Rapid Publication

Modulation of Skeletal Muscle Sodium Channels by Human Myotonin Protein Kinase

J. Paul Mounsey,* Puting Xu,‡ J. Edward John, III,* L. Tyler Horne,* John Gilbert,‡ Allen D. Roses,‡ and J. Randall Moorman*

*Departments of Internal Medicine (Cardiovascular Division) and Molecular Physiology and Biological Physics, University of Virginia Health Sciences Center, Charlottesville, Virginia, 22908; and ‡Departments of Internal Medicine (Neurology) and Neurobiology, Duke University Medical Center, Durham, North Carolina 27706

Abstract

In myotonic muscular dystrophy, abnormal muscle Na currents underlie myotonic discharges. Since the myotonic muscular dystrophy gene encodes a product, human myotonin protein kinase, with structural similarity to protein kinases, we tested the idea that human myotonin protein kinase modulates skeletal muscle Na channels. Coexpression of human myotonin protein kinase with rat skeletal muscle Na channels in Xenopus oocytes reduced the amplitude of Na currents and accelerated current decay. The effect required the presence of a potential phosphorylation site in the inactivation mechanism of the channel. The mutation responsible for human disease, trinucleotide repeats in the 3′ untranslated region, did not prevent the effect. The consequence of an abnormal amount of the kinase would be altered muscle cell excitability, consistent with the clinical finding of myotonia in myotonic dystrophy. (*J. Clin. Invest.* 1995. 95:2379–2384.) Key words: sodium channels • myotonic muscular dystrophy • phosphorylation • protein kinase

Introduction

Myotonic muscular dystrophy (DM),[1] the most common muscular dystrophy in adults, is a multisystem disorder with autosomal dominant inheritance. It is characterized by myotonia, the inability to relax after voluntary contraction. Molecular cloning of the DM gene has revealed that its normal product has sequence homology with protein kinases (1–4), and the product has accordingly been named human myotonin protein kinase (HMPK). Splice variant cDNAs (5) and HMPK protein (5, 6) have been identified in multiple tissues, but the function of the gene product has not been defined. If HMPK is indeed a protein kinase, effects on the regulation of cellular function would be expected, and an alteration in the level of its activity could have widespread consequences. This is in keeping with the protean manifestations of myotonic dystrophy.

The electrophysiologic abnormality in DM is needless repetition of action potentials. Myotonia is present in other human diseases, notably paramyotonia congenita and hyperkalemic periodic paralysis. In these illnesses, molecular cloning studies have identified mutations of muscle Na channels (7, 8). In hyperkalemic periodic paralysis, there are repetitive Na channel openings during a depolarizing stimulus, as if the process of fast inactivation had failed (9). This pattern of Na channel gating is a normal property of muscle Na channels, but normally occurs very rarely (reviewed in reference 10). Patch clamp experiments in skeletal muscle isolated from myotonic dystrophy patients have also revealed frequent late openings of Na channels (11). This caused a very small late Na current, and mathematical modeling shows that this would lead to myotonia or paralysis (12).

In contrast to the other muscle diseases characterized by myotonia, the product of the gene which is abnormal in DM is not itself an ion channel, but rather has homology to protein kinases. This finding substantiates earlier biochemical studies of protein phosphorylation in erythrocytes and muscle isolated from DM patients (13–15). It has therefore been suggested that HMPK normally regulates ion channel function (16, 17). To test this idea, we have measured currents through Xenopus oocyte–expressed skeletal muscle Na channels in the presence of the product of the normal HMPK mRNA. Since the purified product is not available, and since no stimulus of HMPK activity has been identified, we studied its function by coinjecting its mRNA with Na channel mRNA. We find that coexpression of HMPK mRNA reduces Na current amplitude and hastens Na current decay, actions which would reduce muscle excitability.

Defining the function of HMPK does not necessarily clarify the mechanism of DM. The inherited locus of DM is not in the coding region of the gene itself, but in the presence of triplet nucleotide repeats of variable length in the 3′ untranslated region. It has been suggested that the result of the mutation might not be associated with the gene product, but rather have its effect through chromosome distortion (17, 18). The severity of

1. *Abbreviations used in this paper:* DM, myotonic muscular dystrophy; HMPK, human myotonin protein kinase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C.

A

Na channel cDNA in
pALTER-T vector

B
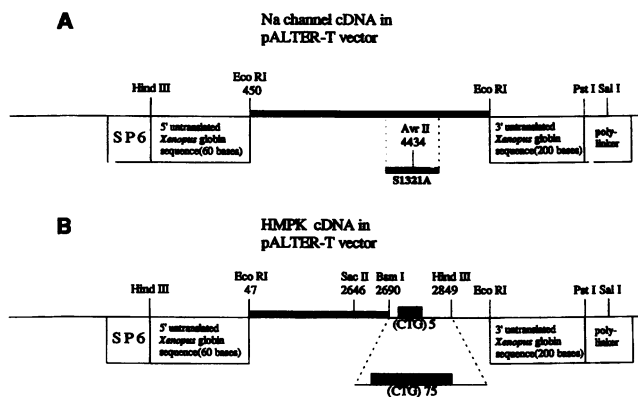
HMPK cDNA in
pALTER-T vector

Figure 1. Molecular clones used in the study. (*A*) Rat skeletal muscle Na channel construct. The coding region and part of the 5' untranslated region beginning at base 450 were inserted into a mutagenesis vector (*pALTER*) containing untranslated frog globin sequence (*T*). A mutation (*S1321A*) simultaneously disabled a phosphorylation site and introduced a unique and silent Avr II restriction site. (*B*) Human myotonin protein kinase construct. The coding region and part of the 5' untranslated region beginning at base 48 were inserted in pALTER-T. A mutation replaced part of the 3' untranslated region containing 5 CTG repeats with an otherwise identical region containing 75 CTG repeats.

the disease correlates with the length of the triplet repeat; generally, longer repeats occur in more severely affected patients (16, 19). We were thus particularly interested in the effect of coinjection of DM mutant HMPK mRNA on Na currents. We tested the effect of both a normal HMPK mRNA—with a short 3' triplet repeat—and a DM mutant HMPK mRNA with a 225-nucleotide repeat. Though a repeat of this length corresponds with only mildly symptomatic DM, cloning of longer repeats is currently problematic. We find that DM mutant HMPK mRNA has qualitatively the same effect on Na currents as normal HMPK mRNA.

Methods

Cloning and mutagenesis. Fig. 1 shows the wild-type and mutant constructs for the Na channel (*A*) and HMPK molecules (*B*). We obtained the full-length rat skeletal muscle Na channel cDNA as an EcoRI insert in pBluescript from Dr. Gail Mandel State University of New York at Stony Brook (20). We obtained the pSP64-T vector, which contains frog hemoglobin 5' and 3' untranslated sequences with an intervening BglII subcloning site, as a HindIII-PstI insert in pSP64 from Dr. Douglas Melton, Harvard University (Cambridge, MA) (21) and Dr. Larry Jones, Indiana University (Indianapolis, IN). By restriction digest, we isolated a HindIII-EcoRI fragment containing the entire frog hemoglobin sequence, and we refer to this fragment as T. The T fragment was subcloned in pALTER-1 (Promega Corp., Madison, WI), a mutagenesis vector. We call this construct pALTER-T. The BglII site in the frog hemoglobin sequence of pALTER-T was mutated to become an EcoRI site, and the EcoRI site in the pALTER-1 polylinker was mutated to become inactive. The EcoRI fragment containing the full-length Na channel sequence was subcloned into the new EcoRI site in pALTER-T. The resulting construct thus has the full-length Na channel cDNA sequence, beginning at nucleotide 450, flanked 5' and 3' by the frog hemoglobin sequence from pSP64T. To construct the S1321A mutation, we used a 50-mer with three base changes using a technique described by Kunkel (22). The nucleotide mutation resulting in the serine to alanine mutation was TCC 4410–4412 GCC. To select mutants, we simultaneously engineered a new, unique AvrII site at bp 4434–4439

with the nucleotide mutation CC 4436/7 TA. Mutant cDNAs, when digested with AvrII and StuI, yielded 7.2-, 2.9-, 1.2-, and 0.1-kb fragments compared with wild-type cDNAs, which yielded 7.2-, 4.1-, and 0.1-kb fragments. The mutation was confirmed by DNA sequencing using standard dideoxynucleotide termination reactions containing 7-deaza dGTP (LARK Sequencing Technologies, Inc., Houston, TX). [^{35}S]dATP was used as the label. Sequencing reactions were analyzed on 6% polyacrylamide wedge gels containing 8 M urea. Wild-type and mutant cDNAs were linearized with SalI and capped RNA was transcribed using the SP6 promoter (Ambion Megascript, Ambion Inc., Austin, TX). The ratio of cap analogue to GTP was 4:1.

HMPK cDNA was isolated from human fetal brain and heart lambda ZAP-II cDNA phage libraries (Stratagene Inc., La Jolla, CA). A mouse myotonic dystrophy kinase cDNA (clone DMR-B15 kindly supplied by Dr. B. Wieringa, University of Nijmegen, the Netherlands) was labeled with [d^{32}P]dCTP and used as a probe to screen cDNA libraries. Inserts were rescued in Bluescript SK vector plasmids using the Exassist/SOLR system (Stratagene Inc., LaJolla, CA, manufacturer's protocol) and used directly for sequencing (Sequenase; US Biologicals, Cleveland, OH) and hybridization. A 3,137-bp HMPK cDNA (lacking the 47 bp at the 5' terminal) containing the coding sequence was spliced together from overlapping cDNA fragments, and the splice positions were confirmed by sequencing. For mutagenesis of HMPK cDNA, an expanded (CTG) n = 75 trinucleotide repeat fragment was amplified from an affected DM patient's genomic DNA by PCR. The mutant HMPK cDNA was obtained by introducing the expanded (CTG) 75 repeat fragment using the BsmI and HindIII restriction sites flanking the (CTG) n repeat region into the normal (CTG) n = 5 repeat region of HMPK cDNA. The normal and mutant HMPK cDNAs were then subcloned into pALTER-T. Normal and mutant RNAs were transcribed during the same reactions, and RNAs from three reactions gave the same results.

Electrophysiology experiments. Stage IV–VI *Xenopus* oocytes were injected with 2.5–50 ng of Na channel RNAs and 20–40 ng of HMPK RNAs, and manually defolliculated the day of the experiment. Whole cell recordings were obtained 1–8 d after injection using a two-microelectrode voltage clamp (OC-725 Warner Instrument Corp., Hamden, CT) at room temperature as previously described (23, 24). Holding potential was −100 mV; voltage steps were applied at 0.5 Hz. For two-pulse protocols to estimate steady state inactivation, conditioning pulses were 0.5 s in duration and were applied at 0.1 Hz. The test pulse was to 10 mV. The bath solution contained (mM) NaCl 150, KCl 4, CaCl$_2$ 1.8, MgCl$_2$ 2, BaCl$_2$ 5, glucose 10, Hepes 10, pH 7.4 (NaOH). Cell-attached patches were obtained using thin-walled capillary glass (7502; Corning Inc., Corning, NY) and conventional means (25, 26). The bath solution was (mM) K aspartate 150, EGTA 10, Hepes 10, pH 7.4 (KOH). The pipette solution was the same as the bath solution in the whole oocyte recordings, and sometimes contained 10–50 μM Gd$_2$O$_3$ to reduce the number of openings of mechanosensitive channels (27). Currents were amplified and low pass filtered (2 kHz; Dagan Corp., Minneapolis, MN), sampled at 50 μs/point (pCLAMP; Axon Instruments Inc., Foster City, CA), and analyzed using PCS (Dr. J. J. Pancrazio, University of Virginia, Charlottesville, VA, 28) and Origin (MicroCal Inc., Amherst, MA). Statistical tests and some plots were made with SigmaStat and SigmaPlot (Jandel Scientific, Corte Madera, CA). Linear components of leak and capacitive currents were corrected by subtracting the scaled mean of traces at other potentials without currents.

Results

Whole oocyte experiments. Two-microelectrode voltage clamp of *Xenopus* oocytes does not accurately resolve activation or inactivation of fast Na currents. We used this technique nonetheless for surveying the amplitudes of currents expressed by multiple kinds and combinations of RNAs. Kinetic analyses were reserved for currents measured in patch clamp experiments.

Fig. 2 shows Na currents in an oocyte expressing the α-subunit of the rat skeletal muscle Na channel alone (*A*) and in
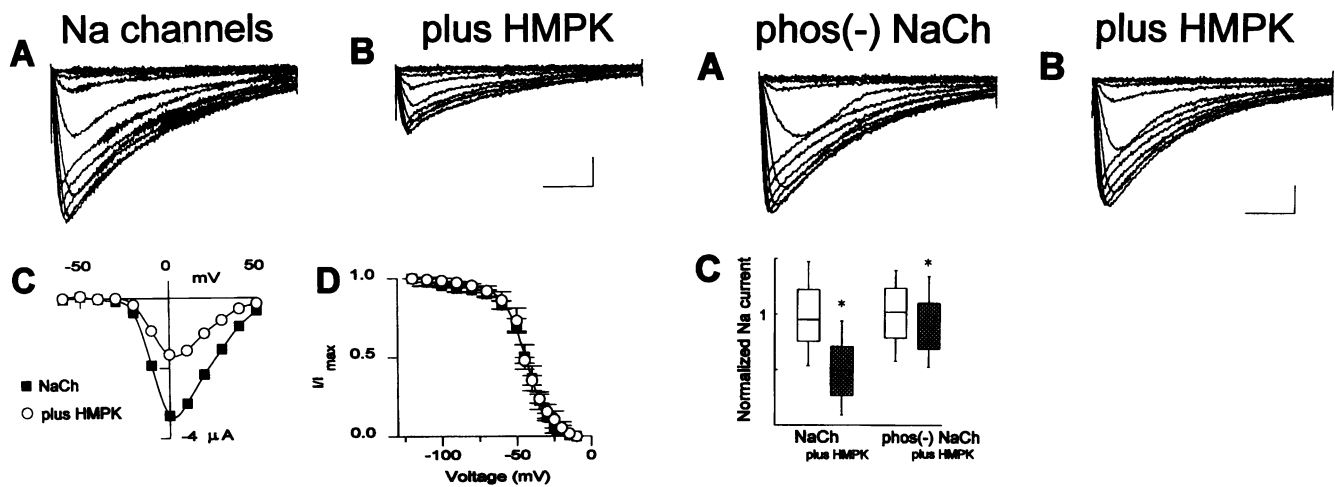
Figure 2. Oocyte currents. (*A* and *B*) Currents during two-microelectrode voltage clamp in a stage IV oocyte expressing only wild-type μ1 rat skeletal muscle Na channels (*A*), and in another oocyte from the same batch coexpressing normal HMPK. Scale bar is 5 ms and 1 μA. (*C*) Peak current voltage relationship from the same oocytes. Na channels alone (■) or coexpressing normal HMPK (○). (*D*) Proportion of available Na channels as a function of the potential of a 500-ms conditioning prepulse (average of two and four stage IV oocytes, including the oocytes in *A* and *B*). The smooth line is a Boltzmann distribution of the form $I/I_{max} = (1 + \exp[\{V + 43.7 \text{ mV}/-7.3\}])^{-1}$. Error bars are SD.

Figure 3. Currents in oocytes expressing the S1321A, or phos(−) mutated, Na channel alone (*A*) and coexpressing normal HMPK (*B*). Scale bar is 5 ms and 1 μA. (*C*) Box plots of peak Na current. In the box plot symbol, the solid horizontal line is the median, the boxes enclose 50% of the data points, and the hatches enclose 80%. All of the currents were normalized to the mean of the currents in oocytes expressing Na channels alone; by definition, NaCh and phos(−) NaCh have means of 1. Values < 1 mean that coexpression of HMPK reduced current. (*P < 0.001, Mann-Whitney *U* test.)

another oocyte from the same batch coexpressing normal HMPK (*B*). Currents were consistently smaller if normal HMPK was coexpressed. Other than amplitude, the currents were qualitatively similar and there was no apparent difference in the voltage dependence of activation or steady state inactivation (*C* and *D*), as assessed in these experiments with stage IV oocytes, where voltage control should be improved over larger stage V and VI oocytes (29).

If HMPK is a protein kinase, its effect on Na channels might not be present in a mutated Na channel that cannot be phosphorylated. One potential phosphorylation site in Na channels is in the cytoplasmic linker between transmembrane domains III and IV (serine 1321). This linking region plays a major role in Na channel inactivation (30–33), and this site has been shown by Catterall et al. to be a substrate for protein kinase C (PKC) in brain Na channels (34). We disabled the site by Ser to Ala mutation (S1321A; phos[−]). The mutated mRNA-induced Na currents (Fig. 3 *A*, phos[−] NaCh) were qualitatively similar to wild-type currents. Importantly, the effect of normal HMPK was much reduced when coexpressed with these mutated Na channels (*B*).

The box plots in Fig. 3 *C* show summary results from 64 to 81 oocytes (seven to nine frogs) expressing wild-type Na channels (*NaCh*) or S1321A-mutated Na channels (phos[−]-NaCh) (*open boxes*), and coexpressing normal HMPK (*hatched boxes*). Within each batch of oocytes, the effect of coinjecting normal HMPK mRNA to reduce current through wild-type Na channels was statistically significant. In contrast, the difference in current amplitudes in oocytes expressing phos(−)-mutated Na channels with or without normal HMPK was much smaller, and the difference was not significant in any given batch. Overall, the average reduction was 48% for wild-
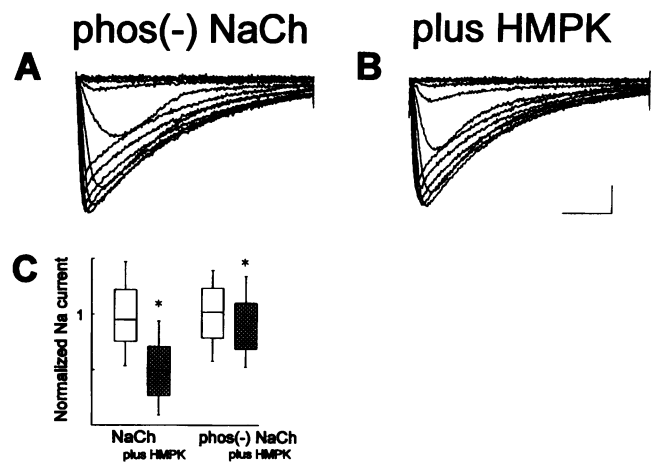
type currents and 8% for phos(−) currents; in these pooled results, both these reductions were statistically significant. This supports the idea that HMPK exerts its effect through phosphorylation, as the effect was considerably reduced after removal of a potential phosphorylation site. As discussed below, the most straightforward explanation is that HMPK is indeed a protein kinase, and that skeletal muscle Na channels are among its substrates.

Patch clamp experiments. For reliable resolution of the peak and the kinetics of these fast currents, we measured Na currents in small patches of oocyte membrane using the cell-attached variant of the patch clamp technique (25). To reduce sources of variability due to sampling, each set of experiments were carried out in oocytes injected within 2 h of each other with mRNAs from the same transcription reaction, and studied in random order within a 12-h period 1–3 d later. In these experiments, we also tested the effect of coexpressing a DM mutant HMPK with 75 CTG repeats in the 3' untranslated region. The 41 patches were of sufficient number for statistical testing of hypotheses about differences in amplitudes and decay rates.

Patches of oocytes expressing only Na channels had larger currents than those coexpressing Na channels and either the normal or DM mutant HMPK; examples are shown in Fig. 4 *A*–*C*. Each panel shows averages of 50–100 traces taken from a single oocyte after a depolarization from a holding potential of −100 mV to 0 mV. The current in Fig. 4 *A* was recorded from an oocyte expressing skeletal muscle Na channels alone; in *B* from an oocyte coexpressing the DM mutant HMPK; and in *C* from an oocyte coexpressing normal HMPK. Despite an equal amount of Na channel mRNA and an equal period of incubation, patches from oocytes coexpressing either form of HMPK had fewer channels apparent than those in oocytes expressing Na channels alone. The change in current amplitude could not be attributed to a change in the voltage dependence of activation gating. In two patches with very large currents for
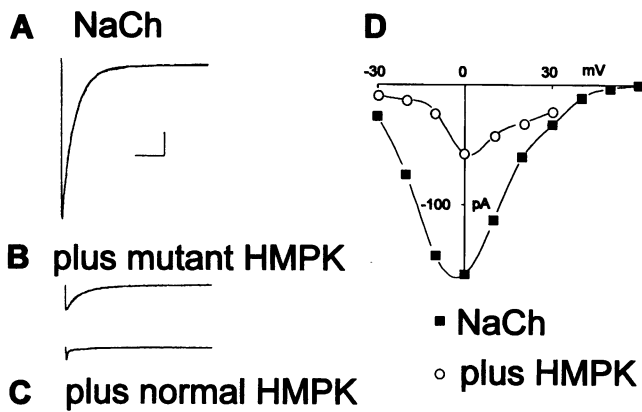
A NaCh

B plus mutant HMPK

C plus normal HMPK

D

■ NaCh

○ plus HMPK

Figure 4. Patch currents. (*A–C*) Average of 50–100 traces in oocytes expressing skeletal muscle Na channels by themselves (*A*), with the DM mutant HMPK (*B*), or with the normal HMPK (*C*). Scale bar is 20 ms and 10 pA. Oocytes from one frog, injected with the same amount of Na channel RNA and tested after equal incubations. (*D*) Peak current voltage relationship for two patches, one in an oocyte expressing only Na channels (■), and in another coexpressing normal HMPK (○).

Figure 5. (*A*) Time to half-decay as a function of peak amplitude for patch currents in oocytes expressing only Na channels (□, $n = 15$), and in oocytes coexpressing normal (●, $n = 16$), and DM mutant (△, $n = 10$) HMPK. (*B*) Box plots of transmembrane charge movement, calculated as the integral of current waveforms. (*$P < 0.005$ for any pair of groups; Mann-Whitney U test).

which a wider range of potentials could be tested, the peak current-voltage relationships showed no shift along the voltage axis (Fig. 4 *D*).

The results are summarized in Fig. 5 *A*. The effect of HMPK to reduce current amplitude was statistically significant ($P < 0.001$, Mann-Whitney U test). The improved voltage control in these patch clamp experiments also allowed assessment of the kinetics of Na current decay. As shown in Fig. 4, currents in oocytes coexpressing either the normal or the mutant HMPK decayed more quickly than those in oocytes expressing Na channels. Because of variability of exponential factors and relative weights of sums of exponentials fits of muscle Na channel inactivation kinetics, we estimated decay rates by measuring the time at which 50% of the current had decayed. The decay rates of Na currents were significantly less if normal HMPK were coexpressed ($P < 0.005$, Mann-Whitney U test), but not if DM mutant HMPK were coexpressed ($P = \text{NS}$).

To assess the effect of changes in both current amplitude and kinetics, we compared the total charge carried across each patch by integrating the current wave form. As shown in the box plots of Fig. 5 *B*, the median charge in patches of oocytes expressing Na channels alone (*open box*) was 15-fold and 5-fold larger than in oocytes coexpressing the normal (*hatched box*) and the DM mutant HMPK (*shaded box*), respectively. The median values are 416 femtocoulombs (Na channels alone), 28 (coexpressing normal HMPK), and 81 (coexpressing DM mutant HMPK). The difference between any pair of groups was significant.

Discussion

We measured Na currents through skeletal muscle Na channels expressed in *Xenopus* oocytes in the presence and absence of mRNA encoding the HMPK gene, which is abnormal in myotonic muscular dystrophy. Our most important findings are (*a*) coexpression of HMPK reduced Na current amplitudes and speeded decay kinetics, (*b*) the effect was greatly reduced in a Na channe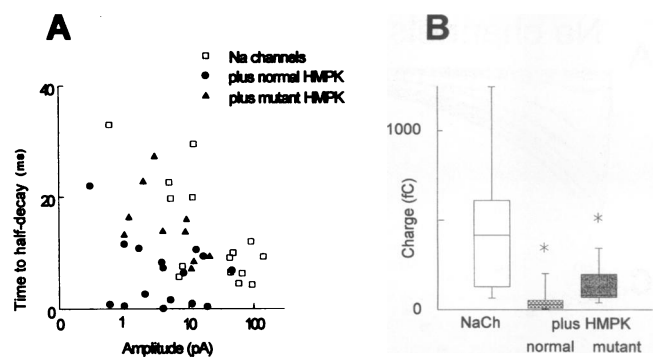l mutant which could not be phosphorylated at a well-conserved site in the inactivation mechanism of the channel, and (*c*) expression of the mutant mRNA which causes human illness, with triplet nucleotide repeats in the 3' untranslated region, had qualitatively similar activity. The simplest explanation of these results is that HMPK is a protein kinase which phosphorylates the Na channel in the inactivation mechanism. Other, equally plausible explanations are that coexpression of HMPK activates an endogenous oocyte protein kinase which phosphorylates Na channels at that site, or that phosphorylation at that site permits the action of HMPK at another site in the molecule. The latter scenario would be similar to the permissive effect of phosphorylation of this site by PKC in brain Na channels, which then allows phosphorylation of other sites by cAMP-dependent protein kinase (PKA) to have a functional effect (35).

This work extends the original observations of Roses and Appel linking DM to membrane protein phosphorylation (13–15) and reconfirms the role of HMPK as the active agent in DM (5, 36, 37). Moreover, these findings link the pathophysiology of DM (altered Na channel kinetics to the genetic abnormality) a mutation in a gene whose product has sequence similarity to protein kinases. The effect of expressing the normal HMPK is dampened excitability: a reduction in its activity may help explain the hyperexcitability of muscle cells in myotonic muscular dystrophy.

These findings link two recently emerging concepts in the regulation of membrane excitability by Na channels. First, modulation of Na channels by phosphorylation has been demonstrated in biochemical and electrophysiological studies. Second, point mutations of Na channels have been identified as the causative abnormality in the human myotonic illnesses paramyotonia congenita and hyperkalemic periodic paralysis. The present work, which shows modulation of Na current amplitude and kinetics by expression of a protein kinase gene which is abnormal in myotonic muscular dystrophy, suggests a new mechanism for human genetic disease: abnormal ion channel modulation leading to abnormal membrane excitability.

Purified muscle Na channels are substrates for PKA (38), and brain Na channels are substrates for both PKA and PKC (39, 40). In brain Na channels, both kinases phosphorylate at multiple sites, and at some common sites (35). The potential phosphorylation site in the III-IV cytoplasmic linker of brain

Na channels was shown first to be a substrate for PKC, and the functional effect of PKC activation in those studies (34, 41) is a slowing of inactivation kinetics. In heart muscle Na channels, on the other hand, the effects of PKC activation by phorbol ester and angiotensin II are different (42–44). The kinetic effects of Na channel phosphorylation appear to be complex and variable, perhaps because the number of potential phosphorylation sites in brain and muscle channels is very different. Brain Na channels have a long cytoplasmic linker between domains I and II with five consensus sites for phosphorylation by PKA, whereas muscle Na channels have a > 200 amino acid deletion in this linker and lose those sites. Since the effect of a protein kinase on Na channel function varies depending on the prior activity of other kinases (35), this loss of phosphorylation sites in muscle Na channels may explain the difference in kinetic effects.

It is possible that phosphorylated channels are not expressed as well, or are less stable. Signal transduction pathways initiated by nerve growth factor lead to increased levels of Na channel expression, an effect which requires activation of PKA (45, 46). In PKA-deficient pheochromocytoma cells, both nerve growth factor and fibroblast growth factor lead to increases in Na channel mRNA level but not to increased protein levels (47). This suggests a role for PKA in the stability of the nascent channel peptide.

How might trinucleotide repeats in the 3' untranslated region of this protein kinase gene cause myotonic dystrophy? In two other diseases characterized by triplet repeat mutations, plausible mechanisms have been postulated (16). In Kennedy's syndrome, the triplet repeat is localized in the coding region of an androgen receptor gene and no receptors can be synthesized. In Fragile X syndrome the coding region of the gene is normal; the triplet repeats are in the 5' untranslated region and abnormal methylation of a promoter region has been observed. In DM, where the coding region of the gene is likewise unaffected, there is no evidence of abnormal DNA methylation. Though no mechanism is known, the levels of HMPK mRNA and protein may be abnormal in DM patients (5, 36, 37).

Our experiments do not demonstrate the mechanism of the disease, as the mutant and normal RNAs had similar kinds of effects on Na channels, and the oocyte expression system lacks the resolution necessary to distinguish small quantitative differences with confidence. Moreover, it is important to note that our experiments used a DM mutant HMPK mRNA with only 75 CTG repeats, or 225 extra nucleotides. This length would be found in mildly symptomatic DM, and more severely affected patients would be expected to have hundreds or thousands of repeats. Our finding of qualitatively similar activity of the DM mutant HMPK might have been different had cloning of longer repeats been possible. The modest reduction of activity we observed may indeed hint at an important mechanism of the disease, or may only be a feature of the expression system we used. For instance, there might have been small differences in stability or quality of the mutant RNA, despite identical conditions for storage and preparation as for the normal RNA, or to different RNA stability or efficiency of translation in the oocyte. Our experiments begin with injection of equal (or nearly equal) amounts of normal and mutant RNAs, and thus cannot address the important issue of transcriptional regulation.

Other questions remain. Not all of the clinical manifestations of DM can be attributed to faulty Na channel modulation, and it is likely that other ion channels and as yet unidentified substrates exist. Moreover, the mechanism by which HMPK is regulated under normal conditions is altogether unknown. Given the diversity and extent of the physiologic abnormalities in DM, HMPK should be viewed as a newly identified and widely used regulator of cellular events.

References

1. Brook, J. D., M. E. McCurrach, H. G. Harley, A. J. Buckler, D. Church, H. Aburatani, K. Hunter, V. P. Stanton, J. P. Thirion, T. Hudson, et al. 1992. Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. Cell. 68:799–808.

2. Aslanidis, C., G. Jansen, C. Amemiya, G. Shutler, M. Mahadevan, C. Tsilfidis, C. Chen, J. Alleman, N. G. Wormskamp, M. Vooijs, et al. 1992. Cloning of the essential myotonic dystrophy region and mapping of the putative defect. Nature (Lond.). 355:548–551.

3. Fu, Y., A. Pizzuti, R. G. Fenwick, J. King, S. Rajnarayan, P. W. Dunne, J. Dubel, G. A. Nasser, T. Ashizawa, P. de Jong, et al. 1992. An unstable triplet repeat in a gene related to myotonic muscular dystrophy. Science (Wash. DC). 255:1256–1258.

4. Mahadevan, M., C. Tsilfidis, L. Sabourin, G. Shutler, C. Amemiya, G. Jansen, C. Neville, M. Narang, J. Barcelo, K. O'Hoy, et al. 1992. Myotonic dystrophy mutation: an unstable CTG repeat in the 3' untranslated region of the gene. Science (Wash. DC). 255:1253–1255.

5. Fu, Y.-H., D. L. Friedman, S. Richards, J. A. Pearlman, R. A. Gibbs, A. Pizzuti, T. Ashizawa, M. B. Perryman, G. Scarlato, R. G. Fenwick, and C. T. Caskey. 1993. Decreased expression of myotonin-protein kinase mRNA and protein in adult form of myotonic dystrophy. Science (Wash. DC). 260:235–238.

6. Brewster, B. S., S. Jeal, and P. N. Strong. 1993. Identification of a protein product of the myotonic dystrophy gene using peptide specific antibodies. Biochem. Biophys. Res. Commun. 194:1256–1260.

7. McClatchey, A., P. Van den Bergh, M. Pericak-Vance, W. Raskind, C. Verellen, D. McKenna-Yasek, K. Rao, J. L. Haines, T. Bird, R. H. J. Brown, et al. 1992. Temperature-sensitive mutations in the III-IV cytoplasmic loop region of the skeletal muscle sodium channel gene in paramyotonia congenita. Cell. 68:769–774.

8. Ptacek, L. J., L. Guow, H. Kwiecinski, P. McManis, J. R. Mendell, R. J. Barohn, A. L. George, R. Barchi, M. Robertson, and M. F. Leppert. 1993. Sodium channel mutations in paramyotonia congenita and hyperkalemic periodic paralysis. Ann. Neurol. 33:300–307.

9. Cannon, S. C., and S. M. Strittmatter. 1993. Functional expression of sodium channel mutations identified in families with periodic paralysis. Neuron. 10:317–326.

10. Moorman, J. R., G. E. Kirsch, A. M. J. VanDongen, R. H. Joho, and A. M. Brown. 1990. Fast and slow gating of sodium channels encoded by a single mRNA. Neuron. 4:243–252.

11. Franke, C., H. Hatt, P. A. Iaizzo, and F. Lehmann-Horn. 1990. Characteristics of Na+ and Cl– conductance in resealed muscle fibre segments from patients with myotonic dystrophy. J. Physiol. 425:391–405.

12. Cannon, S. C., R. H. Brown, and D. P. Corey. 1993. Theoretical reconstruction of myotonia and paralysis caused by incomplete inactivation of sodium channels. Biophys. J. 65:270–288.

13. Roses, A. D., and S. H. Appel. 1973. Protein kinase activity in erythrocyte ghosts of patients with myotonic muscular dystrophy. Proc. Natl. Acad. Sci. USA 70:1855–1859.

14. Roses, A. D., and S. H. Appel. 1974. Muscle membrane protein kinase in myotonic muscular dystrophy. Nature (Lond.). 250:245–247.

15. Roses, A. D., and S. H. Appel. 1975. Phosphorylation of component a of the human erythrocyte membrane in myotonic muscular dystrophy. J. Membr. Biol. 20:51–58.

16. Caskey, C. T., A. Pizzuti, Y. Fu, R. G. Fenwick, and D. L. Nelson. 1992. Triplet repeat mutations in human disease. *Science (Wash. DC)*. 256:784–789.

17. Davies, K. 1992. Too many trinucleotide repeats. *Nature (Lond.)*. 358:91.

18. Jansen, G., M. Mahadevan, C. Amemiya, N. Wormskamp, B. Segers, W. Hendriks, K. O'Hoy, S. Baird, L. Sabourin, and G. Lennon. 1992. Characterization of the myotonic dystrophy region predicts multiple protein isoform-encoding mRNAs. *Nature Genet.* 1:261–266.

19. Harley, H. G., S. A. Rundle, W. Reardon, J. Myring, S. Crow, J. D. Brook, P. S. Harper, and D. J. Shaw. 1992. Unstable DNA sequence in myotonic dystrophy. *Lancet.* 339:1125–1128.

20. Trimmer, J. S., S. S. Cooperman, S. A. Tomiko, J. Zhou, S. M. Crean, M. B. Boyle, R. G. Kallen, Z. Sheng, R. L. Barchi, F. J. Sigworth, et al. 1989. Primary structure and functional expression of a mammalian skeletal muscle sodium channel. *Neuron.* 3:33–49.

21. Krieg, P. A., and D. A. Melton. 1984. Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. *Nucleic Acids Res.* 12:7057–7070.

22. Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA.* 82:488–492.

23. Durieux, M. E., M. N. Salafranca, K. R. Lynch, and J. R. Moorman. 1992. Lysophosphatidic acid induces a pertussis toxin-sensitive Ca^{2+}-activated Cl^- current in *Xenopus laevis* oocytes. *Am. J. Physiol.* 263:C896–C900.

24. Kowdley, G. C., S. J. Ackerman, J. E. John, L. R. Jones, and J. R. Moorman. 1994. Hyperpolarization-activated chloride currents in *Xenopus* oocytes. *J. Gen. Physiol.* 103:217–230.

25. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch. Eur. J. Physiol.* 391:85–100.

26. Methfessel, C., V. Witzemann, T. Takahashi, M. Mishina, S. Numa, and B. Sakmann. 1986. Patch clamp measurements on *Xenopus laevis* oocytes: currents through endogenous channels and implanted acetylcholien receptor and sodium channels. *Pflügers Arch. Eur. J. Physiol.* 407:577–588.

27. Yang, X., and F. Sachs. 1989. Block of stretch-activated ion channels in *Xenopus* oocytes by gadolinium and calcium ions. *Science (Wash. DC)*. 243:1068–1071.

28. Pancrazio, J. J. 1993. PCS: An IBM-compatible microcomputer program for the analysis and display of voltage-clamp data. *Comput. Methods Programs Biomed.* 40:175–180.

29. Krafte, D. S., and H. A. Lester. 1988. Expression of functional sodium channels in stage II-III *Xenopus* oocytes. *J. Neurosci. Methods.* 26:211–215.

30. Vassilev, P. M., T. Scheuer, and W. A. Catterall. 1988. Identification of an intracellular peptide segment involved in sodium channel inactivation. *Science (Wash. DC)*. 241:1658–1661.

31. Stühmer, W., F. Conti, H. Suzuki, X. Wang, M. Noda, N. Yahagi, H. Kubo, and S. Numa. 1989. Structural parts involved in activation and inactivation of the sodium channel. *Nature (Lond.)*. 339:597–603.

32. Moorman, J. R., G. E. Kirsch, A. M. Brown, and R. H. Joho. 1990. Changes in sodium channel gating produced by point mutations in a cytoplasmic linker. *Science (Wash. DC)*. 250:688–691.

33. West, J. W., D. E. Patton, T. Scheuer, Y. Wang, A. L. Goldin, and W. A. Catterall. 1992. A cluster of hydrophobic amino acid residues required for fast Na(+)-channel inactivation. *Proc. Natl. Acad. Sci. USA.* 89:10910–10914.

34. West, J. W., R. Numann, B. J. Murphy, T. Scheuer, and W. A. Catterall. 1991. A phosphorylation site in the Na+ channel required for modulation by protein kinase C. *Science (Wash. DC)*. 254:866–868.

35. Li, M., J. W. West, R. Numann, B. J. Murphy, T. Scheuer, and W. A. Catterall. 1993. Convergent regulation of sodium channels by protein kinase C and cAMP-dependent protein kinase. *Science (Wash. DC)*. 261:1439–1442.

36. Carango, P., J. E. Noble, H. G. Marks, and V. L. Funanage. 1993. Absence of myotonic dystrophy protein kinase (*DMPK*) mRNA as a result of a triplet repeat expansion in myotonic dystrophy. *Genomics.* 18:340–348.

37. Sabouri, L. A., M. S. Mahadevan, M. Narang, D. S. Lee, L. C. Surh, and R. G. Korneluk. 1993. Effect of the myotonic dystrophy (DM) mutation on mRNA levels of the DM gene. *Nature Genet.* 4:233–238.

38. Yang, J., and R. Barchi. 1990. Phosphorylation of the rat skeletal muscle sodium channel by cyclic AMP-dependent protein kinase. *J. Neurochem.* 54:954–962.

39. Rossie, S., D. Gordon, and W. A. Catterall. 1987. Identification of an intracellular domain of the sodium channel having multiple cAMP-dependent phosphorylation sites. *J. Biol. Chem.* 262:17530–17535.

40. Murphy, B. J., and W. A. Catterall. 1992. Phosphorylation of purified rat brain Na+ channel reconstituted into phospholipid vesicles by protein kinase C. *J. Biol. Chem.* 267:16129–16134.

41. Numann, R., W. A. Catterall, and T. Scheuer. 1991. Functional modulation of brain sodium channels by protein kinase C phosphorylation. *Science (Wash. DC)*. 254:115–118.

42. Moorman, J. R., G. E. Kirsch, A. E. Lacerda, and A. M. Brown. 1989. Angiotensin II modulates cardiac sodium channels in neonatal rat. *Circ. Res.* 65:1804–1809.

43. Nilius, B., J. Tytgat, and R. Albitz. 1989. Modulation of cardiac Na channels by angiotensin II. *Biochim. Biophys. Acta.* 1014:259–262.

44. Qu, Y., J. Rogers, T. Tanada, T. Scheuer, and W. A. Catterall. 1994. Modulation of cardiac Na+ channels expressed in a mammalian cell line and in ventricular myocytes by protein kinase C. *Proc. Natl. Acad. Sci. USA.* 91:3289–3293.

45. Kalman, D., B. Wong, A. E. Horvai, M. J. Cline, and P. H. O'Lague. 1990. Nerve growth factor acts through cAMP-dependent protein kinase to increase the number of sodium channels in PC12 cells. *Neuron.* 4:355–366.

46. D'Arcangelo, G., K. Paradiso, D. Shepherd, P. Brehm, and S. Halegoua. 1993. Neuronal growth factor regulation of two different sodium channel types through distinct signal transduction pathways. *J. Cell Biol.* 122:915–921.

47. Ginty, D. D., G. R. Fanger, J. A. Wagner, and R. A. Maue. 1992. The activity of cAMP-dependent protein kinase is required at a posttranslational level for induction of voltage-dependent sodium channels by peptide growth factors in PC12 cells. *J. Cell Biol.* 116:1465–1473.