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Modifications of Human Cardiac Sodium Channel Gating by UVA Light

G.K. Wang, S.Y. Wang
Department of Anesthesia, Harvard Medical School and Brigham & Women's Hospital, 75 Francis St., Boston, MA 02115, USA
Department of Biology, State University of New York, Albany, NY, USA

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Abstract. Voltage-gated Na^+ channels are membrane proteins responsible for the generation of action potentials. In this report we demonstrate that UVA light elicits gating changes of human cardiac Na^+ channels. First, UVA irradiation hampers the fast inactivation of cardiac Nav1.5 Na^+ channels expressed in HEK293t cells. A maintained current becomes conspicuous during depolarization and reaches its maximal quasi steady-state level within 5–7 min. Second, the activation time course is slowed by UVA light; modification of the activation gating by UVA irradiation continues for 20 min without reaching steady state. Third, along with the slowed activation time course, the peak current is reduced progressively. Most Na^+ currents are eliminated during 20 min of UVA irradiation. Fourth, UVA light increases the holding current nonlinearly; this phenomenon is slow at first but abruptly fast after 20 min. Other skeletal muscle Nav1.4 isoforms and native neuronal Na^+ channels in rat GH_3 cells are likewise sensitive to UVA irradiation. Interestingly, a reactive oxygen metabolite (hydrogen peroxide at 1.5%) and an oxidant (chloramine-T at 0.5 mM) affect Na^+ channel gating similarly, but not identically, to UVA. These results together suggest that UVA modification of Na^+ channel gating is likely mediated via multiple reactive oxygen metabolites. The potential link between oxidative stress and the impaired Na^+ channel gating may provide valuable clues for ischemia/reperfusion injury in heart and in CNS.

Key words: Voltage-gated Na^+ channel — UV light — Oxidative stress — Hydrogen peroxide — Oxidant — Chloramine-T

Correspondence to: G.K. Wang; email: wang@zeus.bwh.harvard.edu

Introduction

Ultraviolet radiation at 255–305 nm wavelength causes a continuous reduction in Na^+ currents and their corresponding gating currents (Oxford & Pooler, 1975; Fox et al., 1976; Conti et al., 1988). However, this type of UV irradiation does not affect gating properties of remaining Na^+ currents; their activation and fast inactivation kinetics stay the same. The reduction in Na^+ currents by UVB (290–320 nm) or UVC (190–290 nm) irradiation is supposedly due to direct photodamage of aromatic residues within the Na^+ channel, such as tryptophan, phenylalanine, tyrosine, or histidine. The identity of the photodestruction within the Na^+ channel remains unknown and may depend on many factors, including the UV wavelength, the nature of the excited electronic state, the identity and location of other reactive groups in the vicinity of the chromophore, and the pH (e.g., Middendorf, Aldrich and Baylot, 2000).

Little attention has been given thus far to the possible effects on Na^+ channels of near-visible UV light, the predominant UV species in the earth atmosphere (termed UVA, with wavelength at 320–380 nm). The UVA light does not cause direct photodamage of aromatic amino acids. However, near-visible UV light alone induces a novel ubiquitous calcium-permeable cation current in mammalian cell lines (Mendez & Penner, 1998). These authors proposed that this light-induced cationic current is due to (1) free radical-induced lipid peroxidation and/or (2) protein oxidation. The UVA irradiation causes oxidative stress-induced damage in human lens proteins and in skin tissue, presumably via oxidation of Trp, Tyr, His, Met, and Cys residues, possibly by a free radical/reactive oxygen pathway (e.g., Spector, 1995; Tyrrell, 1991). Such damage in lens proteins and skin tissues is believed to contribute to cataractogenesis and skin aging, respectively. Thus, UVA is an im-

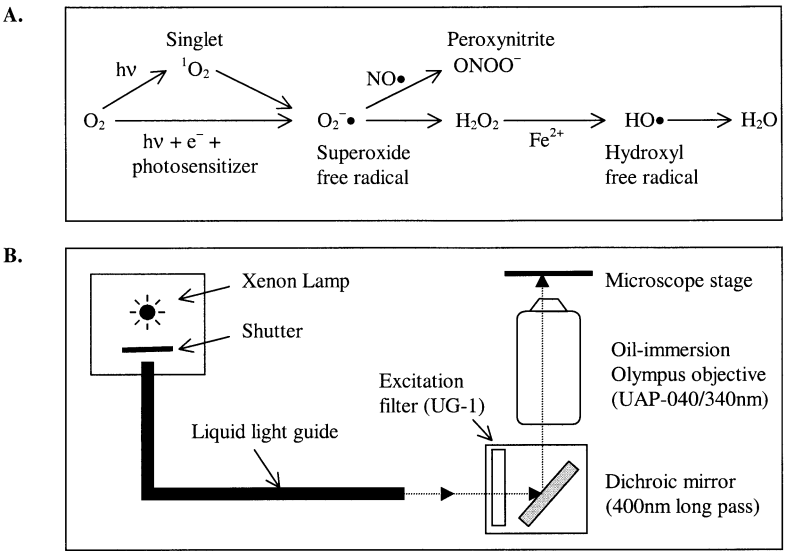

Fig. 1. UVA irradiation, oxygen metabolites, and optical configuration. (*A*) UVA and reactive oxygen species. • depicts the unpaired electron, also known as the free radical. (*B*) Optical configuration for UVA (~340–380 nm) irradiation.

portant factor that evokes oxidative stress in living cells. The action of UVA light and its oxidative cascade involving free radical/reactive oxygen species are outlined in Fig. 1*A*. Oxidative stress also occurs during heart ischemia/reperfusion (Braunwald & Kloner, 1982), which is well known to cause cardiac arrhythmias and apoptosis of myocytes.

We recently attempted to apply UVA light in an unrelated photo-crosslinking study based on a method developed by Horn, Ding & Gruber (2000). Unexpectedly, we found that UVA irradiation irreversibly modifies the Na^+ channel gating in human heart Nav1.5 channels. In particular, fast inactivation of the Na^+ channel is significantly hampered and remains incomplete. Interestingly, like UVA irradiation, both an oxygen metabolite hydrogen peroxide at 1.5% and an oxidant, chloramine-T, at 0.5 mM applied in the external bath solution, inhibit the fast inactivation of Nav1.5 Na^+ currents. The implications of these findings and the possible links among UVA irradiation, oxidative stress, and impaired Na^+ channel gating will be discussed.

Materials and Methods

Cell Culture and Transient Transfection

Human embryonic kidney (HEK293t) cells were cultured and transiently transfected as described (Wang & Wang, 1998). Cells were first grown to ~50% confluence in DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (HyClone, Logan, UT), 1% penicillin and streptomycin (Sigma, St. Louis, MO), 3 mM taurine, and 25 mM HEPES (Invitrogen). Transfection of these cells with Na^+ channel clones (5–10 μg), such as human cardiac Nav1.5 (hH1, Gellens et al., 1992), rat skeletal muscle Nav1.4 (μ1/skm1; Trimmer et al., 1989), and human skeletal muscle Nav1.4 (George et al., 1992), along with reporter plasmid CD8-pih3m (1 μg) was accomplished by a calcium phosphate precipitation method in a Ti25 flask. Several IFM-locus mutants, rNav1.4-M1305I, rNav1.4-M1305A, rNav1.4-M1305L, and rNav 1.4-M1305Q, were created by site-directed mutagenesis as described (Wang & Wang, 1998). Only mutant rNav1.4-M1305I expressed sufficient Na^+ current in HEK293t cells for experiments. Cells were replated 15 hr after transfection on culture dishes (35 mm diameter) each containing three round coverslips (15 mm diameter, 0.15 mm thick; Warner Instruments, Hamden, CT) and 2 ml medium, maintained at 37°C in a 5% CO_2 incubator. Cells on the coverslip were used for experiments after 1–4 days. Transfection-positive cells on the coverslips were identified by immunobeads (CD8-Dynabeads, Lake Success, NY).

Rat pituitary clonal GH_3 cells were maintained in a Ti25 flask as described previously (Wang & Wang, 1992) with the medium described above. For UVA irradiation, neuronal GH_3 cells were also replated on culture dishes, each containing three round coverslips and 2 ml medium. For cell adhesion, all coverslips were pretreated with protamine (Sigma, 1 mg/ml) for 30 min before cell culture.

Whole-Cell Voltage Clamp

The whole-cell configuration of a patch-clamp technique (Hamill et al., 1981) was used to record Na^+ currents in GH_3 cells or in HEK293t cells coated with CD8 immunobeads. Experiments were performed at room temperature (23 ± 2°C). Glass electrodes contained (mM) 100 NaF, 30 NaCl, 10 EGTA, and 10 HEPES adjusted to pH 7.2 with CsOH. The electrodes had a tip resistance of 0.5–1.0 MΩ; access resistance was generally ≤2–3 MΩ. With series resistance compensation of >80%, the voltage error at +50 mV was <4 mV on average. The bath solution contained (mM) 65 NaCl, 85 choline chloride, 2 $CaCl_2$, and 10 HEPES adjusted to pH 7.4 with tetramethyl hydroxide. These ionic conditions resulted in smaller Na^+ currents at voltages from −60 to +10 mV, which in turn minimized the effects of series resistance artifact in the conductance-voltage measurement (Cota & Armstrong, 1989). Tetrodotoxin (TTX) was purchased from Calbiochem-Navabiochem (San Diego, CA). Glutathione (reduced form), bupivacaine, and hydrogen peroxide (30%, w/w) were purchased from Sigma. Reduced glutathione was prepared when needed and included in the pipette solution at a final concentration of 24 mM. Bupivacaine HCl was dissolved in distilled water at 100 mM. The hydrogen peroxide solution was freshly diluted with the external bath solu-

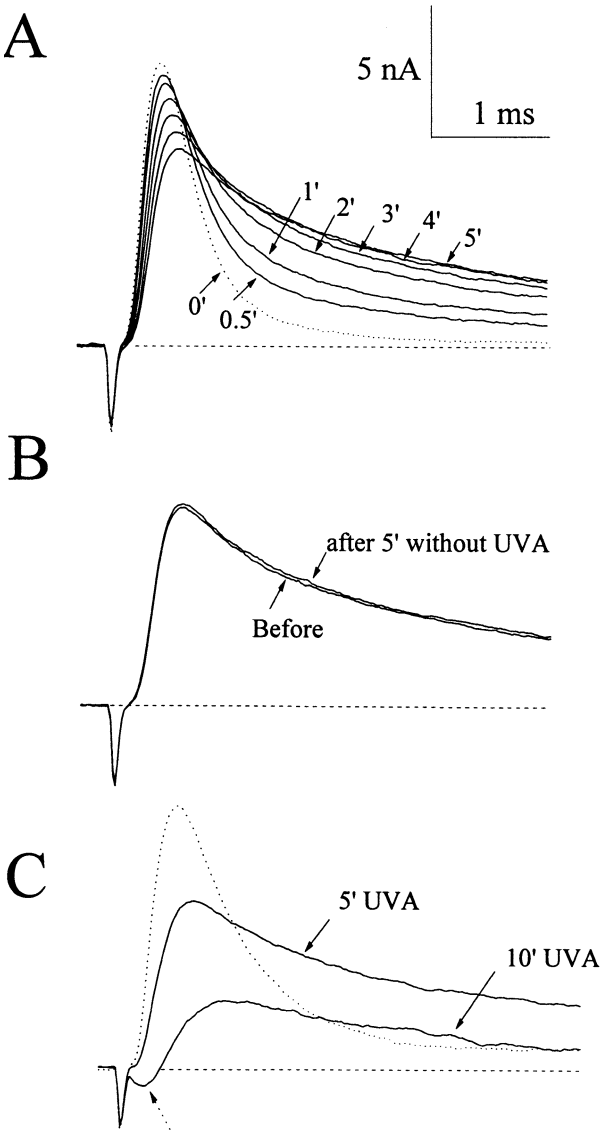

Fig. 2. Modifications of Na^+ current kinetics by UVA irradiation. (*A*) Superimposed Na^+ currents were recorded before (*dashed trace*) and 0.5, 1, 2, 3, 4, and 5 minutes after UVA irradiation; Na^+ currents were generated by a test pulse to +50 mV for 5 msec. Holding potential was −140 mV. (*B*) With the same cell, the Na^+-current trace was first recorded at the time of UVA irradiation for 5 minutes and superimposed with the second Na^+ current trace recorded 5 minutes after UVA light was turned off. No difference in current kinetics was evident. (*C*) Superimposed Na^+ current traces from 5 and 10 minutes after UVA irradiation with a 1.7-fold difference in their τ values. Control current (*dashed trace*) was recorded before UVA irradiation. A different cell than in (*A*,*B*) was used. Notice that the rising phase, the decaying phase, and the peak amplitudes of Na^+ currents are altered by UVA light. Dashed line indicates the zero current base line; dashed arrow indicates the apparent delay in the rising phase of current.

tion to yield a final concentration of 1.5%. Whole-cell currents were recorded with Axopatch 200B, filtered at 5 kHz, and collected by pClamp software (Axon Instruments, Foster City, CA). After gigaohm seal formation and establishment of whole-cell voltage clamp, the cells were dialyzed for ~15 min before data were acquired. The capacitance and leakage current were canceled by the patch-clamp circuitry and further subtracted by the P/−4 method. An unpaired Student's *t*-test was used to evaluate estimated parameters (mean ± SEM); p values of < 0.05 were considered statistically significant.

Light Source, Microscope Configuration, and Recording Chamber for UVA Irradiation

A 75 W continuous Xenon light source was purchased from Ion-Optix Corp. (Milton, MA). The Xenon light source was equipped with an electronic shutter (Fig. 1*B*) controlled by a manual switch. The light source was directed to a 1-meter flexible liquid light guide with a microscope adapter, which was positioned at the Epi-illumination filter block of an inverted microscope (Nikon Diaphot 300). Within the filter block the light was first filtered by a UG-1 (1 mm thick, Chroma Technology, Brattleboro, VT) and was then reflected by a dichroic mirror (400DCLP, Chroma Technology). The transmission spectrum of UG-1 has one major peak around 360 nm and a minor peak around 750 nm, while the 400DCLP dichroic mirror reflects wavelengths ≤400 nm. In a few experiments, light was bandpass-filtered between 340 and 380 nm by a D360/40× exciter filter (Chroma Technology). We found that the results with this filter were comparable to those with the UG-1 filter. UVA light then entered an oil-immersion 40× Olympus objective (UAPO-40×, 340 nm; 1.3 N.A.; Optical Analysis, Nashua, NH) before it finally reached the specimen. Under this optical configuration, wavelengths from 340–380 nm are preferentially transmitted. Individual round coverslips were mounted near the center of a recording chamber (model RC-25F, Warner Instruments). A small drop of immersion oil was applied onto the objective lens and the bottom of the coverslip. The recording chamber with a volume of about 150 µl was continuously superfused with the bath solution. During UVA irradiation we readjusted the leak subtraction, when possible, using the Axopatch 200B device before the pulse protocol.

Results

General Description of UVA Effect on Human Cardiac Nav1.5 Na^+ Channels

Upon UVA irradiation the kinetics of the hNav1.5 Na^+ current were altered within the first 30 sec of application without apparent delay. Figure 2*A* shows the superimposed current traces at a test pulse of +50 mV during the first 5-min UVA irradiation. Four distinct UVA-induced characteristics were identified. First, there were rapid changes in fast inactivation gating of human cardiac Nav1.5 Na^+ channels after UVA application. A significant portion of the Na^+ current was maintained at the end of the pulse; this maintained current increased during UVA irradiation and reached a maximal quasi steady-state level within 5 to 7.5 min before it began to decline. The time constants of decaying phases of Na^+ currents at +50 mV were measured as 0.65 ± 0.13 msec (mean ± SE, $n = 5$) and 2.53 ± 0.33 msec ($n = 5$) before and 5 min after UVA irradiation, respectively (Fig. 2*A*). The difference in the time constant values was ~3.9 fold ($p < 0.05$).

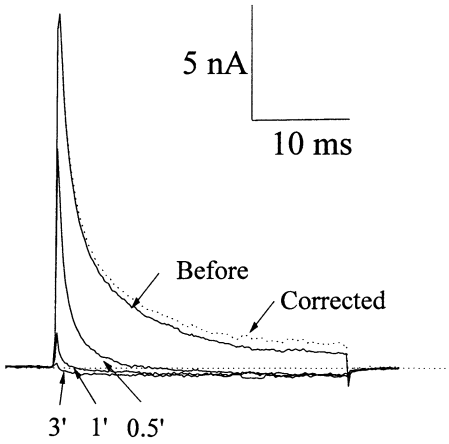

Fig. 3. UVA-modified Na^+ currents before and after 1 mM bupivacaine. After UVA irradiation for a period of 3 minutes, Na^+ currents were subsequently recorded before and 0.5, 1, and 3 minutes after 1mM bupivacaine perfusion of the recording chamber. The superimposed Na^+ currents were elicited by a +50-mV test pulse for 30 msec. The holding potential was −140 mV. The dashed straight line indicates the zero current baseline, whereas the dashed current trace (corrected) is obtained after the subtraction of the 3′ trace from the control (*before*) trace.

It is noteworthy that turning off the UVA light immediately stops the gating modifications (Fig. 2*B*). This result suggests that the UVA action, presumably due to reactive oxygen metabolites, is not through "diffusible" metabolites generated distantly. Rather, these reactive oxygen metabolites have very short half-life times, small diffusion constant values, and diminutive effective range (Hoshi & Heinemann, 2001).

Second, the activation time courses of Na^+ currents were also affected by UVA irradiation without apparent delay (Fig. 2*A*). The apparent slowing of activation kinetics could in part be due to an indirect effect of slowed inactivation. However, the sigmoid rising phase was retarded progressively and did not reach steady state during the 5-min irradiation period. The half times to reach the peak current amplitude of the Na^+ currents from the beginning of the +50-mV pulse were measured 0.32 msec before and 0.40 msec after 5-min UVA irradiation, respectively, a 1.25-fold difference. The half-time values continued to increase during UVA irradiation and showed no evidence of reaching steady state (Fig. 2*C*).

Third, the peak current amplitude is progressively reduced. Figures 2*A* and 2*C* show the reduction of the peak Na^+ current by UVA irradiation from 0 to 5 min in one cell and from 5 to 10 min in another cell. The reduction of the peak current amplitude was 32.6 ± 3.4% ($n = 5$) for the first 5 min. This reduction in the peak current continued at about the same rate for the next 5–15 min. As a result, UVA irradiation abolished most Na^+ currents after ~20 min application. Fourth, the holding current is continuously increased during prolonged UVA irradiation. The increase in the holding current was nonlinear with a slow time course initially (generally $< |-0.5|$ nA after 10 min UVA irradiation) followed by a rapid rise in holding current, and finally reached the maximum (−20 nA) of the patch-clamp device after 20–30 min irradiation. The nonlinear nature of the rise of the holding current by UVA light has been described before in untransfected HEK cells (Mendez & Penner, 1998). We found that with prolonged UVA irradiation, the apparent decaying phases of the Na^+ currents may be further slowed (Fig. 2*C*). This could be due to additional UVA modification of the fast inactivation gating process and/or the continuous increase of UVA-induced cationic currents and the slowing of the activation (Fig. 2*C*, dashed arrow).

To confirm that the slow decaying current is, indeed, carried by TTX-insensitive Nav1.5 channels, we first irradiated the cell with the UVA light for 3 min. After the irradiation, we recorded UVA-modified Na^+ currents and then perfused the recording chamber with the external solution containing 1mM bupivacaine. Figure 3 shows that bupivacaine at 1mM blocks UVA-modified Nav1.5 Na^+ currents effectively. Within 3 minutes of drug perfusion all the peak and the slowly-decaying currents were completely eliminated. A small inward current becomes evident in Fig. 3 after bupivacaine application (trace 3′). The Na^+ current after subtracting this inward current is shown in Fig. 3 as a dashed trace. Most of the Nav1.5 Na^+ current returned after drug wash-off for 15 minutes. Thus, the slowly-decaying currents after the UVA irradiation were, indeed, carried by Nav1.5 Na^+ channels. The inward current is likely a result of the leak subtraction protocol, which will elicit UVA-light induced calcium influx (Mendez & Penner, 1998).

Conductance-Voltage Curves and Steady-State Inactivation Before and After UVA Irradiation

To characterize the current-voltage relationship, we first recorded the current families at various voltages before and after 5-min UVA irradiation. Figures 4*A* (before) and *B* (after) show the superimposed current traces under these conditions. Evidently, the decaying phases were slowed at all voltages after UVA irradiation (Fig. 4*C*). The peak current-voltage relationship was shifted to the depolarizing direction by 18 mV after UVA irradiation with $V_{0.5}$ values of −35.3 ± 4.9 mV *vs.* the control value of −53.3 ± 2.7 mV (Fig. 4*D*; mean ± SE by a Boltzmann equation; $n = 6$; $p < 0.05$). In addition, the slope factor became less steep by ~2.1 fold with a k_a

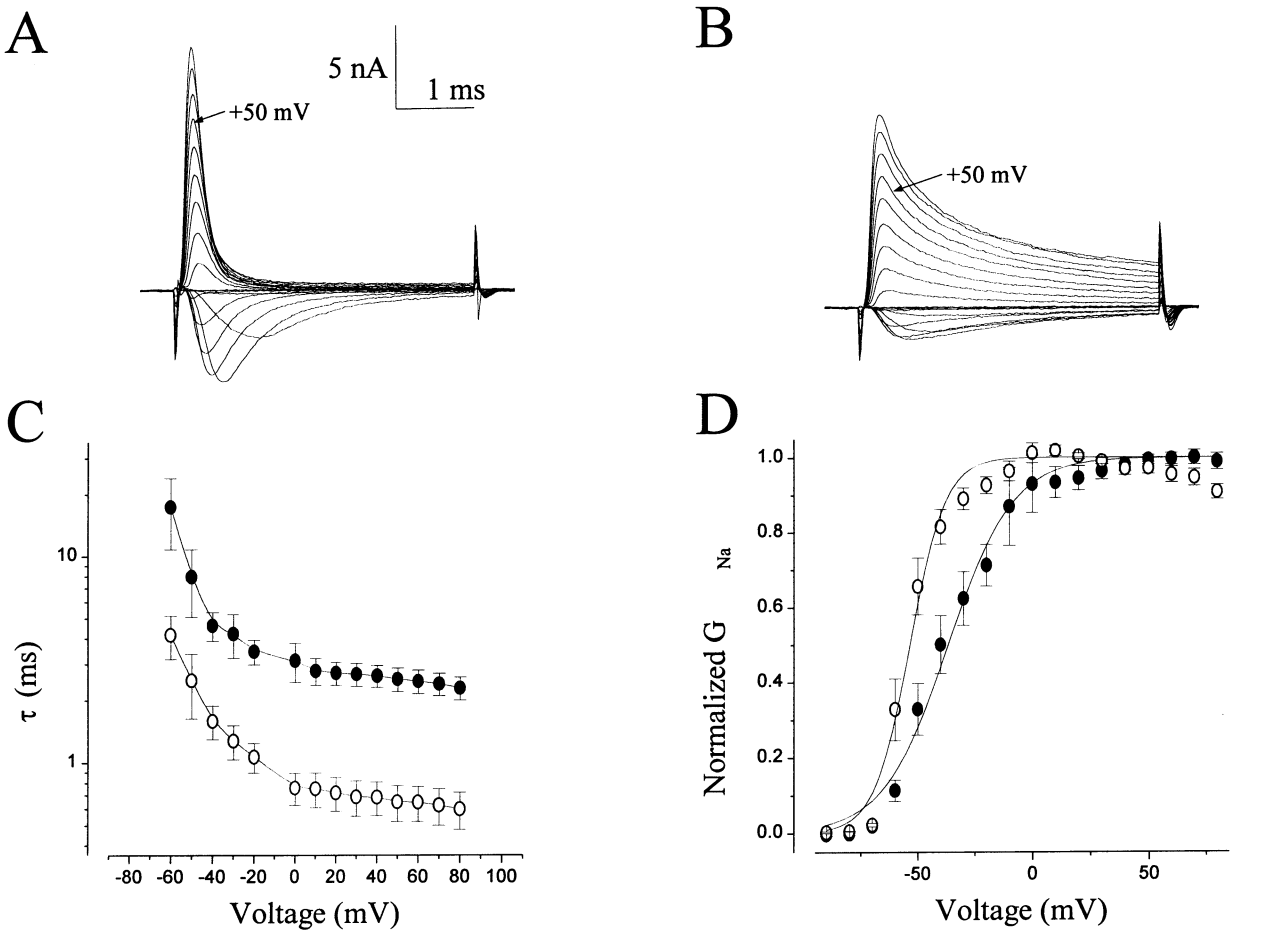

Fig. 4. Current-voltage relationship before and after UVA irradiation. Superimposed current traces were recorded (*A*) before and (*B*) 5 min after UVA irradiation at various test voltages from −90 mV to +80 mV in 10-mV step increments. Current traces elicited by +50 mV were labeled. The decaying phases of Na^+-current families were well fitted by a single-exponential function and their time constants before (*empty circles*) and 5 min after (*filled circles*) UVA irradiation were plotted against the test voltage (*C*). The peak amplitudes of Na^+-current families were also measured before (*empty circles*) and 5 min after (*filled circles*) UVA irradiation, converted to conductance, normalized with respect to maximal conductance, and plotted against the test voltage (*D*). Conductance was estimated by an equation, $G_{Na} = I_{Na}/[E_m/E_{Na}]$, where I_{Na} is the peak current elicited by the test pulse, E_m is the amplitude of the test pulse, and E_{Na} is the reversal potential. Data were normalized with respect to the maximal G_{Na} and fitted to a Boltzmann equation (solid line): $y = 1/[1 + \exp(V_{0.5.} - V)/k_a]$, where V is the test voltage, $V_{0.5}$ is the voltage at which $y = 0.5$, and k_a is the slope factor. The mean values ± SE of individually fitted data are presented in the text.

value of 6.7 ± 0.9 mV *vs.* 13.8 ± 1.9 mV ($n = 6$; $p < 0.05$).

Conventional steady-state inactivation (h_∞) was measured by a test pulse of +50 mV with various prepulse voltages from −170 mV to −25 mV for 100 msec. Superimposed current traces were shown under these conditions before (Fig. 5*A*) and after 5-min UVA application (Fig. 5*B*). The analyzed results are presented in Fig. 5*C*. The $V_{0.5}$ value of the h_∞ curve was shifted in the depolarizing direction by only 3.2 mV (−90.0 ± 2.4 mV *vs.* −86.8 ± 2.3 mV, respectively, mean ± SE; $n = 5$; $p = 0.36$). The degree of this rightward shift was not statistically significant but was likely larger than −3.2 mV since we did not take into account the spontaneous leftward shift of 0.135 mV/min found for hH1 channels (Wang, George & Bennett, 1996). More important was a non-inactivating component (9.8 ± 2.8 %, mean ± SE, $n = 5$) present at voltages higher than −50 mV after UVA irradiation. This non-inactivating component was 3.3 times as large as that of control (3.0 ± 1.0 %; $p < 0.05$). Irradiation with UVA light apparently created a rather significant quantity of Na^+ currents that are non-inactivating even after 100-msec conditioning pulses. In addition, the slope factor was significantly less steep (1.7-fold) than that of control (12.3 ± 0.7 mV *vs.* 7.1 ± 0.3 mV; mean ± SE, $n = 5$; $p < 0.05$). We did not measure the detailed gating parameters beyond 5-min UVA irradiation because holding currents, which were nonlinear during UVA irradiation, often interfered with these measurements.

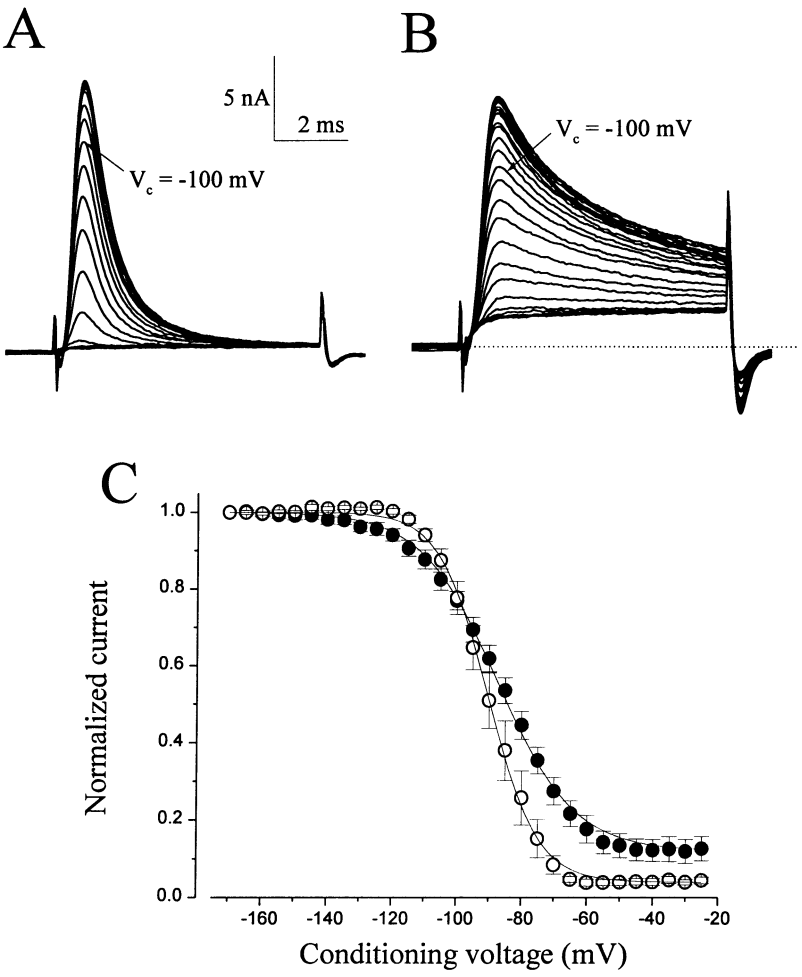

Fig. 5. Steady-state Na^+-channel inactivation before and after 5-minute UVA irradiation. The h_∞ curve was measured by a two-pulse protocol. The conditioning pulse was varied in 5-mV step increments from −170 mV to −25 mV, followed by a constant test pulse of +50 mV. A 0.08-msec gap at −140 mV was inserted between the conditioning pulse and the test pulse to reset the activation gate. Superimposed Na^+ current traces during the test pulse were recorded before (*A*) and after (*B*) 5 minutes of UVA irradiation. Current traces with a conditioning voltage of −100 mV were labeled. Notice that a maintained current is evident in (*B*), which is non-inactivating even with a conditioning pulse of −25 mV for 100 msec. Peak Na^+ currents were measured within 1 msec of the test pulse, normalized against the amplitude with a conditioning pulse of −170 mV, and plotted against the conditioning voltage (*C*). Data were fitted to a Boltzmann equation (solid line): $y = 1/[1 + \exp(V_{pp} - V_{0.5})/k_h]$, where V_{pp} is the conditioning voltage, $V_{0.5}$ is the voltage at which $y = 0.5$, and k_h is the slope factor. The mean values ± SE of individually fitted data are presented in the text.

Modifications of Nav1.4 Isoforms and Native Neuronal Na^+ Channels by UVA Light

To address whether other α-subunit Na^+ channel isoforms and native Na^+ channels are also sensitive to UVA irradiation, we selected the human skeletal muscle Nav1.4 isoforms and the native neuronal Na^+ channels in rat GH_3 cells for testing. Figures 6*A* and *B* show that hNav 1.4 and neuronal native Na^+ channels, respectively, are also sensitive to the UVA irradiation. During the first 10-min UVA application, the decaying phases of Nav1.4 currents were slowed considerably and the maintained currents reached steady state within this period. The times to reach the peak current amplitude of the rising phases were lengthened, and generally the peak amplitudes were reduced progressively without reaching steady state. In some cells, peak Nav1.4 current amplitudes remained the same or slightly increased during the first 2–3 min UVA application (Fig. 6*A*). This may be due to the fact that inactivation gating, if slowed significantly, will increase the peak current amplitude (Gonoi & Hille, 1987) despite the simultaneous reduction of peak current amplitude by UVA. It generally took 7.5–10 min for the maintained current to reach its maximal quasi steady state during UVA irradiation.

Application of UVA likewise modified the fast inactivation gating for native neuronal Na^+-channel gating in GH_3 cells. Again, the maintained current generally took 7.5–10 min to reach its maximal quasi steady state during UVA irradiation (Fig. 6*B*). During this period, the time to reach the peak current amplitude was progressively slowed and the peak amplitude decreased. In some GH_3 cells the peak current amplitude held steady or increased slightly during the initial two minutes of the UVA application, as seen in some cells transfected with hNav1.4 channels. Thus, the UVA effects on the channel gating of hNav1.4 and native neuronal channels were very similar to each other and comparable to those of Nav1.5 channels. We likewise found similar results in

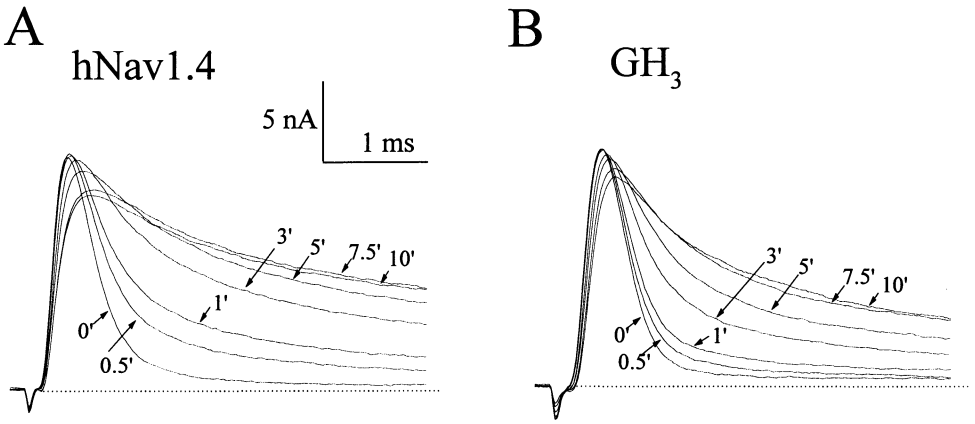

Fig. 6. UVA irradiation in cells expressing hNav1.4 and native neuronal Na^+ channels. Superimposed Na^+ currents were recorded in Hek293t cells with human skeletal muscle Nav1.4 channels (*A*) and in GH_3 cells with native neuronal Na^+ channels (*B*) before (trace 0′) and after UVA irradiation for 0.5, 1, 3, 5, 7.5, and 10 minutes. The pulse protocol was the same as in Fig. 4*A*. Holding potential was set at −140 mV.

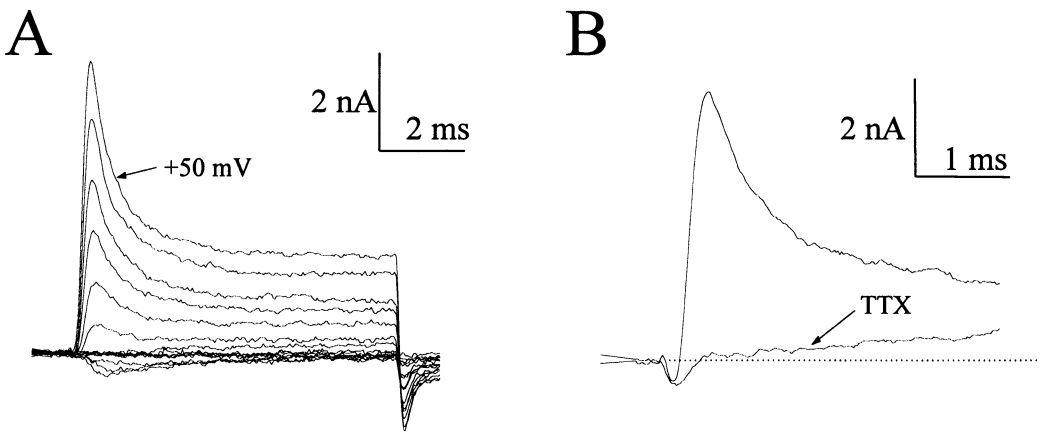

Fig. 7. UVA irradiation of intact neuronal GH_3 cells. GH_3 cells were first selected under the microscope, positioned at the center of the microscope field as usual, and irradiated with UVA light for 5 minutes. Subsequently, the whole-cell configuration was established to determine if the level of Na^+ currents was stable for further recording. Superimposed traces of a Na^+-current family (*A*) were recorded at various test voltages as described in Fig. 4*A*. These Na^+ currents were sensitive to TTX as shown in (*B*) with superimposed traces of Na^+ currents before and 2 minutes after application of 20 μl of TTX at 10 μM directly to the well. Also found in untransfected cells, the small TTX-resistant current is probably the residual outward current (Wang & Wang, 1998) and the UVA-induced cation current (Mendez & Penner, 1998).

rat skeletal muscle Nav1.4 channels after UVA irradiation. Taken together, the above results demonstrate that the molecular target sites of UVA irradiation are mostly conserved within Na^+ channel isoforms.

UVA Irradiation of Intact Cells

We asked whether cellular ingredients might protect the UVA effect on the Na^+ channels in intact cells, whereas the whole-cell configuration might remove such ingredients after dialysis by the pipette solution. To address this question, we applied UVA irradiation to intact GH_3 cells before making the whole-cell configuration with pipette electrodes. After UVA irradiation for 5 minutes, the GH_3 cells were voltage-clamped as described above. The UVA-irradiated GH_3 cells were generally leaky (i.e., required a larger holding current) under voltage-clamp conditions. However, Na^+ currents with retarded inactivation were found under various test pulses (Fig. 7*A*). Generally, inward Na^+ currents in GH_3 cells were small; these neuronal Na^+ channels were activated only by depolarization larger than −40mV (Cota & Armstrong, 1989), about 20–30 mV more positive than that needed to activate Nav1.5 currents in HEK293t cells (Fig. 2*A*). These UVA-modified Na^+ currents in GH_3 cells remained sensitive to TTX (Fig. 7*B*). We concluded that the cellular ingredients in intact cells were unable to protect the Na^+ channels

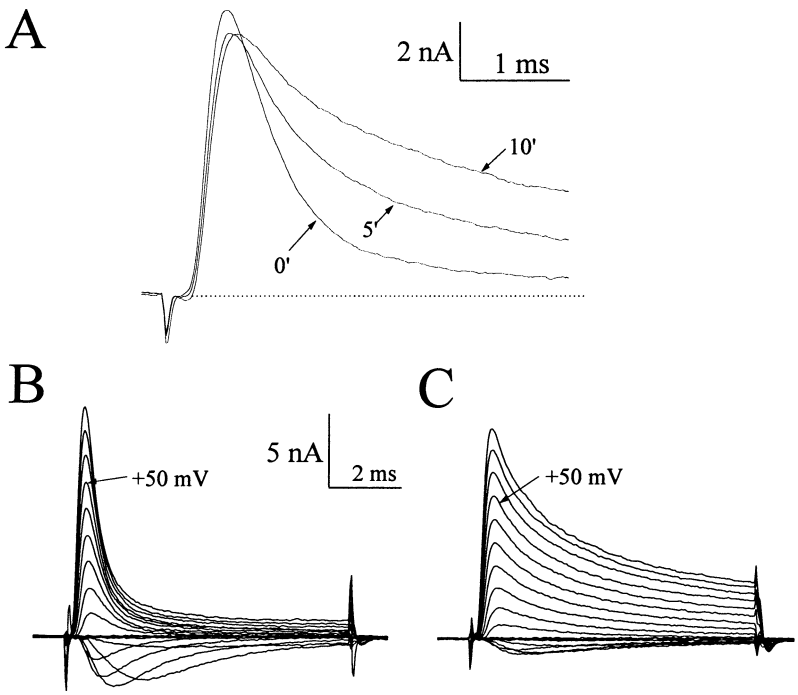

Fig. 8. Modifications of human Nav1.5 Na^+ channel gating by external 1.5% H_2O_2. (*A*) Superimposed Nav1.5 Na^+ current traces were recorded before (0′), 5, and 10 minutes after treatment with external 1.5% H_2O_2. The pulse protocol was the same as shown in Fig. 2*A*. Notice that a greater portion of Na^+ currents was maintained at the end of the pulse than in the UVA-irradiated cells. Families of Na^+ currents at various voltages were recorded before (*B*) and 10 minutes after (*C*) treatment with external 1.5% H_2O_2. The pulse protocol was the same as shown in Figs. 4*A* and *B*. Current traces elicited by +50 mV were labeled.

from UVA irradiation. Thus, oxygen metabolites generated by the UVA irradiation either exceed the protective capacity of antioxidants found in intact cells, or these metabolites are inaccessible to cellular antioxidants.

Effects of H_2O_2 on Na^+ Channel Isoforms

To test whether oxygen metabolites were indeed involved in the UVA effects, we first applied H_2O_2 externally to the transfected cells expressing human Nav1.5 channels. H_2O_2 at 1.5% inhibited fast-inactivation gating conspicuously. There was a slight slowing of the rising phase of Na^+ currents during the first 10 minutes of H_2O_2 application, but no further changes were found thereafter. The peak Na^+ current was also reduced but in a variable degree, from $<5\%$ in some cells to up to 30% in other cells. The H_2O_2 concentration (1.5%) was half that reported to induce a cationic current in HEK293 cells (Mendez & Penner, 1998). The effects of H_2O_2 on the Na^+ current decaying phase reached steady state after 10–15 min of this drug application. Fig. 8*A* shows the superimposed current traces recorded before, 5 min and 10 min after the H_2O_2 treatment. The time constants of the decaying phases were measured as 0.56 msec and 1.49 msec before and 10 min after the H_2O_2 treatment, respectively, with a difference of 2.7-fold. Thus, the effects of UVA irradiation and H_2O_2 treatment on the slowing of the decaying phase of the Na^+ currents at +50 mV are similar (Fig. 2*A* *vs.* Fig. 8*A*). Figures 8*B* and 8*C* show the current families before and 10 min after the H_2O_2 treatment. It was clear that the decaying phases of the outward Na^+ currents were slowed significantly, as expected. Furthermore, peak inward currents were reduced more than outward currents were, a phenomenon similar to that seen after UVA irradiation (Fig. 4).

Further experiments showed that human Na^+ channel Nav1.4 channels ($n = 5$) and native neuronal Na^+ channels in rat GH_3 cells ($n = 5$) are also sensitive to external 1.5% H_2O_2, and their phenotypes after modifications are also similar to those of hNav1.5 isoforms. Taken together, our data again demonstrate that the target site for H_2O_2 is conserved within Na^+ channel isoforms. Since these results suggest a linkage between UVA irradiation, oxidative stress, and impaired Na^+ channel gating, we subsequently asked whether internal glutathione in its reduced form protects the Na^+ channel from UVA modifications or from the H_2O_2 treatment. We found that neither the time course, nor the level of modification of the Na^+ channel kinetics were affected by internal 24 mM glutathione after UVA irradiation ($n = 5$). Irradiation was performed ~20 min after internal dialysis with 24 mM glutathione included in the pipette solution. Likewise, results were identical after external H_2O_2 treatment, whether the intracellular solution contained 24 mM glutathione or not. These data indicate that either the amount of glutathione applied may be insufficient or the compound may not have access to the membrane-bound Na^+ channel for its protection from modification by hydrogen peroxide or UVA irradiation. Because of the large H_2O_2 concentration required in these experi-

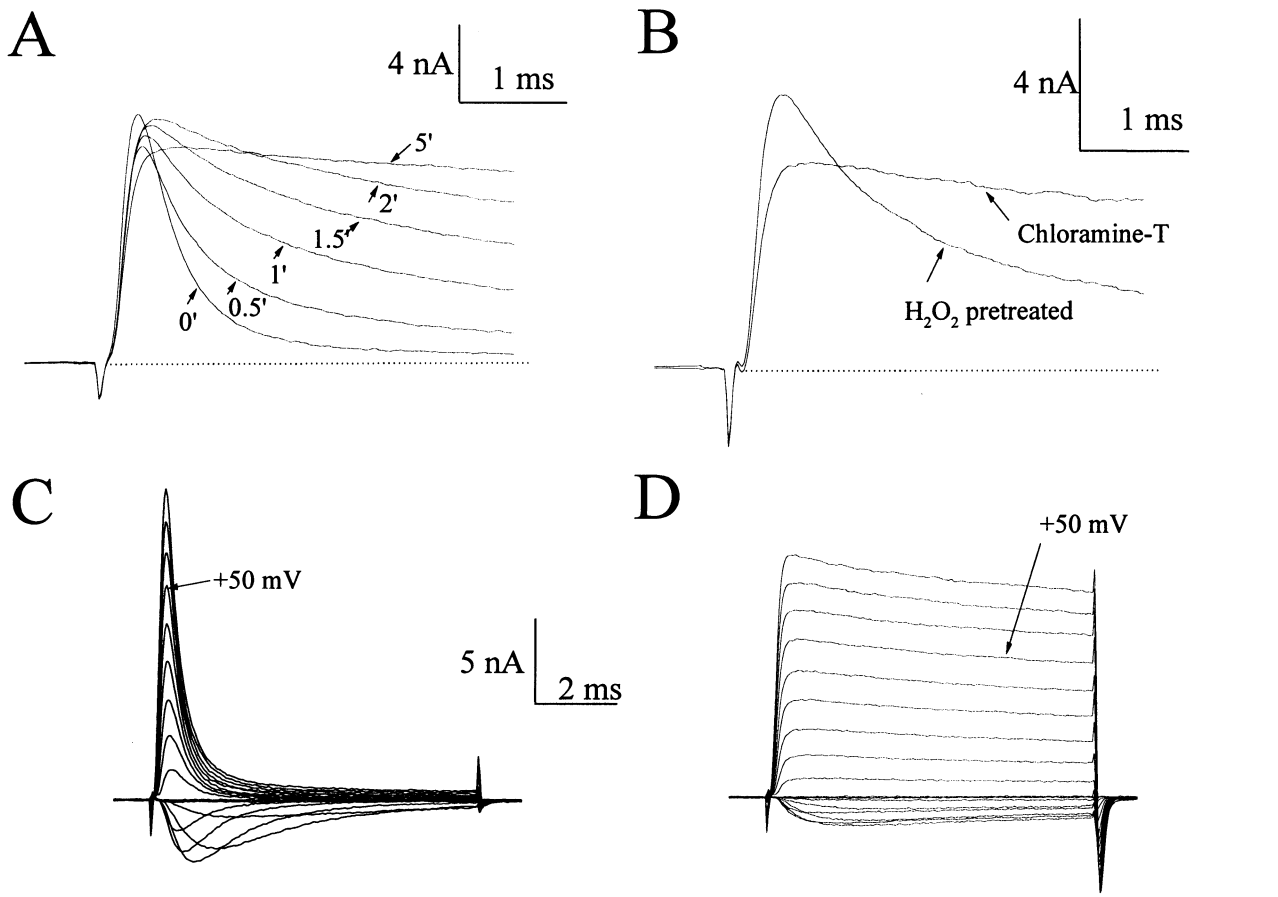

Fig. 9. The effects of oxidant chloramine-T on human Nav1.5 Na^+ channels. (*A*) Superimposed human Nav1.5 Na^+ currents were elicited at the test pulse of +50 mV and recorded before (0′), 0.5, 1, 1.5, 2, and 5 minutes after the treatment of external chloramine-T at 0.5 mM. In a different experiment, human Nav1.5 Na^+ currents were first modified by external 1.5% H_2O_2 for 10 minutes and then washed with the external solution for 2 minutes. (*B*) The current trace was subsequently recorded after this external H_2O_2 pretreatment and superimposed on the current trace taken after 2 minutes of the further external treatment by chloramine-T at 0.5 mM. Notice that the H_2O_2 pretreatment does not protect from the effects elicited by chloramine-T treatment. For comparison, human Nav1.5 current families were recorded before (*C*) and after (*D*) 5 minutes of treatment by chloramine-T at 0.5 mM. The pulse protocol is the same as shown in Fig. 4*A*. The level of maintained currents was higher in chloramine-T-treated cells than in UVA-irradiated or in H_2O_2-treated cells, as shown in Figs. 4*B* and 8*C*, respectively.

ments, the physiological significance (if any) of H_2O_2 modifications of the Na^+ channel fast inactivation gating remains unclear *in vivo*. Nonetheless, our findings provide direct evidence that intrinsic oxygen metabolites, such as H_2O_2, could have drastic effects on Nav1.5 Na^+ channel inactivation. In contrast, activation was less affected by H_2O_2, suggesting that other oxygen metabolites besides H_2O_2 are involved in the slowing of the activation time courses caused by UVA irradiation.

Effects of Oxidant Chloramine-T on Human Nav1.5 Channels

To date, the effects of chloramine-T on hNav1.5 channels have not been studied. To test whether different oxidants modify Na^+ currents differently, we applied oxidant chloramine-T externally to cells transfected with the hNav1.5 clone. Chloramine-T is known to cross the cellular membrane (Wang, Brodwick & Eaton, 1985). Figure 9*A* shows that external chloramine-T at 0.5 mM rapidly removed the fast inactivation during the test pulse, usually after 2.5- to 5-min application. The time constants for traces were measured as 0.42 msec, 2.29 msec, and 5.82 msec before, 2.5 min and 5 min after chloramine-T treatment, respectively. Notice that most Na^+ currents were maintained during the pulse after 5-min treatment with chloramine-T. There was also an initial small slowing and reduction in Na^+ currents during the first 0.5–1 min of application. This initial change was followed by an increase in peak amplitude until most fast inactivation was removed. Thereafter, prolonged application of chloramine-T increased the leak (holding) current and continued to reduce the peak current amplitude with

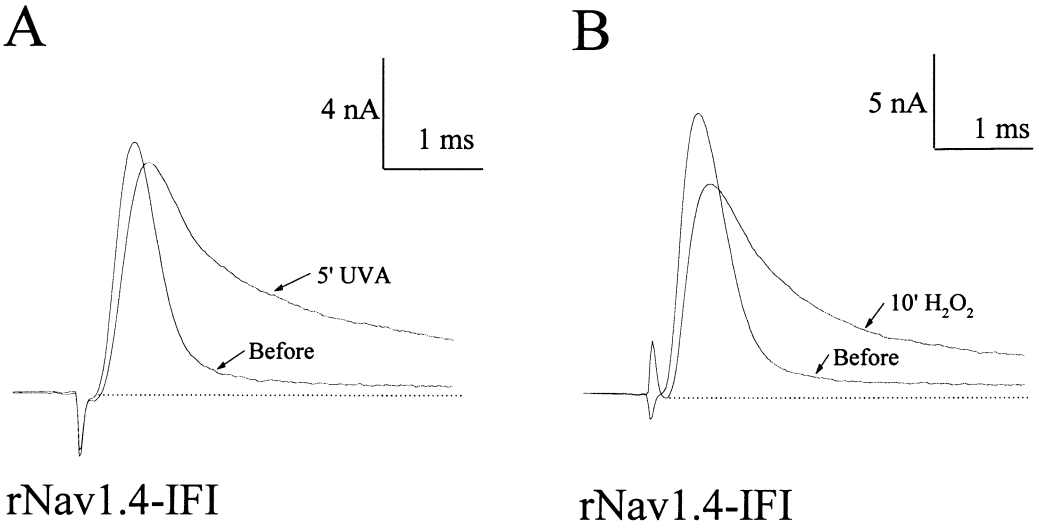

Fig. 10. UVA irradiation and H_2O_2 effects on rat skeletal Nav1.4-IFI mutant channels. Superimposed Na^+ currents of rat Nav1.4-IFI were elicited by a test pulse of +50 mV and recorded before and 5 minutes after UVA irradiation (*A*) or before and 10 minutes after treatment with external H_2O_2 at 1.5% (*B*). Two different cells were used in (*A*) and (*B*), as the UVA and H_2O_2 effects could not be reversed by washing. Notice that the fast-inactivating Na^+ current of the rat Nav1.4-IFI mutant channel before UVA or external H_2O_2 treatment is comparable to that of the wild-type human Nav1.4 channel shown in Fig. 6*A*.

little effect on the rising phase of Na^+ currents. For comparison, Figures 9*C* and *D* show the superimposed Na^+-current families before and 5 min after chloramine-T treatment. Clearly, chloramine-T was far more effective in modifying the fast inactivation of hNav1.5 channels than were UVA and H_2O_2. Finally, pretreatment with H_2O_2 at 1.5% did not protect the channel from further modification by chloramine-T, as shown in Fig. 9*B*. These observations demonstrate that different oxidants modify Na^+ currents differently, dependent on their reactivity and their final reaction products. Furthermore, the slight slowing of the activation time course in hH1 channels is isoform-specific, since such an effect is not evident in other Na^+ channels (e.g., Wang, 1984a).

IFM Motif and UVA Irradiation

Finally, we asked whether the methionine residue within the IFM motif, a conserved triplet sequence known to be essential for fast inactivation gating (West et al., 1992), is a molecular target of UVA irradiation or of H_2O_2 treatment. It has been reported that oxidation of the synthetic IFM peptide (S-oxomethionine) also reduced its potency in restoring fast Na^+ inactivation of IFM → QQQ mutant channels (Eaholtz et al., 1999). We found that the rat mutant Nav1.4-IFI channel (with IFM → IFI mutation) remained sensitive to UVA irradiation and also to H_2O_2 or chloramine-T treatment. The current kinetics of mutant rNav1.4-IFI channels appeared the same as those of the wild type, indicating that M-to-I substitution does not significantly alter the Na^+ channel fast inactivation. Figures 10*A* and *B* show that UVA irradiation and H_2O_2 treatment both modified the Na^+ current kinetics in mutant rNav1.4-IFI channels in the same way they modified the kinetics of wild-type channels. Each of these experiments was repeated in three cells with similar results. Thus, the methionine residue within the IFM motif is not the molecular target responsible for the observed effects of UVA irradiation and H_2O_2 treatment.

Discussion

This report demonstrates significant modifications of human Nav1.5 Na^+ channel gating by UVA irradiation. Fast inactivation is strongly inhibited within a few minutes of UVA irradiation; Na^+ channel activation is retarded albeit at a slower time course. Switching off the UVA light stops the modification of Na^+ channel gating instantly. To our knowledge, these phenomena have not been described before, even though UVA irradiation has been frequently used for fluorescence imaging (e.g., fura-2 or indo-1 as Ca^{2+} indicators) and for photocrosslinking experiments (e.g., Horn et al., 2000). Possible irreversible modifications of Na^+ channel gating should be considered while using UVA light on excitable cells, particularly when an exposure time of more than 30 sec is applied (Fig. 2*A*). Implications and physiological significance of our findings are discussed below.

Possible Linkage of UVA Light, Oxygen Metabolites, and Impairment of Na^+ Channel Gating

During in vivo electron transport, one oxygen molecule (O_2) accepts four electrons and four H^+ ions to

form two H_2O. This respiratory reaction is catalyzed by cytochrome oxidase within the mitochondria of eukaryotic cells. With UVA light, the oxygen (in aerated solution, ~0.2 mM) is instead activated by hν to form a spin-inverted singlet oxygen ($^1O_2$; lifetime ~4 μsec in water). Singlet oxygen may also be formed through the quenching of the triplet states of photosensitizers, including endogenous UVA sensitizers such as flavins, quinones, and porphyrins, by ground-state oxygen (Valenzeno & Tarr, 1992). In vivo singlet oxygen via its reactive cascade can generate a variety of peroxides and other byproducts (Fig. 1*A*), including various free radicals and their subsequent radical chain reactions. Alternatively, by acquiring an electron from endogenous UVA-excited sensitizers, oxygen itself may also become the free radical superoxide anion ($\cdot O_2^-$). The superoxide radical subsequently forms hydrogen peroxide by enzyme superoxide dismutase ($\cdot O_2^- + e^- + 2H^+ \rightarrow H_2O_2$), which is then decomposed to water and oxygen by the enzyme catalase or glutathione peroxidase (Tyrrell, 1991). Simultaneous generation of superoxide anion and free radical nitric oxide (NO·) may form a toxic reaction product, peroxynitrite anion ($OONO^-$; Fig. 1*A*; Cuzzocrea et al., 2001; Hoshi & Heinemann, 2001). In the presence of Fe^{2+}, hydrogen peroxide can also form a hydroxyl radical (·OH) by the Fenton reaction pathway ($H_2O_2 \rightarrow 2 \cdot OH$).

Oxygen metabolites, including singlet oxygen, superoxide radical, peroxynitrite, hydrogen peroxide, endoperoxides, lipid peroxides, hydroperoxides, and hydroxyl radical, are highly toxic to cells if not removed. In mammalian cells, oxygen metabolites are known to attack proteins, DNA, and lipid membranes. As a working hypothesis, we view that each of these oxygen metabolites could in theory modify the activation and/or fast inactivation of the Na^+ channel. Consistent with this view, Oxford, Pooler & Narahashi (1977) reported that, upon visual light irradiation, the Rose Bengal sensitizer blocks and impairs Na^+ channel inactivation when applied internally. They suggested that these effects are caused by the action of reactive oxygen metabolites, possibly via singlet oxygen. Another oxygen metabolite, hydrogen peroxide, is shown here for the first time to modify fast inactivation gating. External H_2O_2 at a concentration of 1.5% (or 440 mM) readily modified fast inactivation gating. The alteration of Na^+ channel inactivation by hydrogen peroxide was not reversible after washing, indicating the occurrence of covalent chemical modifications. Contrary to this positive result, several earlier reports showed that hydrogen peroxide does not modify fast inactivation gating directly (e.g., Wang, 1984b; Rack, Rubly & Waschow, 1986; Barrington, Martin & Zhang, 1997). Nonetheless, Ward and Giles (1997) found that hydrogen peroxide produced a marked prolongation of the action potentials and a significant enhancement of late opening events in rat ventricular myocytes. Since these authors applied hydrogen peroxide via an amphotericin-perforated patch method, and since direct intracellular hydrogen peroxide application yielded negative results, they suggested that protein kinase C is the intracellular second messenger for the observed H_2O_2 effects. We note that all of these previous studies used a much lower concentration of hydrogen peroxide (≤20 mM). One explanation for the lack of direct modification of Na^+ channel fast inactivation by ≤20 mM H_2O_2 is that cells in these studies contain the enzymes catalase and glutathione peroxidases, which may efficiently remove the applied hydrogen peroxide. Consistent with this hypothesis, little or no modification of hNav1.5 inactivation gating was detected when an external H_2O_2 concentration of 44 mM (0.15%) was applied. Nonetheless, it is also possible that the reactivity of H_2O_2 to its Na^+ channel target may be relatively low and that hydroxyl free radical byproduct may be generated from H_2O_2 (Fig. 1*A*), which in turn modifies fast inactivation of Na^+ channels. These various possibilities deserve further investigation. We did not attempt to apply H_2O_2 in the pipette solution as this neutral molecule penetrates the membrane readily when applied externally.

Possible Molecular Targets of UVA Irradiation of the Human Cardiac Na^+ Channel

Oxidants such as chloramine-T and H_2O_2 are capable of inhibiting the fast inactivation gating of the Nav1.5 Na^+ channels. Since chloramine-T modifies both methionine and cysteine residues specifically (Shechter, Burstein & Patchornik, 1975; Hoshi & Heinemann, 2001), we therefore suggest that reactive oxygen radicals and their metabolites act as oxidants in modifying the fast Na^+ channel inactivation, i.e., via the modification of methionine or cysteine residues. We surmise that cysteine residues are less likely to be the target site for the fast inactivation, since the cysteine-modifying reagents, such as N-ethylmaleimide and 5,5′-dithiobis-2-nitrobenzoic acid do not significantly modify the inactivation gating of the wild-type Na^+ channels (Wang, 1984b). Thus, UVA, H_2O_2, and chloramine-T probably modify the fast inactivation of Na^+ channels via common methionine targets. Additional experiments showed that the methionine residue on the IFM triplet motif, important for fast inactivation gating, is not the direct molecular target of UVA or hydrogen peroxide (Fig. 10). There are 68 additional methionine residues in the human Nav1.5 Na^+ channel. One or several of them may be the target of UVA irradiation. Further site-directed mutagenesis is required to resolve the precise target sites in Na^+ channels pertinent to reactions elicited by UVA and hydrogen peroxide.

As for the slower modifications of the Nav1.5 activation gating by UVA, we suggest that molecular targets other than those responsible for the fast inactivation gating must be involved. This is because the time courses in their modifications are different and because neither H_2O_2 nor chloramine-T modifies the activation gating as significantly as UVA does. It is likely that (*a*) other oxygen metabolites besides H_2O_2 modify the activation gating via different reactions and that (*b*) multiple UVA targets (such as tyrosine, tryptophan, histidine, cysteine, and methionine residues) are involved in the activation gating.

The modifications of gating by oxidants are not unique to the Na^+ channel. Oxidants are also able to modify other ion channels, such as large-conductance Ca^{2+}-activated K^+ channels (Dichiara & Reinhart, 1997), *Shaker* K^+ channels (Ciorba et al., 1997), and various other K^+ channels (Kv1.3, Kv1.4, Kv1.5, Kv3.4, IRK3; Duprat, et al., 1995). Methionine has been implicated as the molecular target in some of these studies (for review, *see* Hoshi & Heinemann, 2001).

Oxidative Stress And Impaired Fast Na^+ Channel Gating in vivo?

Based on our UVA results, we hypothesize that reactive oxygen metabolites will strongly affect the function of the Na^+ channel in vivo. The linkage of the oxidative stress and the Na^+ channel function, if established, will undoubtedly have significant implications. A number of reports show that a small number of Na^+ channels are present with slowly-inactivating kinetics in cells transfected with Na^+ channel α-subunit isoforms; up to 3% of Nav1.4 Na^+ channels are slowly-inactivating (e.g., Ukomadu, et al., 1992). Non-inactivating persistent Na^+ currents are also commonly detected in the central nervous system (Crill, 1996) and in ventricular myocytes (Saint, Ju & Gage, 1992). The precise origin of non-inactivating Na^+ channels remains unclear. It is possible that a portion of this non-inactivating current is derived from oxidation of normal Na^+ channels via reactive oxygen metabolites under physiological conditions. However, a sudden spurt of reactive oxygen metabolites can be detected during oxidative stress such as myocardial ischemia-reperfusion, CNS trauma, inflammatory injury, shock, and organ failure. In fact, even hypoxia alone has been shown to seriously compromise the proper function of Na^+ channels (Ju, Saint & George, 1996; O'Reilly, Cummins & Haddad, 1997; Hammarstrom & Gage, 1998). Since Na^+ channels are critical for neuronal, skeletal, and cardiac function, the repercussion of impaired activation- and fast inactivation-gating under these pathological conditions could be life threatening. Even small alterations in Na^+ channel gating can elicit dangerous complications in individuals, as has been documented for many genetic diseases caused by mutations associated with the Na^+ channel protein (Ashcroft, 2000). Future experiments to identify the exact oxygen metabolites involved in modifying Na^+ channel gating and their corresponding molecular targets will help design better strategies for possible pharmacological intervention during ischemia-reperfusion.

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