Chronic high inspired CO2 decreases excitability of mouse hippocampal neurons

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Abstract

To examine the effect of chronically elevated CO$_2$ on excitability and function of neurons, we exposed mice to 8% and 12% CO$_2$ for 4 weeks (starting at 2 days of age), and examined the properties of freshly dissociated hippocampal neurons obtained from slices. Chronic CO$_2$-treated neurons (CC) had a similar input resistance ($R_m$) and resting membrane potential ($V_m$) as control (CON). While treatment with 8% CO$_2$ did not change the rheobase ($64\pm11$ pA, n=9 vs. $47\pm12$ pA, n=8 for CC 8% vs. CON), 12% CO$_2$ treatment increased it significantly ($73\pm8$ pA, n=9, $p=0.05$). Furthermore, the 12% CO$_2$ but not the 8% CO$_2$ treatment decreased the Na$^+$ channel current density ($244\pm36$ pA/pF, n=17, vs. $436\pm56$ pA/pF, n=18, for CC vs. CON, $p=0.005$). Recovery from inactivation was also lowered by 12% but not 8% CO$_2$. Other gating properties of Na$^+$ current, such as voltage-conductance curve, steady state inactivation, and time constant for deactivation, were not modified by either treatment. Western blot analysis showed that the expression of Na$^+$ channel types I, II and III was not changed by 8% CO$_2$ treatment but their expression was significantly decreased by 20-30% ($p=0.03$) by the 12% treatment. We conclude from these data and others (Gu et al. 2004) that 1) neuronal excitability and Na$^+$ channel expression depend on the duration and level of CO$_2$ exposure and 2) maturational changes occur in early life regarding neuronal responsiveness to CO$_2$. (This work was supported by NIH grants PO1 HD-32573, RO1 NS-35918 and RO1 HL-66327).

Key Words: Na$^+$ channels, excitability, CO$_2$
Introduction

Hypercapnia has been linked to many diseases, such as obstructive sleep apnea and chronic obstructive lung disease. Some of these conditions affect millions of adults and children. Untreated sleep apnea can lead to higher blood pressure and a higher risk for stroke (Otsuka 2000).

The effects of hypercapnia have long been studied (Brown 1930; Sieker and Hicham 1956), but the effects on neural properties are not very well understood. Although we have previously studied neurons from chronically elevated CO₂ (8%) (Gu et al. 2004), we do not know to what extent the magnitude and duration of this elevated CO₂ will have on neuronal function. We therefore asked 1) how would neuronal properties change if we increase the duration of CO₂ exposure? 2) how would 8% CC treatment compare with a higher level of CO₂, such as 12%? and 3) what is the effect of chronic hypercapnia on neuronal function, such as excitability? In this work, we undertook this task and used 8% and 12% CO₂ treatment with a duration of 4 weeks to extend our previous results. We used electrophysiological and Western blot methods to determine the cellular mechanisms that were altered in response to hypercapnia. We focused our work on the hippocampus since our previous work was done on the same neurons and in order to compare with results from the literature (Gu et al. 2000).
Methods

**Chronic CO₂.** A computer-controlled chamber (OxyCycler, Reming Bioinstruments, Redfield, NY) was used for the induction and maintenance of chronic CO₂. The CO₂ level was monitored and controlled by the software from OxyCycler at constant concentration of 8% or 12%. Animals exposed to CO₂ are referred to here as CC 8% or CC 12% CO₂. Oxygen in the chamber was kept at constant concentration of 21% balanced by Nitrogen. Mice were placed in the chamber at the age of 2 days with their dam and were exposed to CO₂ for about 25-30 days until the time of sacrifice. They hyperventilated but they did not seem to be in any distress. All exposed mice, survived the period of exposure. Control mice were born and raised in our animal facilities. Pregnant mice (CD1) were purchased from Charles River Laboratories, Wilmington, MA.

**Preparation of CA1 cells.** Mice at age of 26-31 days were used and sacrificed after inhalation of isoflurane (Baxter, Deerfield, IL or VedCo, MO). Their hippocampi were quickly removed in ice-cold dissection solution and sliced into transverse sections with thickness of 400 μm. The slices were immediately transferred to a home-made container with 10 ml of fresh, oxygenated, and slightly stirred HEPES buffer at room temperature. After 30 minutes of exposure to protease (0.1%, Sigma) digestion, and 20 minutes of trypsin (0.08%, type XI, Sigma) the slices were washed with HEPES buffer and left in the oxygenated and stirred HEPES solution. The CA1 region was then dissected out and triturated in a small volume (0.25 ml) of HEPES buffer with 3 pipettes of gradually reduced
openings. The Albert Einstein College of Medicine Animal Care and Use Committee and University of California San Diego IACUC (Institutional Animal Care and Use Committee) have approved these studies.

Electrophysiological recording and solutions. Electrodes for whole-cell recording were pulled on a Flaming/Brown micropipette puller (Model P-87, Sutter Instrument Co., Novato, CA) from filamented borosilicate capillary glass (1.2 mm OD, 0.69 mm ID, World Precision Instruments, Sarasota, FL). The electrodes were fire-polished, and resistances were 2 - 5 MΩ for voltage clamp experiments and 7 - 9 MΩ for current clamp experiments measured in the solutions below. Membrane potentials (V_m) and action potentials (APs) were recorded in the current-clamp mode. Input resistance (R_m) was calculated at -70 mV as 1/slope of the current trace evoked by a ramp voltage from -160 to 100 mV in the voltage-clamp mode. The slope was derived from least-square regression analysis for 100 data points between voltage and current. Current traces in voltage clamp were leak-subtracted. Liquid junction potentials were nulled for each individual cell with the Axopatch 1C amplifier.

The HEPES-buffered solutions for the enzymatic preparation and trituration of the CA1 cells as well as the external HEPES solution used for the current clamp experiments contained (in mM) and: 130 NaCl, 3 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose and pH was adjusted to 7.4 with NaOH. The pipette solution contained: 138 KCl, 0.2 CaCl₂, 1 MgCl₂, 10 HEPES (Na⁺ salt), 10 EGTA and pH was adjusted to 7.4 with Tris. The external solutions for the voltage clamp experiments contained similar reagents as in the current clamp experiments, except for 10 mM TEA chloride, 5 mM 4-AP, and 0.1 mM CdCl₂ and
reducing NaCl from 130 mM to about 117 mM. The internal pipette solution for the voltage clamp experiments was also similar to the internal solution for the current clamp experiments, except for the use of either CsF instead of KCl (we had previously shown that there was no difference in whole-cell Na$^+$ current recorded when CsF or CsCl was used (O’Reilly et al. 1997)). Osmolarity of all solutions was adjusted to 290 mOsm. All recordings were performed at room temperature (22-24°C). One-tailed Student t test was performed for comparisons. Significances were indicated if p<0.05 assuming two groups had an equal variance. Numbers in the text and in the figures were given as means±S.E. All chemicals were purchased from Sigma.

**Recording criteria.** These criteria have been previously used, as detailed in our previous publications (Oreilly et al. 1997). Briefly, we have used morphologic and electrophysiological criteria. a) **Morphologic criteria:** CA1 cells were used if they had a smooth surface, a three-dimensional contour, and were pyramidal shape. Similar criteria have been used by us (Gu and Haddad 2003; Gu et al. 2000) and others (Hamill et al. 1981) on freshly dissociated neurons. The CA1 neurons studied were obtained from 12-17 or 26-31 day old mice. b) **Electrophysiological criteria:** i) Neurons were considered for recording if the seal resistance was > 1 GΩ. ii) Only neurons with a holding current of < 0.1 nA (command potential -100 mV) were used in the study. iii) Series resistance was less than 10 MΩ in neurons studied. The series resistances were compensated at 90% level with the Axopatch 1C amplifier (Axon Instruments). Under these conditions, the error caused by uncompensated series resistances was < 0.6 mV. The error was not corrected. To obtain adequate voltage clamp and minimize the space clamp problem, only neurons of small size
with short processes were used in the current measurement. In addition, only cells with
current-voltage curves that were smoothly graded over the voltage range of activation (~−50
to -10 mV) were used, as we have done in the past (Gu and Haddad 2003; Gu et al. 2000).

**Immunoblotting.** Hippocampi from four different animals, from each of the 3 groups
(CON, 8% CC and 12% CC) each group had 4 samples) to lysis buffer (200 mM
mannitol, 80 mM HEPES, 41 mM KOH, 1 tablet/50ml of Complete protease inhibitor
cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany), 230 μM phenylmethylsulfonyl fluoroide, pH 7.5). Samples were homogenized and the homogenate
was centrifuged at 1,000 g at 4°C for 10 min. The supernatant was then centrifuged at
100,000 g at 4°C for 1.5 h. The pellet was re-suspended in lysis buffer, and protein
concentration was determined with the use of the Bio-Rad Dc Protein Assay kit.
Membrane proteins (30 μg) were resolved on 4–12% precast NuPAGE Bis-Tris gels
(Invitrogen, Carlsbad, CA) and electro-transferred onto polyvinylidene difluoride
membranes (Immobilin-P; Millipore, Bedford, MA). Affinity-purified rabbit polyclonal
antibodies against Na⁺ channel type I (1:600 dilution), type II (1:300), and type III (1:80)
(Sigma, Saint Louis, MO) were applied. Protein signals were detected with the use of an
enhanced chemiluminescence system (ECL; Amersham, Chalfont, UK). For
normalization, membranes were stripped and re-probed with affinity-purified goat
polyclonal antibody to actin at 1:500 dilution (Santa Cruz Biotechnology, Santa Cruz,
CA). Scanning densitometry of immunoblot films was performed on a Personal
Densitometer SI scanner (Molecular Dynamics, Sunnyvale, CA) and analyzed with the
aid of ImageQuant image analysis software (Molecular Dynamics, Sunnyvale, CA). Data are presented as ratios of protein to corresponding actin.

**Statistical Analysis.** Student's $t$-test or the Wilcoxon rank sum test was used for comparisons. All values represent means ± SE. Differences in means are considered significant if $p < 0.05$. 
Results

Membrane excitability

To determine whether CO₂ affects neuronal excitability, we measured the rheobase. Stepping from the same Vₘ (-75 mV) in the current clamp mode, the current required to generate one AP was 64±11 pA (n=9) at 8% and 73±8 pA (n=9) at 12%, values that were 36% and 55% higher than those of CON (47±12 pA, n=8) respectively (Fig. 1A, B and C), with p values of 0.27 and 0.046 when CON was compared with 8% and 12% (Fig. 1D). Samples of voltage traces from CON, 8% and 12% CC are shown in Fig. 1.

Passive Neuronal properties

Since CC treatment decreased neuronal excitability in 12%-treated neurons, we then investigated the potential factors that caused the lowering of excitability. We began with the passive neuronal properties such as resting membrane potential, Vₘ and input membrane resistance, Rₘ. CC-treated animals (8% and 12%) for 28 days did not show any effect on Vₘ. Indeed, CA1 neurons had similar membrane potentials as those of CON neurons (Vₘ: -37±4 mV, n=10, and -36±3 mV, n=17 for CC at 8% and 12% CO₂ versus -42±2 mV, n=10, for CON). Furthermore, CC treatment did not have any effect on Rₘ and whole cell capacitance.
Na$^+$ current magnitude

Since 12% CO$_2$ treatment significantly changed neuronal excitability but did not change passive neuronal properties of CA1 hippocampal neurons, we then asked whether the Na$^+$ channel properties had been affected by the treatment. We first examined the effect of CC treatment on the Na$^+$ current of both CON and CC neurons. Steps from a holding potential of -130 mV to -20 mV evoked an inward current that reached a peak in <1 ms and decayed quickly to zero current. TTX (1 µM) blocked that current. After 28 days of CC treatment, the average peak Na$^+$ current was 1971±662 pA (n=17) for 8% as compared to control (1465±209 pA, n=22, p=0.21). CC treatment at 12% level, however, dramatically suppressed the peak Na$^+$ current (882±129 pA, n=17) as compared to the Na$^+$ current in CON (p=0.02). To eliminate the role of cell membrane surface area, we divided the peak current by cell capacitance. After 4 weeks of chronic CO$_2$ exposure, the current density at 8% (424±107 pA/pF) was similar to that of control (436±56 pA/pF, p=0.56). However, the current density at 12% (244±36 pA/pF) was substantially reduced when compared to the control group (p=0.005) (Fig. 2E).

Na$^+$ current gating properties

Since CC at 12% treatment decreased the Na$^+$ current density, we then asked whether other Na$^+$ current gating properties have been affected. We examined the gating properties of the Na$^+$ current to determine whether other properties could have played a role in determining the difference in excitability.

i) Activation.


Holding neurons at -130 mV and stepping commands from -70 mV to 10 mV in 10 mV increments, we obtained a family of curves of Na\(^+\) currents at different V\(_m\). Their IV curves are illustrated in Fig. 3. When the relative conductance-voltage curves were plotted and fitted to the Boltzmann equation, we found that after 4 weeks of CC treatment, the curve was shifted slightly in a depolarization direction at 8% and 12% as compared to that in the control group. Neither the mid-point of the curves nor the slope factors for the CO\(_2\) treatment were different from those of control (Fig. 3).

**ii) Steady-state inactivation.** We also studied the steady-state inactivation of the Na\(^+\) current, as described in Figure 3. We found that the mid-points of the inactivation curve at both 8% and 12% were almost identical to that of control (-68±2 mV, slope factor = 6±1 (n=11), -69±2 mV, slope factor = 6±1 (n=6) and -67±2 mV, slope factor = 6±0.4 (n=17) for CC 8%, CC 12% and CON respectively) (Fig. 3D).

**iii) Recovery from inactivation.** When we examined the recovery from inactivation, the currents evoked by the second pulse were smaller than the those of first one with their amplitude increasing with the increased intervals between the two pulses (Fig. 4). The time constants (τ\(_h\)) for recovery from inactivation could be fitted by a first order exponential equation. When τ\(_h\) from both 8% and 12% neurons were compared to control, these were not different from those for CON (5.7±0.9 ms, n=11, 7.2±2.0 ms, n=5, and 5.8±0.5 ms, n=14, for CC at 8%, CC at 12% and CON neurons respectively) (Fig. 4D). However, when we compared individual data points with each other, our data (< 20 ms) showed significant differences between 12% CO\(_2\) and control (Wilcoxon Rank Sum test).
iv) Deactivation characteristics. We further examined the deactivation properties, the transition from the open to the resting closed state without the inactivation, for both groups of neurons. We held CA1 neurons at -100 mV, depolarized them for 1 ms to -10 mV and repolarized to -100 mV. The mean time constant for deactivation, $\tau_d$ at -100 mV, could be fitted by a first order exponential equation. After 4 weeks of treatment, $\tau_d$ was smaller, albeit not significantly, in both CC 8% and 12% neurons as compared with the CON.

Na$^+$ Channel Expression

We further examined whether the decrease in Na$^+$ current density is due to a decrease in Na$^+$ channel expression, and, if so, which Na$^+$ channel subtype might be involved. Since there are three major neuronal subtypes of Na$^+$ channels in the central nervous system (CNS), we used specific antibodies against each neuronal isoform of Na$^+$ channel and performed immunoblotting assays. Mice exposed to 8% CO$_2$ for 4 weeks did not show any change in the hippocampal membrane protein expression of any of Na$^+$ channel subtypes type I, II and III Na$^+$ channel subtypes. However, exposure to 12% CO$_2$ selectively decreased Na$^+$ channel subtype I and III protein level by 27% and 20% respectively ($n=4$; $p=0.026$ and $p=0.029$) (Fig. 5). It is interesting to note that the down-regulation of Na$^+$ channel expression was less than the decrease in Na$^+$ channel current density, i.e., reduced to 44% of control, in contrast to approximately one-half of this decrease in the expression of membrane Na$^+$ channel.
Discussion

In order to extend our previous observations on the effect of CO₂ on neuronal excitability, we exposed mice to a longer duration of CO₂ treatment (e.g., 4 weeks) and also increased the severity of CO₂ (12% CO₂). We have shown in this work that 1) the excitability of neurons from animals chronically exposed to 8% CO₂ for 4 weeks was similar to control; 2) neurons from animals chronically exposed to 12% CO₂ had a lower excitability than control and than those exposed to 8% CO₂; 3) except for recovery from inactivation, most gating properties of Na⁺ channels were not modified by either 8% or 12% treatment; 4) Na⁺ channel current density was not modified by 8% but decreased by 12% CO₂ treatment and 5) Western blot experiments confirmed our electrophysiological results, i.e. Na⁺ channels had decreased expression by 12% but not by 8% treatment. The difference in excitability was related to Na⁺ channel expression and properties and not related to Vₘ, since a) the rheobase experiments were performed at the same voltage (-75 mV) and b) their inherent Vₘ were similar in magnitude. Furthermore, the difference in excitability was also not related to Rₘ since both CON and CC neurons had similar Rₘ.

Since the Na⁺ channel gating properties, with the exception of the slow-down of the recovery from inactivation did not change by CO₂ treatment, the decreased excitability at 12% is most likely due to the lower Na⁺ current density CO₂ treatment induced. One hypothesis for this decrease in Na⁺ channel density is that the Na⁺ channel protein expression is decreased. This is borne out by our findings of decreased expression of Na⁺ channel subtypes (I and III).

It is important to highlight that the difference in excitability is most likely not related to the major Na⁺ channel gating properties since these have not been altered by the CO₂
treatment. The slowing down of recovery from inactivation could be only a partial explanation as firing is diminished. However, we do not believe that this can explain the difference in excitability without considering Na⁺ channel expression. In addition, if the Na⁺ current density is a product of Na⁺ channel expression, channel open probability (Pₒ), single channel amplitude and conductance, then it is still possible that Pₒ, or single channel amplitude might change and could play a role. By itself, the change in Na⁺ channel expression can account for a major part of the change in cellular excitability but this does not rule out other factors.

Another important conclusion from this work is that different results have been obtained from different CO₂ treatments. Indeed, this is not surprising as different concentrations of CO₂ have been reported to have totally opposite effects on Kᵢr4.1 and Kᵢr5.1 channels. For example, 3% CO₂ enhanced Kᵢr currents and caused hyperpolarization and 8% CO₂ suppressed Kᵢr currents and caused depolarization (Cui et al. 2001). Opposite effects on field EPSPs were also reported with different concentrations of CO₂ in hippocampal slice preparations: 2% CO₂ is an enhancer while 10% and 20% were inhibitors of field EPSPs (Dulla et al. 2005). This idea does not seem to be unique for the brain as different concentrations of CO₂ have different effects, for example, on lung development. We have recently shown that 8% CO₂ has a major effect on gene regulation, such as for surfactant proteins A and D, but 12% CO₂ does not (Li et al. 2006). Studies dissecting the effect of various CO₂ levels on various organs would be necessary in the future.

How this decreased excitability actually occurs after a 4-week treatment of 12% CO₂ is not clearly understood. A number of factors could have contributed. First, although acute hypercapnia is not well compensated (Bracket et al. 1965), chronic hypercapnia is generally
Therefore, we believe that the effects of chronic hypercapnia on hippocampal neurons are most likely due to either CO$_2$ or HCO$_3^-$.

Second, it is possible that the effect of CO$_2$ is either direct, such as on neuronal membranes, (Balestrino and Somjen 1988) or via neuromediators or modulators. Third, if the effect is indirect, CO$_2$ effect could be induced by alterations in adenosine and ATP (Dulla et al. 2005), or alterations in growth factors (e.g., platelet activating factor, Simakajornboon et al. 1998). Effects of CO$_2$ via pH are controversial since it has been reported that a) short term treatment with CO$_2$/HCO$_3^-$ has its effect via HCO$_3^-$ and not from CO$_2$ or pH (Bruehl and Witte 2003), and b) the effect of CO$_2$ on K$_{ir1.1}$ channels was secondary to changes in pHi and pHo (Zhu et al. 2000). Fourth, although we used HEPES as buffer in our experiment (pH 7.4), this might not have been the pH that exposed mice had. It is possible that their pH sensitivity might have been altered. Using CO$_2$/HCO$_3^-$ as buffer will affect neuronal excitability and Na$^+$ channel gating properties as we have previously shown (Gu et al. 2000).

We have demonstrated in this work that chronic 12% CO$_2$ treatment decreased the current density by 44%, and decreased Na$^+$ channel types I and III expression by 27% and 20%, respectively. The discrepancy between current density and protein expression might be due to several factors. For example, 1) Na$^+$ channel expression using Western blot analysis was underestimated. Na$^+$ channel type II expression was also decreased by the treatment (as a trend with no statistical significance) and type IV, known to be in the hippocampus (Shaller et al. 1995), was not tested in our experiments.

2) The current density was based on the peak currents of whole cell recording. This peak current is not equivalent to Na$^+$ channel expression by Western blot analysis since it is a product of the number of the Na$^+$ channels involved, the single channel amplitude, the
single channel conductance and the open probability of the Na\(^+\) channels. Therefore, it is possible that the open probability of the channel, for example, contributes to the peak current.

Our previous data (Gu et al. 2004) and our current results lead us to conclude that CO\(_2\) stimulates the expression of Na\(^+\) channels when CO\(_2\) exposure is early in life. By so doing, CO\(_2\) increases excitability of neurons (Gu et al. 2004; Xia and Haddad, 1994). In our previous paper (Gu et al. 2004), we found that the 8\% CO\(_2\) treatment over a relatively short period of time (2 weeks) increased neuronal excitability over that of age-matched control neurons. In this current study, we found that a longer period of the same treatment (4 weeks) did not further increase this excitability.

In summary, we have shown that 12\% but not 8\% CO\(_2\) decreases neuronal excitability when mice are exposed for four weeks to this stress. This decreased excitability is secondary, by and large, to a decrease in Na\(^+\) channel subtype I and II expression.

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References


**Figure Legends**

**Figure 1.** Voltage traces of evoked action potentials (APs) from control (CON) (A), CC (chronic CO₂ treated) 8% (B) and CC 12% (C) neurons. Evoked APs were collected in the current clamp mode at -75 mV with 10 depolarizing currents, starting with 10 pA (A) using 10 pA increments, from bottom and follow upward incrementally. Scale represents 50 mV and 25 ms. In D, the minimum currents used to evoke an AP were averaged. * Represents significantly different means at p<0.05.

**Figure 2.** Comparison of Na⁺ current densities, expressed as ratio of normalized peak Na⁺ current over whole cell capacitance in control (CON), 8% CO₂ and 12% CO₂. * Represents significantly different means at p<0.05.
**Figure 3.** Voltage-conductance relationship of the Na\(^+\) current. In A (CON), B (CC 8%) and C (CC 12%), current traces were collected from -70 to 0 mV with 10 mV increment from a holding potential of -130 mV. Voltage protocol is shown under the trace of A. In D, voltage-conductance relationship was expressed as normalized conductance (g/g\(_{\text{max}}\)) against V\(_{\text{m}}\). Curves were fitted by the Boltzmann equation. Steady-state inactivation of the Na\(^+\) current. In E (CON), F (CC 8%) and G (CC 12%), current traces were collected at -20 mV from prepulse potentials ranging from -130 to -20 mV with an increment of 10 mV and a duration of 502 ms. Voltage protocol is shown under the trace of E. The scales in E, F and G represent 1 nA and 10 ms. I/I\(_{\text{max}}\) was plotted against the prepulse potential in H.

**Figure 4.** Recovery from inactivation of the Na\(^+\) current. In A (CON), B (CC 8%) and C (CC 12%), two identical pulses were delivered with increasing intervals (2.7 ms) (t) in between each pair of pulses. Voltage protocol is shown under the traces of C. The scales in A, B and C represent 1 nA and 10 ms. In D, recovery from inactivation of the Na\(^+\) current was plotted as the ratio of the peak of the second current over that of the first (I\(_{\text{peak2}}\)/I\(_{\text{peak1}}\)) against the intervals (t) in a two-pulse voltage protocol. * Represents significantly different means at p<0.05 with Wilcoxon rank sum test.

**Figure 5.** Differential regulation of Na\(^+\) channel subtypes I (A), II (B), and III (C) in the 4-week chronic CO\(_2\)-exposed mice. Immunoblotting method was used to compare hippocampal membrane protein levels of Na\(^+\) channel subtypes I, II, and III between chronic CO\(_2\)-exposed mice (both 8% and 12% CO\(_2\)) and their age-matched, controls. In
A–C, top, representative immunoblots of Na\(^+\) channel proteins and their corresponding actins; bottom, densitometric analyses of the protein signals. In each graph, the x-axis represents experimental conditions; the y-axis depicts the relative level of Na\(^+\) channels as a ratio of Na\(^+\) channel protein to actin density per 30 \(\mu\)g of total membrane protein. Values are means ± SE (n = 4 for each group). * Represents significantly different means at p<0.05.