

A Sodium Channel Defect in Hyperkalemic Periodic Paralysis: Potassium-Induced Failure of Inactivation

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Summary

Hyperkalemic periodic paralysis (HPP) is an autosomal dominant disorder characterized by episodic weakness lasting minutes to days in association with a mild elevation in serum K^+ . In vitro measurements of whole-cell currents in HPP muscle have demonstrated a persistent, tetrodotoxin-sensitive Na^+ current, and we have recently shown by linkage analysis that the Na^+ channel α subunit gene may contain the HPP mutation. In this study, we have made patch-clamp recordings from cultured HPP myotubes and found a defect in the normal voltage-dependent inactivation of Na^+ channels. Moderate elevation of extracellular K^+ favors an aberrant gating mode in a small fraction of the channels that is characterized by persistent reopenings and prolonged dwell times in the open state. The Na^+ current, through noninactivating channels, may cause the skeletal muscle weakness in HPP by depolarizing the cell, thereby inactivating normal Na^+ channels, which are then unable to generate an action potential. Thus the dominant expression of HPP is manifest by inactivation of the wild-type Na^+ channel through the influence of the mutant gene product on membrane voltage.

Introduction

Hyperkalemic periodic paralysis (HPP), also known as adynamia episodica hereditaria or Gamstorp's disease, is one member of a class of heritable paralyses that occur transiently in association with mild changes in serum K^+ concentration ($[K^+]$; Gamstorp, 1956). In HPP the weakness can be focal or generalized and is precipitated in affected patients by cold, rest after exercise, fasting, or K^+ ingestion. The latter three raise serum $[K^+]$, typically from the basal level of 3.5–4.5 mM to 5–7 mM. At least three subtypes of HPP have been delineated clinically: with persistent spontaneous action potentials in myofibrils after voluntary muscle contraction (myotonia), with normal relaxation after

contraction, and with cold-induced stiffness (paramyotonia). Affected members within a family show the same variant (for review, see Rüdél and Ricker, 1985).

In vivo measurements of membrane potentials from muscles of patients during spontaneous and K^+ -induced attacks demonstrate a marked depolarization of the resting potential (Creutzfeldt et al., 1963). During an induced attack, a rise in serum $[K^+]$ from 3.75 to 6.9 mM shifted the resting potential from -69 to -46 mV, 7 mV in excess of the shift predicted for a membrane permeable to K^+ alone. Creutzfeldt and co-workers suggested that the membrane of HPP muscle must have an abnormally elevated permeability to other ions and showed that a 3-fold increase in the ratio of Na^+ to K^+ permeability could, for example, account for the large depolarization. In experiments with acutely dissociated HPP muscle, Lehmann-Horn and others (1983, 1987) showed that the excessive depolarization is blocked by tetrodotoxin. Under voltage clamp, they found an abnormal steady inward current at potentials more positive than -60 mV that is blocked by tetrodotoxin. They also showed that raising extracellular $[K^+]$ from 3.5 to 10 mM causes a dramatic decrease in twitch tension over several minutes, which is reversed by acidification of the high $[K^+]$ bath with CO_2 . They concluded that a noninactivating Na^+ conductance exists in HPP muscle and that elevated serum $[K^+]$ depolarizes the resting potential enough to turn on this persistent inward current. This further depolarizes the cell and renders it incapable of firing an action potential. However, a Hodgkin-Huxley analysis of whole-cell currents measured in myoballs cultured from patients with HPP showed disappointingly little difference in activation and inactivation time constants or in the voltage dependence of inactivation (h_∞ curve) between normal and HPP muscle (Rüdél et al., 1989).

Recently, we have shown that the gene for the Na^+ channel α subunit from adult human muscle and that for the human growth hormone are tightly linked to the expression of HPP within a large kindred (Fontaine et al., 1990). The multipoint lod score is about 7, indicating that there is less than a 1 in 10^7 chance that these probes and the disease coinhere by chance. Most likely, the defect in HPP is in the Na^+ channel itself. To confirm that the channel is defective and to understand the functional defect in physiological terms, we have studied Na^+ channels in HPP muscle with single-channel recording. We find that high external $[K^+]$ induces steady, repetitive opening of these channels, consistent with a failure of inactivation, but at low probability. K^+ acts directly on the channel, rather than indirectly by depolarizing the muscle. This abnormality provides a pathophysiological explanation for the weakness in HPP.

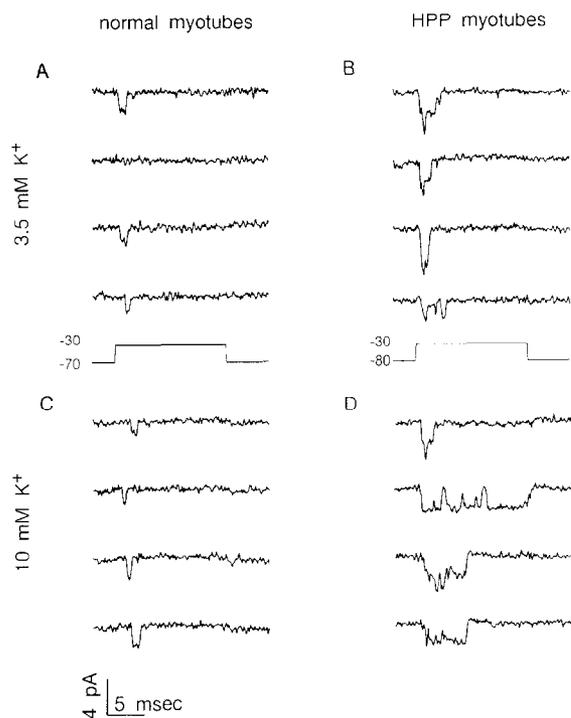


Figure 1. Na^+ Channel Gating in Normal and HPP Myotubes
Each of the four groups (A–D) shows the response for four consecutive depolarizing steps of 15 ms duration. Channel openings in excised, outside-out patches are shown as downward deflections. (A) and (B) show the response in 3.5 mM external K^+ . (C) and (D) show the response for the same two patches after changing to an extracellular solution with 10 mM K^+ . Current amplitude and time base scale bars apply to all traces.

Results

Single-channel Na^+ currents were measured in myotubes cultured from a patient with the myotonic form of HPP and from a human control with no neuromuscular disease. Recordings were obtained from both excised and cell-attached membrane patches (Hamill et al., 1981). Depolarizing voltage steps activated inward currents in both normal and HPP myotubes (Figure 1). In excised patches with an “outside-out” orientation, this current was completely and reversibly blocked by adding 100 nM tetrodotoxin to the bath. Since the juvenile form of the voltage-dependent Na^+ channel is considerably less tetrodotoxin sensitive than the adult form (Gonoi et al., 1985; Weiss and Horn, 1986), this indicates that the observed currents were conducted through the adult form of the channel. The single-channel conductance, measured by the currents evoked by depolarizations to between -40 and -20 mV, was about 19 pS and was not significantly different in HPP ($n = 2$ patches) and normal ($n = 3$) myotubes between either the high or the low $[\text{K}^+]$ bath.

A striking difference, however, was found in the gating of the unitary Na^+ currents in the two types of muscle. Single-channel currents were measured with

3.5 mM (Figure 1A) or 10 mM (Figure 1C) K^+ in the bath solution for the same outside-out patch containing 2 Na^+ channels from normal muscle. At both normal and high external $[\text{K}^+]$, the channel(s) enters an inactive state within about 4 ms of the depolarizing step, and there are no further openings. The transition from a resting or closed state(s) to the inactive state may be either through the open state (Figure 1A, traces with openings) or directly from the closed to the inactive state (traces without openings). The right side of Figure 1 shows the response of Na^+ channels in a patch excised from an HPP myotube. In 3.5 mM external K^+ , most channels also inactivate within 4 ms, as shown in Figure 1B. This patch contained 4 channels, and the multiple openings within each trace may be caused by separate channels opening with different latencies, rather than 1 channel reopening. In 3.5 mM K^+ , the mean open time was independent of membrane potential for potentials more positive than -50 mV, and the mean determined from 3 excised HPP patches ($0.85 \pm .14$ ms, SEM) was comparable to that of the channels from normal myotubes ($0.76 \pm .03$ ms, $n = 7$ patches). When the $[\text{K}^+]$ of the bath was raised to 10 mM, channels in the patch changed to a dramatically altered gating mode. Typically, one of the channels in a patch would switch to a noninactivating mode, as evidenced by persistent openings throughout the depolarizing step. Since we seldom observed simultaneous openings late in the step, and the probability of being in the open state was high in the noninactivating mode, this persistent current is most likely due to reopening of the same channel (see below). The dwell time in the open state was also increased, at times up to tens of milliseconds (see Figure 2).

Patch excision may alter the kinetics of Na^+ channel gating. In neuroblastoma cells, the process of pulling off a patch of membrane shifts the voltage dependence of inactivation in the depolarizing direction and causes longer open times (Aldrich and Stevens, 1987). Na^+ channels in outside-out patches excised from rat cardiac muscle have markedly increased open times and bursts of multiple reopenings (Kirsch and Brown, 1989). We observed excessive reopenings and slightly increased open times in 2 of 9 patches excised from normal myotubes. Therefore, we used cell-attached patches for quantitative comparison of gating in Na^+ channels from normal and HPP myotubes. The K^+ concentration of the electrode solution, on the external face of the membrane, was either 0 or 10 mM. In this recording configuration the intracellular $[\text{K}^+]$ remained normal, 150 mM.

Figure 2 shows Na^+ current observed for long depolarizing steps in a cell-attached patch from an HPP myotube. Depolarizations in which a channel failed to inactivate are obvious and tend to cluster in consecutive traces (Figure 3). Note that the time scale in Figure 2 is 4 times longer than that in Figure 1. We seldom observed more than 1 noninactivating channel open late in the depolarization, even in patches containing many Na^+ channels. Furthermore, when noninactiva-

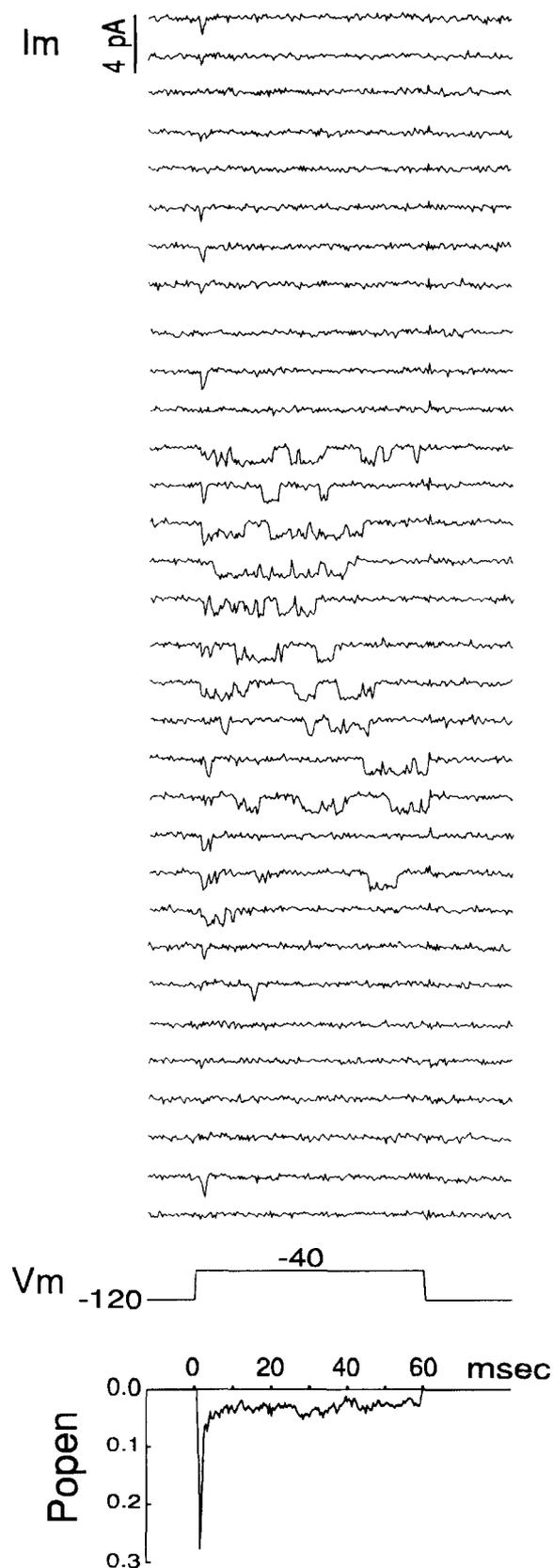


Figure 2. Clusters of Na⁺ Channel Gating in the Noninactivating Mode
 Thirty-two consecutive traces show the Na⁺ current recorded

tion occurred the open probability was high. These two properties imply that the probability of a channel entering the abnormal, noninactivating mode of gating is low and that the *stationary* open probability of a channel in the noninactivating mode (see ensemble-averaged current at the bottom of Figure 2) can be estimated from the proportion of time a channel is in the open state late in the depolarization (see Experimental Procedures). For the patch from which the records in Figure 2 were obtained, the open probability in the noninactivating mode, P_{oni} , was 0.58. The average P_{oni} from 6 cell-attached patches with 10 mM K⁺ in the electrode by 0.51 ± 0.05 (95% confidence limits, SEM).

With the assumption that half of the Na⁺ channels in myotubes cultured from a patient with HPP are from the mutant gene, the probability that an abnormal Na⁺ channel is in the noninactivating mode, P_m , can be estimated from the fraction of depolarizing steps that contain a noninactivating channel (see Experimental Procedures). This technique relies on the ability to detect the failure of inactivation unambiguously. Most of our data are from depolarizing steps of 15 ms duration. Therefore we designated traces with a noninactivating Na⁺ channel as those depolarizations for which the integral of the late current, between 5 and 15 ms, exceeded a threshold. Figure 3 shows the value of this integral for individual depolarizations of cell-attached patches from normal and HPP myotubes, all with high [K⁺] in the pipette. Notice that Na⁺ channels in HPP myotubes failed to inactivate for both mild (-40 mV; Figure 3B) and stronger (0 mV; Figure 3C) depolarizations. For the 9 cell-attached patches in HPP myotubes for which there were sufficient data to complete the quantitative analysis, the likelihood of inactivation failure had no clear voltage dependence over a 40 mV range of test potentials. The dashed line in Figure 3 illustrates the threshold we used to define depolarizing steps that failed to inactivate. For this threshold value, the mean probability of an HPP Na⁺ channel entering the noninactivating mode of gating, P_m , was 0.058 ± 0.027 ($n = 9$ patches, data pooled for all depolarizing potentials). Since P_m is an operational definition of the probability of abnormal gating that depends on a somewhat arbitrarily defined threshold, occasionally normal myotubes evidenced enough late openings to exceed the threshold and yield a nonzero estimate of P_m . This effect became more pronounced in patches with many (>4) normal

during individual 60 ms depolarizations, presented at 1.25/s, from a cell-attached patch on an HPP myotube with 10 mM K⁺ in the electrode. Capacitance transients have been subtracted; the brief downward deflection at the beginning of many traces is the current from a normally gated Na⁺ channel. The prepulse and test potentials were -120 mV and -40 mV, respectively (both relative to the cell's resting potential). The trace at the bottom shows the single-channel open probability computed from the 256 traces in this run, based on the maximum likelihood estimate that there was only 1 channel in this patch.

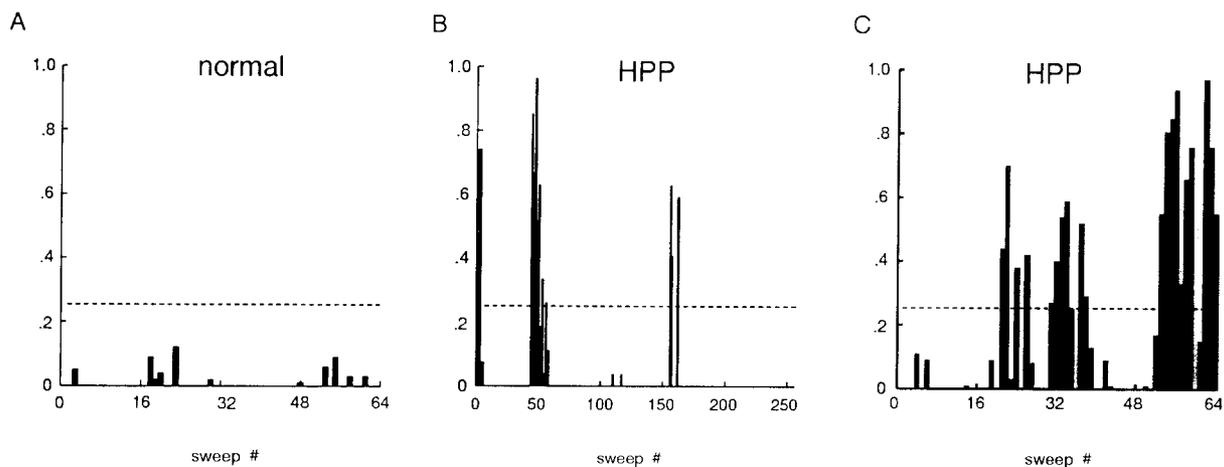


Figure 3. Loss of Na⁺ Channel Inactivation in Cell-Attached Patches

Channels entered and left the noninactivating mode with a time course of several seconds. Each vertical bar depicts the time-averaged number of channels open in the period between 5 and 15 ms after the onset of a single depolarizing voltage step. Late openings are rare in normal myotubes (A), but occur in clusters for HPP myotubes (B and C). The clusters of K⁺-induced loss of inactivation in HPP muscle usually occur at low probability (in [B], $P_m = 0.056$, same patch as in Figure 2, which shows sweeps 33–64), but may be more frequent (in [C], $P_m = 0.22$, different patch). The dashed line shows the threshold criterion used to define a sweep that failed to inactivate. All recordings were obtained from cell-attached patches with 10 mM K⁺ in the electrode; sweeps were presented at 1.25/s. Clamp potentials (relative to the cell's resting potential) were (A) normal, $V_{\text{hold}} = -80$ mV, $V_{\text{test}} = -30$ mV; (B) HPP, $V_{\text{hold}} = -120$ mV, $V_{\text{test}} = -40$ mV; (C) HPP, $V_{\text{hold}} = -100$ mV, $V_{\text{test}} = 0$ mV.

Na⁺ channels. Values of P_m measured in patches from 9 HPP and 5 normal myotubes are shown in Figure 4 for low (0 mM) and high (10 mM) external [K⁺]. The probability of entering the noninactivating mode clearly increased with external [K⁺] for HPP (Figure 3; triangles) but not for normal (circles) myotubes.

How does K⁺ cause HPP Na⁺ channels to enter the noninactivating mode? Does K⁺ act directly on the extracellular portion of the channels, or does a second messenger system convey the signal to the intracellular domain, where the inactivation process is thought to reside? Two lines of evidence favor the former explanation. First, cell-attached patches were formed on HPP myotubes with 0 mM K⁺ in the electrode, and myotubes were then superfused with 10 mM K⁺. Under these circumstances, in which high [K⁺] did not reach the extracellular face of the channels, we did not observe an increase in the probability of late opening. Second, Na⁺ channels in excised outside-out patches did show late openings when the bath [K⁺] was raised (Figure 1). This implies that the influence of external [K⁺] on Na⁺ channel inactivation is not mediated by a freely diffusible intracellular second messenger. The local [K⁺] at the external face of an HPP Na⁺ channel (or perhaps in the pore) apparently governs the mode of inactivation.

Lowering pH was found to reverse the K⁺-induced decrease of twitch tension measured *in vitro* from HPP muscle (Lehmann-Horn et al., 1987). We consequently measured the influence of pH on the gating of Na⁺ channels. When the pH of the high [K⁺], cell-attached electrode was decreased from 7.4 to 6.8 with

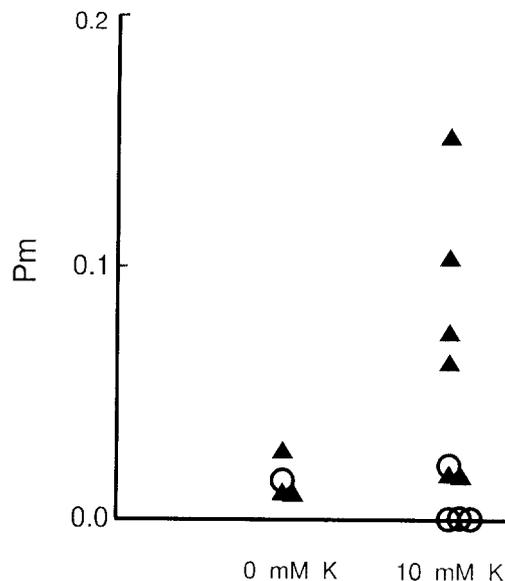


Figure 4. K⁺ Dependence of Inactivation Loss

Each symbol represents the average response of a single cell-attached patch, measured from hundreds of depolarizing steps. P_m , the probability of a channel entering the abnormal gating mode, represents the fraction of traces that exceeded our threshold for qualifying as a noninactivating Na⁺ current, normalized by the number of channels in the patch (see Experimental Procedures). For Na⁺ channels in HPP myotubes, elevated external [K⁺] strongly increased the probability of switching into a noninactivating mode (triangles). Na⁺ channels from normal myotubes (circles) inactivated quickly and therefore rarely crossed the threshold for detecting a noninactivating channel, regardless of external [K⁺]. The cells were bathed in saline containing 3.5 mM K⁺, thus the membrane under the patch pipette containing "0 mM K⁺" had been previously exposed for hours to 3.5 mM K⁺.

HCl, reopenings with long open times still occurred in 2 of 3 patches from HPP myotubes. Thus a 4-fold elevation of extracellular [H⁺] did not influence the ability of K⁺ to induce the noninactivating mode of HPP Na⁺ channels; the pH effect may be more complicated than a competitive interaction with K⁺.

Discussion

Linkage and Single-Channel Data Implicate a Defective Na⁺ Channel in HPP

We have demonstrated a highly anomalous form of Na⁺ channel gating in myotubes cultured from a patient with HPP. The muscle donor is a member of the family in which our previous study (Fontaine et al., 1990) demonstrated a strong linkage between the gene for the human Na⁺ channel α subunit and the expression of HPP. The direct single-channel evidence of abnormal Na⁺ channel function supports the linkage data and strengthens the hypothesis that the defect is in the function of the Na⁺ channel itself. The lack of evidence that the K⁺-induced failure of inactivation is mediated by a diffusible second messenger system further supports this hypothesis.

HPP is, to our knowledge, the first genetic disease in humans shown to be caused by a defect in an ion channel of the plasma membrane. Cystic fibrosis is attributed to a defect in ion transport across the plasma membrane, but the defective gene (Riordan et al., 1989; Drumm et al., 1990) codes for the "cystic fibrosis transmembrane regulator" (Welsh and Liedtke, 1986; Schoumacher et al., 1987), which is probably a cAMP-dependent modulator of a Cl⁻ conductance, rather than the ion channel itself. Malignant hyperthermia has been shown to be genetically linked to the gene for the ryanodine receptor, the Ca²⁺ release channel of the sarcoplasmic reticulum (MacLennan et al., 1990). This channel is defective in a porcine model of malignant hyperthermia (Fill et al., 1990), and although the physiological defect has not yet been confirmed in humans, the human defect is likely to be the channel as well.

An Extracellular Influence on Na⁺ Channel Inactivation

The mechanism of the external K⁺-dependent loss of inactivation in HPP remains unknown, but is particularly intriguing because proteases (Armstrong et al., 1973), N-bromoacetamide (Oxford et al., 1978; Patlak and Horn, 1982), and antibodies capable of destroying or slowing Na⁺ channel inactivation (Vassilev et al., 1988) all act at a cytosolic domain of the channel protein. Oocyte expression of altered mRNA for the rat brain Na⁺ channel II has shown that the sequence coding for the cytoplasmic linkage between repeats III and IV is necessary for at least one component of voltage-dependent inactivation (Stühmer et al., 1989). How then does 5–10 mM K⁺ in the extracellular solution alter a function of the channel thought to be intracellular, when the normally high intracellular [K⁺]

(~150 mM) has no effect? There is no obvious answer. To date, the only evidence for an extracellular cation influence on inactivation occurs in the presence of a peptide neurotoxin. Neurotoxins from scorpion, sea anemone, and coral inhibit Na⁺ channel inactivation from the extracellular face of the membrane. Slowing of inactivation by the coral-derived goniopora toxin is increased by extracellular monovalent cations, including K⁺; but unlike the case in HPP myotubes, Na⁺ was the most effective cation (Gonoi et al., 1986).

We have presented two lines of evidence suggesting that the loss of Na⁺ channel inactivation is not mediated by a diffusible second messenger and that extracellular K⁺ directly alters the probability of channel gating in the abnormal mode. First, the probability of observing a noninactivating Na⁺ channel increased with bath [K⁺] in excised, outside-out patches as shown in Figure 1. In this configuration of single-channel recording, the cytoplasmic face of the patch is exposed to the electrode solution, which was a high [Cs⁺] solution to block K⁺ channels in these patches. Any diffusible second messenger and internal K⁺ were presumably dialyzed to negligible concentrations by exchanging with the electrode solution. The ability of the Na⁺ channel to enter the noninactivating mode must be independent of these factors. Second, Na⁺ channels within a cell-attached patch electrode that did not contain K⁺ did not show an increased probability of noninactivating, even when the remainder of the cell was subsequently superfused with 10 mM K⁺ bath solution.

We consequently view the gating of the HPP Na⁺ channel as a switch between two modes. Studies on many tissues in several species have demonstrated an altered mode of Na⁺ channel gating with bursts of reopenings and increased open times. A noninactivating Na⁺ current occurs in frog skeletal muscle (Patalak and Ortiz, 1986, 1989), cardiac Purkinje fibers (Reuter, 1968), squid axon (Shoukimas and French, 1980), and hippocampal neurons (French et al., 1990), but only in about 0.02% of the depolarizing steps. Disruption of the normal internal milieu by patch excision in either the outside-out or the inside-out configuration increases tremendously the probability of long open times and bursts of reopenings (Kirsch and Brown, 1989). In the cell-attached mode when the extracellular face of the channel is exposed to low [K⁺], the HPP Na⁺ channel has a high probability (>99%) of gating in a normal mode (able to inactivate), which appears to be indistinguishable from the behavior of wild-type Na⁺ channels. At higher external [K⁺], the HPP Na⁺ channel has a small (5%–10%) but functionally significant probability of switching to an aberrant mode of gating for which inactivation is defective. Many kinetic details, however, remain to be clarified about this abnormal mode of gating.

The possibility of modal changes in Na⁺ channel gating and the novel influence that external [K⁺] has on this process may provide a unique opportunity to examine structure-function relationships in Na⁺ chan-

nels. Because we have shown a strong genetic linkage between HPP and the adult form of the Na⁺ channel α subunit (Fontaine et al., 1990), one expects that sequence analysis of the Na⁺ channel gene cloned from a family with HPP may reveal a defect in the coding region, thereby providing a structural basis from which to explore the influence of K⁺ on inactivation gating. Without specific sequence data, however, it is not possible to exclude the possibility of another defective gene, closely linked to, but separate from the α subunit locus on chromosome 17. Expression of functional Na⁺ channels in oocytes injected with mRNA transcribed from the cDNA of the α subunit (Trimmer et al., 1989) or high molecular weight mRNA isolated from rat brain (Auld et al., 1988) produces macroscopic currents with abnormally slowed transients and single-channel currents with prolonged open times and bursts of reopenings. The normally rapid termination of the macroscopic Na⁺ current can be restored by coinjection with a low molecular weight brain mRNA, which suggests that a small protein distinct from the α subunit may participate in the inactivation process (Auld et al., 1988). Injection of the α subunit alone into a somatic cell line also results in the expression of Na⁺ channels with normal kinetics (Scheuer et al., 1990). More importantly, Moorman et al. (1990) have shown that oocyte expression of a single mRNA from type III Na⁺ channel derived from rat brain can produce two dramatically distinct modes of gating. In approximately 30% of the depolarizing steps, the channel does not enter an absorbing inactive state; rather, bursts of reopenings occur with a 2-fold increase in mean open time. Their patches contained multiple channels, but on a statistical basis it appears that an individual channel can gate in either a normally inactivating or a noninactivating mode. The majority of the records were obtained in cell-attached patches for which the resting potential was reduced by using a 150 mM K-aspartate bath solution. It is not clear what role the elevated [K⁺] may have had in producing the abnormal mode of gating.

Inactivation Failure and the Pathogenesis of HPP: A Novel Mechanism for Dominant Inheritance

The mechanism whereby one mutant autosomal gene in a pair of alleles results in the dominant expression of a heritable disease is often obscure. In HPP we propose that when extracellular [K⁺] is raised, the mutant gene product inactivates both the wild-type Na⁺ channels and the HPP Na⁺ channels operating in the normal mode by its effect on membrane potential. Modest elevation in extracellular [K⁺], for instance in response to exercise, may cause a small proportion of HPP Na⁺ channels to switch into a noninactivating mode of gating (approximately 3%–15%; see Figure 4). The electrophysiological consequence of this small fraction of abnormally functioning channels would be great. K⁺-induced depolarization opens them, but they fail to inactivate. The persistent inward Na⁺ current would further depolarize the cell's resting potential. Depolarization causes an increased outward K⁺ current,

which may raise the external [K⁺] and further amplify the effect by positive feedback. Eventually the depolarizing shift in membrane potential may be large enough to inactivate a large fraction of the Na⁺ channels functioning in their normal mode. In other words, the few noninactivating Na⁺ channels would inactivate the normal ones through an effect on the resting potential. The end result would be a moderately depolarized cell incapable of generating an action potential. Notice that raised extracellular [K⁺] plays two roles in this mechanism: mild depolarization of the resting potential, as approximated by the Nernst equation, which opens Na⁺ channels; and increased probability of an HPP Na⁺ channel gating in the noninactivating mode, which further depolarizes the cell. This hypothesis provides a novel mechanism for the dominant expression of a heritable disorder and explains why the defect was not evident from the whole-cell voltage-clamp recordings of Rüdél et al. (1989). In their experiments, the voltage clamp held the membrane at a potential that restored function of the normal channels and the small, noninactivating inward current was obscured by the current through normal channels.

A mutation that caused a smaller proportion of channels to be in the noninactivating mode might cause the resting potential to approach the voltage threshold of an action potential without inactivating a significant proportion of channels, thereby causing hyperexcitability with spontaneous activity, as seen in myotonic states. Noninactivating Na⁺ channels have been observed in myotonic dystrophy (Franke et al., 1990), paramyotonia congenita (Lehmann-Horn et al., 1987), and chondrodystrophia myotonica (Lehmann-Horn et al., 1990), suggesting that Na⁺ channel mutations could be a general feature of the myotonias. Thus the electrical state of the muscle could be at either extreme—unexcitable or spontaneously active—depending on the relative position and steepness of the voltage dependence of activation and inactivation of the normally gated Na⁺ channels.

Experimental Procedures

Biopsy and Culture of Human Muscle

HPP muscle was obtained from a biopsy of one member of the family for which the genetic linkage between HPP and the α subunit of the Na⁺ channel was established (Fontaine et al., 1990). A control specimen was obtained from a patient with no known neuromuscular disorder who was undergoing surgery. The biopsy material was dissociated, and primary myogenic cultures were established as previously described (Levi et al., 1987). Myoblasts were passaged with trypsin-EDTA, plated on coverslips that had been cleaned with KOH-ethanol, and cultured for 2–3 weeks in Dubecco's modified Eagle's medium with 5% fetal calf serum. A coverslip was then transferred to the recording chamber on the stage of a Zeiss IM-35 microscope, where gigaohm seals were formed with patch pipettes without the need for enzymatic treatment to loosen cells from the growth substrate.

Patch-Clamp Recording

Standard procedures were used for pipette fabrication, seal formation, and single-channel recording in the cell-attached and excised patch modes (Hamill et al., 1981). Patch-clamp electrodes were constructed from microcapillary glass (VWR Scientific),

pulled in a two-stage process to a tip internal diameter of approximately 1 μm. The electrode was then coated with Sylgard to reduce capacitance and fire-polished to a final tip size with a bubble number of 5, corresponding to a DC resistance of 5 MΩ with standard electrode and bath solutions.

Electrode and bath solutions were designed to isolate Na⁺ currents and to vary the concentration of K⁺ on the external face of the membrane. Cell-attached electrodes contained Na⁺ as the current carrier and Cd²⁺ to block Ca²⁺ channels. Methane sulfonate was substituted for Cl⁻ as the major anion to eliminate the possibility of conduction through Cl⁻ channels (140 mM Na⁺, 140 mM CH₃SO₄⁻, 2 mM Ca²⁺, 0.1 mM Cd²⁺, 5 mM HEPES, 3 mM dextrose). In the outside-out, excised patch configuration, the electrode solution bathes the cytoplasmic surface of the membrane and a Cs⁺ solution was used to block the outward K⁺ current (140 mM Cs⁺, 2 mM Mg²⁺, 100 mM F⁻, 44 mM Cl⁻, 4 mM EGTA, 5 mM HEPES). The recording chamber was filled with standard mammalian saline with variable amounts of K⁺ (140 mM Na⁺, 2 mM Ca²⁺, 2 mM Mg²⁺, 5 mM dextrose, 148 mM Cl⁻, 5 mM HEPES, with 0, 3.5, or 10 mM KCl). Rapid change of the solution bathing the external aspect of the cell or excised patch was achieved by pressure ejection of bath solution from a separate micropipette. All experiments were performed at room temperature (21°C–23°C).

Single-channel currents were measured with a Yale MarkV patch-clamp amplifier using a 10G feedback resistor in the head-stage. The amplifier output was filtered at 3 kHz (-3 dB, 8-pole Bessel) and sampled at 10 kHz with a PDP 11/73 computer (INDEC). Voltage-clamp command potentials were issued by the computer. Between test depolarizations, the membrane potential was maintained at a holding potential of -80 mV for excised patches and 80 mV negative to the cell's resting potential for cell-attached patches. A prepulse potential with a large negative amplitude (see individual records for specific value) was applied for 300 ms prior to the test potential to remove slow inactivation of Na⁺ channels. Finally, the test potential was presented for 15–60 ms. The entire depolarization sequence was repeated every 800 ms. Capacitive transients were determined from the average of 25 test depolarizations negative to the holding potential. A linearly scaled leak trace was then subtracted on-line from the current evoked during a test depolarization.

Data Analysis

All statistical analyses were performed on idealized records, which were constructed as follows. Secondary leak subtraction was performed by adding all the traces with no openings and subtracting the mean from individual traces with openings. The amplitude of the single-channel Na⁺ current at a given test potential was measured by using a cursor to select the beginning and end of long, unequivocal single openings manually. The unitary current amplitude equals the mean current measured over tens of these selected intervals. A threshold of half the unitary current amplitude was used to detect openings. The dwell time between threshold crossings and the number of channels open during the dwell were stored as the idealized current record. The mean open time was computed directly from the average of open dwell times when they were nonoverlapping throughout the depolarization. For patches with many channels, overlapping was extensive, and the mean open time was computed by the method of Fenwick et al. (1982).

Even though most of the patches contained more than 1 Na⁺ channel, it was possible to estimate the possibility of an HPP Na⁺ channel entering the noninactivating mode of gating, P_m, and the open probability when in this mode, P_{oni}. Because there were no late events with more than 1 channel open simultaneously in single traces where inactivation failed, and because the open probability was high (see Figure 2), it is reasonable to assume that at most 1 abnormal channel in a patch entered the noninactivating mode during a single depolarization. Therefore the probability of an HPP Na⁺ channel entering the noninactivating mode equals the fraction of depolarizing steps that failed to inactivate divided by the number of HPP Na⁺ channels in the patch, N_{HPP}. In a patch with more than 1 Na⁺ channel, however, N_{HPP} is not known. Using the maximum likelihood technique of Patlak and

Horn (1982), we estimated the total number of Na⁺ channels in the patch, N_T, from the early portion of the depolarization when both types of channel may open. We then assumed equal expression of wild-type and HPP Na⁺ channels in heterozygotes, so that on average, N_{HPP} is half of N_T. Thus our final estimate for P_m is

$$P_m \cong \left(\frac{2}{N_T} \right) \frac{\# \text{ traces with noninactivation}}{\text{total } \# \text{ traces}}$$

A single trace was considered to contain a noninactivating channel if the time-averaged current late in the depolarization was greater than a threshold, specifically, if

$$\frac{1}{15-5} \int_{.5}^{15} i(t) dt > 0.25$$

Since the current contributed by normal Na⁺ channels after 5 ms is very small, the ensemble average of this integral over all the traces containing a noninactivating channel is a good estimate of P_{oni}, the time-averaged open probability (5 < t < 15 ms) of an HPP Na⁺ channel when it is operating in the noninactivating mode.

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