup>8</sup> The rat Na<sub>v</sub>1.4-R1441C mutant (ortholog of human R1448C) was provided by F. Bezanilla (University of Chicago) in the pBSTA vector. Throughout this article, the rat Na<sub>v</sub>1.4 mutants are referred to by their corresponding human Na<sub>v</sub>1.4 numbering for ease of comparison to the literature on human HypoPP mutations. cRNA was synthesized by in vitro transcription using the mMessage mMachine kit (Ambion, Austin TX).

**Expression in oocytes.** Frogs (*Xenopus laevis*) were housed in an AAALAC-accredited facility, and all procedures were performed within guidelines established by the UTSW Institutional

Animal Care and Use Committee. Stage V oocytes were injected with 50 ng of cRNA encoding either WT or mutant rNa<sub>v</sub>1.4, along with 50 ng (10-fold molar excess) of cRNA encoding the human sodium channel  $\beta$ 1 subunit.<sup>13</sup>

**Electrophysiology.** Currents were recorded from oocytes voltage-clamped in the cut-open configuration, using the CA-1B amplifier (Dagan Corporation, Minneapolis, MN), as described previously.<sup>8</sup> The recording chamber was modified to allow gravity-flow perfusion to exchange the solutions on both the extracellular and intracellular faces of the oocyte membrane. The external bath to approximate the mammalian physiologic monovalent cation gradient contained (in mM) 112 NaOH, 3 KOH, 1.5 CaOH<sub>2</sub>, 4.5 BaOH<sub>2</sub>, 10 HEPES, pH 7.4 with methane sulfonic acid. For ionic substitution experiments, NaOH was replaced by KOH or N-methyl-D-glucamine (NMDG<sup>+</sup>). All external solutions contained 1  $\mu$ M tetrodotoxin (TTX, Tocris Chemicals) to block ionic currents through the Na<sub>v</sub>1.4 pore. The internal solution contained (in mM) 103 KOH, 7 NaOH, 10 EGTA, 10 HEPES, pH 7.4. For the ionic selectivity experiments in figure 3B, the internal KOH and NaOH were replaced

**Figure 2** Currents recorded from voltage-clamped oocytes expressing Na<sub>v</sub>1.4 constructs



(A) The currents elicited by a series of step depolarizations of 400 msec duration over a range of  $-140$  mV to  $40$  mV, in  $10$ -mV increments from a holding potential of  $-100$  mV. No leak subtraction has been performed; rapid transients are clipped. Only the HypoPP mutant R1132Q (black, middle trace) has large inward currents elicited at negative test potentials. Notice the R1132Q current offset at the holding potential of  $-100$  mV. Wild-type (blue) and PMC R1448C mutants (red) have only small background leakage currents. (B) Current responses for a series of brief 15-msec depolarizations from  $-140$  mV for the same oocytes. The linear background currents (leakage and capacitance) have been subtracted digitally to yield the gating charge displacement current. The area under the rapid current transient is an index of channel expression, and these data show that all 3 Na<sub>v</sub>1.4 constructs had comparable levels of expression at the surface membrane.

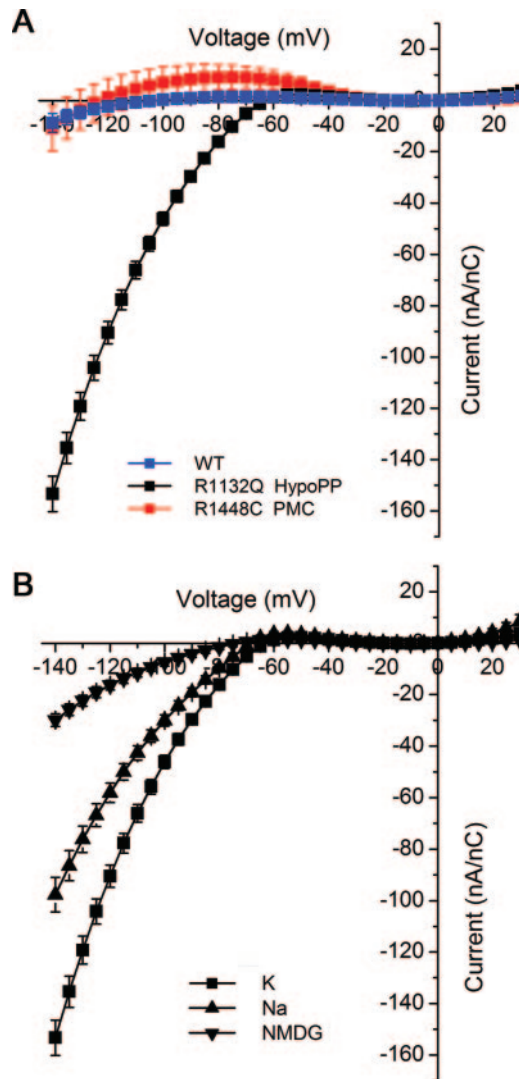
by  $110$  mM NMDG<sup>+</sup>. Currents were filtered at  $5$  kHz and sampled at  $100$  kHz for measurement of gating charge displacement, or  $1$  kHz and  $10$  kHz respectively for measurement of steady-state currents. Application of command pulse sequences and data acquisition were performed with pClamp 7.0 (Molecular Devices, Sunnyvale, CA). A  $-P/8$  subtraction protocol was used to remove the linear leakage and capacitance currents from the gating charge displacement measurements. No on-line leak subtraction was performed during the measurement of ionic gating pore currents. Recordings were performed at room temperature ( $22^{\circ}\text{C}$ ).

**RESULTS** A sustained inward (negative) current was observed in oocytes injected with cRNA encoding the HypoPP mutant R1132Q, but not for those injected with WT or the PMC mutant R1448C. The top row of figure 2 shows currents elicited in response to a series of voltage steps from  $-140$  to  $+40$  mV from a holding potential of  $-100$  mV. The small-amplitude proportionately spaced current responses for WT and the PAM mutant R1448C show the passive background leakage currents. For the HypoPP mutant

R1132Q, however, progressively larger amplitude current responses were observed for test potentials more negative than  $-60$  mV. These features are typical of anomalous gating pore currents, as reported for HypoPP mutations at arginines in positions R669 and R672.<sup>6,7</sup>

The absence of a detectable gating pore current in WT or PMC-R1448C channels is not caused by a failure of channel expression at the plasma membrane. The expression level of functional sodium channels can be quantified by recording the small current transients that arise from movement of the channel voltage sensors in response to step changes in membrane potential. These so-called gating charge displacement currents are revealed by linear subtraction of background capacitance current and linear ionic leakage currents, as shown in the tracings on the bottom row of figure 2. The area under these current transients reflects the number of charges that moved, and will saturate for large-amplitude voltage steps. The maximal charge displacement,  $Q_{\text{max}}$  in

**Figure 3** Gating pore current for the hypokalemic periodic paralysis (HypoPP) R1132Q mutant



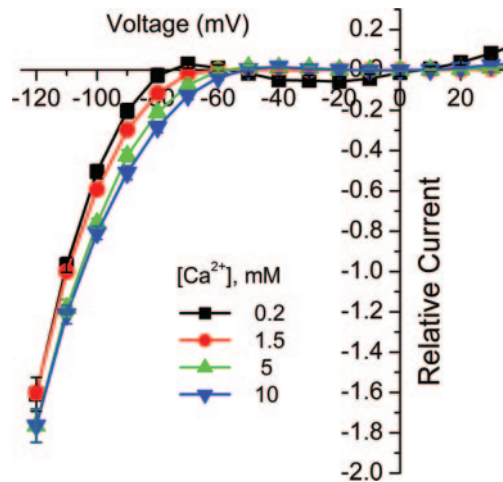
(A) Steady-state current recorded at the end of a 400-msec pulse for test potentials between  $-140$  mV and  $+30$  mV. The HypoPP R1132Q mutant passes an inward gating pore current at membrane potentials less than  $-60$  mV (inward rectification), whereas WT and the paramyotonia congenita (PMC) R1448C mutant do not ( $n = 4$  oocytes for WT and 5 for each mutant). Linear background currents have been subtracted by fitting the I-V response between  $-20$  mV and  $+20$  mV. (B) Selectivity of the R1132Q gating pore by measuring currents for a variety of test cations. The permeability sequence is  $K^+ > Na^+ > NMDG^+$  (N-methyl-D-glucamine). Under physiologic conditions, the primary charge carrier would be inward movement of  $Na^+$  ions.

nanocoulombs, is proportional to the channel expression level. Measurement of  $Q_{max}$  gives experimental verification of channel expression at the membrane and can be used to normalize the ionic currents recorded from different oocytes, thereby adjusting for variations in expression level and allowing data to be pooled from many oocytes.

The average gating pore current, normalized by expression level  $Q_{max}$ , is shown as a function of test potential in figure 3A. In these summary plots, the background nonspecific leakage currents have been removed by subtraction of a linear fit to the passive currents observed over a voltage range from  $-10$  mV to  $+10$  mV. Robust gating pore currents are observed for the HypoPP-R1132Q mutant, but not for WT or PMC-R1448C channels. To gain insight on which ions are permeating the channel to produce the gating pore current, we substituted the extracellular  $K^+$  by  $Na^+$  or the large organic molecule N-methyl-D-glucamine ( $NMDG^+$ ). The current amplitude was reduced by about 30% in  $Na^+$ , figure 3B, but was still detectable when the only major extracellular cation was  $NMDG^+$ . In contrast, the gating pore currents are insensitive to cation substitution for the histidine HypoPP mutations in domain II (R669H and R672H) because those gating pore currents are carried by protons. These data imply a rank order in relative permeability of  $K^+ > Na^+ \gg NMDG^+$ . Under physiologic conditions in vivo, the electrochemical gradient would favor inward  $Na^+$  flow as the major charge carrier for the anomalous gating pore current in HypoPP R1132Q channels.

Small molecules or ions that could block the anomalous gating pore current would be ideal therapeutic agents for reducing the severity and frequency for attacks of weakness in HypoPP. Consequently, much interest has been generated by reports that  $Ca^{2+}$  is a low-affinity blocker of gating pore currents.<sup>6,14</sup> In principle, this effect could underlie the beneficial effect of elevated extracellular  $Ca^{2+}$  to stabilize the resting membrane potential of HypoPP fibers studied in vitro,<sup>15</sup> although clinical improvement by the administration of calcium gluconate has been reported only in hyperkalemic periodic paralysis.<sup>16</sup> We measured the current-voltage response for gating pore currents from HypoPP-R1132Q mutant channels in a range of external  $Ca^{2+}$  from 0.2 mM to 10 mM. As shown in figure 4, external  $Ca^{2+}$  did not appreciably block the R1132Q gating pore current, although there was an apparent rightward (depolarized) shift in the I-V relation at high  $Ca^{2+}$  as would be expected for surface charge screening effects by the divalent cation.<sup>17</sup> For comparison to prior reports,<sup>14</sup> we also tested for  $Ca^{2+}$  block of gating pore currents in HypoPP-R672G channels, but again detected none (data not shown). We tested for block of HypoPP-R1132Q by other divalent cations at a concentration of 3.5 mM and a test potential of  $-100$  mV. No block was detected for  $Ba^{2+}$  (relative current of  $99 \pm 3.2\%$ ,  $n = 3$ ), and modest block was observed for  $Ni^{2+}$  ( $65 \pm 4.3\%$ ,  $n = 3$ ) and  $Zn^{2+}$  ( $53 \pm 4.1\%$ ,

**Figure 4** Calcium does not block the R1132Q gating pore

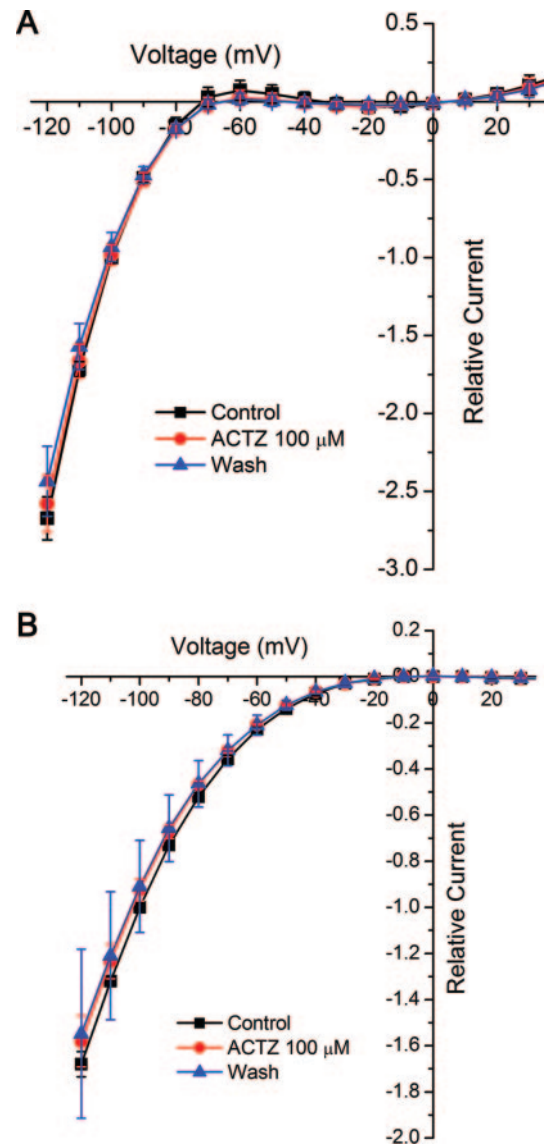


Hypokalemic periodic paralysis (HypoPP) R1132Q gating pore currents were recorded in high external  $K^+$  (100 mM) as the  $Ca^{2+}$  was varied from 0.2 to 1.0 mM. Each oocyte served as its own control for expression level, as exposure to all 4  $Ca^{2+}$  levels was performed and current amplitudes were normalized to the response at a test potential of  $-110$  mV in 1.5 mM  $Ca^{2+}$  ( $n = 3$  oocytes). The rightward shift of the I-V curve for higher  $[Ca^{2+}]$  is caused by an apparent shift in the voltage dependence of channel gating due to screening of surface charges on the plasma membrane.

$n = 3$ ). The carbonic anhydrase inhibitor acetazolamide is the preferred prophylactic agent for reducing the risk of an attack of weakness in HypoPP.<sup>18</sup> We therefore tested for block of gating pore currents in HypoPP-R1132Q and HypoPP-R672G mutant channels by acetazolamide. As shown in figure 5, 100  $\mu$ M acetazolamide did not block gating pore currents, across all voltages tested from  $-140$  mV to  $+40$  mV.

**DISCUSSION** A growing set of experimental data and modeling studies support the notion that an anomalous gating pore current is a fundamental mechanism for the loss of sarcolemmal excitability during attacks of weakness in HypoPP (reviewed in<sup>5</sup>). All 8 of the mutations in  $Na_v1.4$  associated with HypoPP are missense substitutions at arginine residues in the voltage sensor domains.<sup>19</sup> Prior studies have shown that all 5 mutations in the voltage sensor of domain II are capable of producing a gating pore current.<sup>6-8</sup> The present study now extends these observations to an arginine in the domain III voltage sensor (R1132Q). Moreover, we show specificity by demonstrating that a PMC mutation of an arginine in a homologous position for the voltage sensor of domain IV (R1448C) does not result in the creation of a gating pore. Rather, the PMC-R1448C mutation has a gain-of-function defect that is manifest as a slower rate of inactivation for the Na current through

**Figure 5** Acetazolamide (ACTZ) does not block gating pore currents in  $Na_v1.4$  hypokalemic periodic paralysis (HypoPP) mutants



Current-voltage plots show exposure to 100  $\mu$ M acetazolamide did not block the gating pore current for the HypoPP mutants  $Na_v1.4$ -R1132Q (A) or  $Na_v1.4$ -R672G (B). The current-voltage relation for the R672G mutant did not show saturation as we reported before,<sup>8</sup> because the oocyte was maintained at a holding potential of  $-30$  mV between test pulses.

the conventional pore.<sup>20</sup> Additional missense mutations at R1448 associated with PMC (leucine, proline, or serine) were tested, and we did not detect gating pore currents. The confidence was lower, however, to exclude these mutations as permissive for conducting gating pore currents because the expression level was lower ( $Q_{max} < 0.5$  nC, data not shown). Nevertheless, we propose it is unlikely that any PMC mutations at R1448 produce gating pore currents. This conclusion is based on the clear

absence of gating pore currents for R1448C and the fact that we have never encountered a mixed setting wherein some disease-associated substitutions at a particular S4 arginine produce a gating pore current while others do not. Curiously, a set of 3 missense mutations at the third arginine in domain II produce gating pore currents that are activated by depolarization and are closed at the resting potential.<sup>21</sup> These mutations (R657G/Q/W) cause potassium-sensitive normokalemic periodic paralysis,<sup>22</sup> for which the mechanistic link to a depolarization-activated gating pore current remains to be established. A variety of gain-of-function changes for the Na<sup>+</sup> current (impaired inactivation or enhanced activation) have been observed for more than 20 PMC and hyperkalemic periodic paralysis mutations studied to date,<sup>23</sup> but conversely have never been observed in studies of Na<sub>v</sub>1.4-HypoPP mutants.<sup>24,25</sup>

Computer simulations<sup>9</sup> and recordings from dissociated muscle fibers<sup>10</sup> have demonstrated that the gating pore current is sufficient to cause the instability of the muscle resting potential that produces a susceptibility to attacks of weakness in HypoPP. The gating pore current is active at the resting potential, and thereby depolarizes the fiber, but only by a few mV in normal external [K<sup>+</sup>]. More importantly, when external [K<sup>+</sup>] is reduced to ~3.0 mM or lower, then the affected fiber undergoes a sustained depolarization that renders the muscle inexcitable.<sup>5,9</sup> This depolarization in the setting of reduced external [K<sup>+</sup>] is paradoxical, in that normal fibers would hyperpolarize. For normal fibers, this paradoxical depolarized shift of V<sub>rest</sub> can occur, but only at an extremely low [K<sup>+</sup>] of ~1.5 mM.<sup>9</sup> The mechanism for the paradoxical depolarization in low [K<sup>+</sup>] is a failure of the inward rectifier K conductance to balance the depolarizing currents from the gating pore and leakage conductance.<sup>5</sup> As a result, the fiber depolarizes until the delayed rectifier K channels become active at about -50 mV.

In principle, a drug that could block the gating pore but otherwise not affect Na channel function would be an ideal therapy for HypoPP. We tested 2 agents, Ca<sup>2+</sup> and acetazolamide, that have been used to abort or prevent attacks of weakness in periodic paralysis. Our voltage-clamp studies did not reveal significant block of the gating pore current by either, and so presumably they must act through other mechanisms. Others have reported low-affinity block by Ca<sup>2+</sup> of the gating pore current for the HypoPP-R672G mutant.<sup>6,14</sup> We attribute this difference to experimental methodology. We used a rapid perfusion system to add and wash out test reagents, for which no discernable block occurred (figure 4). In prior studies, however, the high Ca<sup>2+</sup> was added to

the external media in an equal-volume 2× concentration and the currents were recorded after minutes of equilibration.<sup>6,14</sup> We have often observed a nonspecific rundown of the gating current amplitude and propose that this time-dependent effect produced an apparent block.

The most commonly affected gene in HypoPP is *CACNA1S*,<sup>3</sup> which encodes the Ca<sub>v</sub>1.1 calcium channel that serves as the voltage sensor for excitation-contraction coupling. Six of the 7 mutations in Ca<sub>v</sub>1.1-HypoPP are missense mutations at arginines in the voltage sensor domains. Sodium and calcium channels share many structural similarities (figure 1), including the S4 voltage-sensor domains. The possibility that mutations in the voltage-sensor domains of Ca<sub>v</sub>1.1 also create gating pore currents provides a unifying hypothesis for how mutations of 2 channels with very different functions in skeletal muscle, Na<sub>v</sub>1.4 and Ca<sub>v</sub>1.1, may result in a common clinical phenotype of HypoPP. Experimental demonstration of gating pore currents for HypoPP Ca<sub>v</sub>1.1 mutations is needed, since important functional differences exist between the homologous voltage-sensor domains, even within the Na<sub>v</sub>1.4 channel. The domain IVS4, for example, is coupled to inactivation gating and R1448C does not support a gating pore current, whereas domain IIS4 is coupled to activation and HypoPP mutations at 2 arginines in this S4 (R669, R672) are permissive for gating pore currents. Attempts to detect a gating pore current in Ca<sub>v</sub>1.1-HypoPP mutant channels have been hampered by the relatively poor expression level of the channel at the membrane in heterologous systems.

## AUTHOR CONTRIBUTIONS

David Francis performed the experiments, data analysis, and edited the manuscript. Volodymyr Rybalchenko performed the studies on channel blockers. Arie Struyk assisted with the experimental design and performed the mutagenesis. Stephen Cannon assisted with experimental design, data analysis, and wrote the manuscript.

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## DISCLOSURE

Dr. Francis and Dr. Rybalchenko report no disclosures. Dr. Struyk is an employee of Merck & Co., Inc. and receives compensation in the form of salary and stock; his spouse is an employee of Johnson & Johnson and receives compensation in the form of salary and stock; and receives research support from Merck & Co., Inc., Johnson & Johnson, and Muscular Dystrophy Association. Dr. Cannon serves on the scientific advisory board for Telethon (Italy); serves on the editorial advisory board of the *Journal of General Physiology*; and receives research support from the NIH/NIAMS.

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