ERK Integrates PKA and PKC Signaling in Superficial Dorsal Horn Neurons. II. Modulation of Neuronal Excitability

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Submitted 8 April 2003; accepted in final form 7 May 2003

Hu, Hui-Juan and Robert W. Gereau IV. ERK integrates PKA and PKC signaling in superficial dorsal horn neurons. II. Modulation of neuronal excitability. J Neurophysiol 90: 1680–1688, 2003. The sensation of pain, like other sensory modalities, is subject to modification by experience. In particular, pain sensitivity can be dramatically increased after an injury or in response to prolonged activation of nociceptive primary afferent neurons. A large body of literature suggests that this nociceptive sensitization can occur at the level of the spinal cord dorsal horn, and further that this so-called central sensitization is induced by the activation of a number of protein kinases, including protein kinase A (PKA), protein kinase C (PKC), and extracellular signal-regulated kinase (ERK) families. Recent studies in other brain regions have revealed that modulation of A-type currents can alter both first-spike latency and spike frequency (McCorquodale and Huguenard 1992; Yang et al. 2001). In the present study, we examine the modulation of neuronal excitability by these kinases, with a particular eye to evaluating the impact of kinase-mediated inhibition of IA on neuronal firing properties. We report that activation of PKA, PKC, and ERK lead to a decrease in first-spike latency, and an increase in spike frequency in response to direct current injection. Furthermore, the effects of PKA and PKC activation appear to require activation of ERK, given that blocking ERK activation prevents the modulation of firing properties induced by activators of PKA or PKC. This mirrors nicely the interaction of these kinases in the modulation of IA (Hu et al. 2003). We suggest that ERK phosphorylation of channels underlying IA leads me to at least the alteration in first-spike latency we have observed here, and further that the effects of PKA and PKC are attributed to their ability to activate ERK signaling.

METHODS

Cell culture

Primary cultures of spinal cord superficial dorsal horn neurons were prepared from 5- to 10-day-old ICR mice using a procedure similar to a previously described method (Hugel and Schlichter 2000). Briefly, after decapitation a laminectomy was performed and the spinal cord was carefully removed. Spinal cord superficial dorsal horn was isolated by cutting through lamina III with a surgical blade, and cut into 1- to 2-mm fragments. The tissue fragments were incubated for 45 min at 37°C in Hanks’ balanced salt solution (HBSS; Invitrogen Life Technologies, Carlsbad, CA) (in mM: 137 NaCl, 5.4 KCl, 0.4 KH2PO4, 1 CaCl2, 0.5 MgCl2, 0.4 MgSO4, 4.2 NaHCO3, 0.3 Na2HPO4, 5.6 glucose) containing papain (15 U/ml; Worthington Biochemical, Lakewood, NJ), rinsed 3 times with HBSS, and then replaced with culture medium containing Neurobasal medium (Gibco), fetal calf serum (5%, Gibco), heat-inactivated horse serum (5%, Gibco), L-glutamax-1 (2 mM, Gibco), B-27 (2%, Gibco), and glucose (20 mM, Sigma). The fragments were mechanically dissociated by gently triturating with a fire-polished Pasteur pipette. The resulting cell suspension was plated onto 12-mm poly-d-lysine- and collagen-coated coverslips, and cultured for 6 to 8 days (for action potential recordings) or 3 days (for Na+ and Ca2+ current recordings) in humidified air with 5% CO2 at 37°C.

INTRODUCTION

The sensation of pain, like other sensory modalities, is subject to modification by experience. In particular, pain sensitivity can be dramatically increased after an injury or in response to prolonged activation of nociceptive primary afferent neurons. A large body of literature suggests that this nociceptive sensitization can occur at the level of the spinal cord dorsal horn, and further that this so-called central sensitization is induced by the activation of a number of protein kinases, including protein kinase A (PKA), protein kinase C (PKC), and extracellular signal-regulated kinases (ERKs) (Ji and Woolf 2001; Karim et al. 2001).

This and the companion study are concerned with understanding the changes in neuronal excitability in the dorsal horn that occur in response to activation of PKA, PKC, or ERKs. We have shown that activation of these protein kinases leads to an inhibition of transient outward potassium currents (known as A-type currents or IA) in mouse superficial dorsal horn neurons from mice (Hu et al. 2003). Previous studies in other brain regions have revealed that modulation of A-type currents can alter both first-spike latency and spike frequency (McCormick and Huguenard 1992; Yang et al. 2001). In the present study, we examine the modulation of neuronal excitability by these kinases, with a particular eye to evaluating the impact of kinase-mediated inhibition of IA on neuronal firing properties. We report that activation of PKA, PKC, and ERK lead to a decrease in first-spike latency, and an increase in spike frequency in response to direct current injection. Furthermore, the effects of PKA and PKC activation appear to require activation of ERK, given that blocking ERK activation prevents the modulation of firing properties induced by activators of PKA or PKC. This mirrors nicely the interaction of these kinases in the modulation of IA (Hu et al. 2003). We suggest that ERK phosphorylation of channels underlying IA leads me to at least the alteration in first-spike latency we have observed here, and further that the effects of PKA and PKC are attributed to their ability to activate ERK signaling.

METHODS

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**Electrophysiological recording**

Whole cell current clamp recordings were made by standard procedures at room temperature using either a Patch Clamp PC-501A amplifier (Warner Instrument, Holliston, MA) and CLAMPEX 8.0 software (Axon Instruments, Union City, CA), or an EPC-10 amplifier and Pulse software (HEKA Elektronik, Lambrecht, Germany). Electrodes were pulled from filamented borosilicate glass and fire-polished. The electrode solution contained (in mM): 140 potassium methylsulfate, 2 MgCl₂, 1 EGTA, 10 HEPES, 3 Na₂ATP, 0.3 Na₂GTP, pH adjusted to 7.4 with KOH. Pipette resistances were 4–7 MΩ. Most neurons had series resistances around 10–14 MΩ (range, 8–20 MΩ). The bath solution was HBSS. Recordings of Na⁺ currents and Ca²⁺ currents were performed under voltage-clamp configurations, and the membrane voltage was held at −80 mV. All neurons included in this study had resting membrane potentials < −50 mV and had stable input resistance. For Na⁺ current recordings, the extracellular solution contained (in mM) 95 NaCl, 5 KCl, 10 CsCl, 20 TEA-Cl, 1 MgCl₂, 1 CoCl₂, 1 CaCl₂, 5 HEPES, and 10 glucose. The intracellular pipette solution contained (in mM) 120 mM CsCl, 20 TEA-Cl, 1 MgCl₂, 1 EGTA, 10 HEPES, 3 Na₂ATP, and 0.3 Na₂GTP. The same intracellular solutions were used for Ca²⁺ current recordings, and the extracellular

**FIG. 1.** Based on their firing properties in response to depolarizing current injection, neurons in mouse spinal cord superficial dorsal horn can be divided into four groups: repetitive, delayed firing, phasic (cells showing spike frequency adaptation), and single spike. Current injection protocols are shown below voltage traces, and percentage of cells demonstrating this firing profile are given in parentheses above traces. All neurons are manually clamped at −70 mV by current injection.

**FIG. 2.** 4-Aminopyridine (4-AP) modulates action potential firing and membrane properties of spinal dorsal horn neurons. A: representative examples of action potentials (generated by current injection protocol shown below voltage traces) and A-type currents recorded from same cell before and after 1.5-min bath-application of 4-AP (1 mM). Insets: A currents obtained by subtraction of sustained current (outward current remaining after prepulse to −10 mV) from total current induced by depolarizing step to 40 mV from −80 mV. B: summary of changes in action potential firing and membrane properties induced by 4-AP. Values are means ± SE; n = 6. *P < 0.05, paired t-test, compared with predrug control.
solution was the same except that 1 μM TTX was added, CoCl₂ was omitted, and 2.5 mM BaCl₂ was used instead of 1 mM CaCl₂.

**Drug application**

4-Aminopyridine (4-AP) was purchased from Sigma (St. Louis, MO) and dissolved in water as stock solution. Phorbol 12-myristate 13-acetate (PMA), PD98059, 8-Br-cAMP, PKI(6–22) amide, forskolin (FSK), IBMX, and Ro31-8220 were purchased from Biomol (Plymouth Meeting, PA) and prepared as concentrated stock solutions in DMSO. All of these were diluted to final concentrations in extracellular solution. Coverslips were placed in a small laminar flow perfusion chamber and continuously perfused with extracellular solution or various pharmacological manipulations at about 2 ml/min.

**Data analysis**

Off-line evaluation was done using Clampfit (Axon Instruments), Pulsefit (HEKA Elektronik), and Origin 5.0 software (Microcal, Northampton, MA). Data are expressed as original traces or as means ± SE. Treatment effects were statistically analyzed by paired or independent Student’s t-test. Error probabilities of $P < 0.05$ were considered statistically significant.

**FIG. 3.** Activation and inhibition of protein kinase C (PKC) modulates action potential firing and membrane properties of spinal dorsal horn neurons. 

**A**: representative examples of action potentials (generated by current injection protocols shown below voltage traces) recorded before and after 5-min bath-application of PMA (5 μM) and during wash-out. 

**B**: representative examples of action potentials recorded before, after 5-min bath-application of Ro32-8220 (1 μM), and after application of PMA + Ro32-8220. 

**C–E**: summary of changes in action potential firing and membrane properties induced by PMA (C), Ro32-8220 (D), and PMA in presence of Ro32-8220 (E). Values are means ± SE; n = 6 neurons each. *$P < 0.05$, paired t-test.
RESULTS

In our conditions, neurons grown in culture for <3 days were not able to fire action potentials in response to current injection. This is likely attributable to a low density of Na+ channels (Wolff et al. 1998). After 3 days in culture, most neurons have longer neurites, and a small percentage of these cells can generate single spikes with relatively high thresholds. These action potentials are TTX sensitive (data not shown). After 6 days in culture, most neurons were able to generate multiple spikes in response to current injection from a holding potential of −70 mV. Among the 78 neurons tested after 6 days in culture, the population can be divided into the following four categories based on their firing properties (Fig. 1): repetitive (49%), delayed firing (29%), phasic (those neurons showing spike frequency adaptation, 18%), and single spike (4%, not included in present study).

These firing properties resemble those described for rat and hamster neurons in slices (Grudt and Perl 2002; Hochman et al. 1997). Under voltage clamp, all neurons we recorded displayed A-type currents. In many cell types, A-type currents are more sensitive to 4-AP blockade than sustained K currents (Hille 1992). Therefore in an effort to evaluate the impact of A-type currents on neuronal excitability, we first tested the effect of 4-AP on action potential firing and membrane properties. Multiple action potentials were generated by a step of depolarizing current injection at a holding potential of −70 mV (maintained by adjusting current injection in this and the remaining experiments). In response to bath-application of 1 mM 4-AP for 1–2 min, first-spike latency (the time from initiating current injection to the first action potential) was decreased by 71.1 ± 6.5%, spike frequency (defined as the inverse of the interval between the first two spikes) was increased by 51.5 ± 11.5%, action potential amplitude was increased by 13.5 ± 2.9%, action potential threshold was reduced from −39 ± 1.2 to −46 ± 2.0 mV, resting membrane potential was increased from −58 ± 1.6 to −52 ± 1.5 mV, and input resistance was slightly increased by 9.3 ± 3.5%. The effects of 4-AP were reversible. Under voltage clamp, 4-AP reduced A-type currents by 59.2 ± 1.8% (Fig. 2). Although not conclusive, these data suggest that A-type currents may regulate action potential firing in superficial dorsal horn neurons.

FIG. 4. Protein kinase A (PKA) activation modulates action potential firing and membrane properties of spinal dorsal horn neurons. A: representative examples of action potentials (generated by current injection protocol shown below voltage traces) recorded before and after 5-min bath-application of 50 μM forskolin (FSK) with IBMX (50 μM) and during washout. B and C: summary of changes in action potential firing and membrane properties induced by FSK + IBMX (B) or bath-application of 1 mM 8-br-cAMP (C). Values are means ± SE; n = 5–7 neurons each. *P < 0.05, paired t-test, compared with predrug control.
PKC effects on excitability of neurons in superficial dorsal horn

We demonstrated that PKC activation modulates A-type K\textsuperscript{+} channels (Hu et al. 2003). To determine whether PKC modulates neuronal excitability, we investigated the effects of PMA, a PKC activator, on action potentials evoked by direct current injection. PMA (5 \mu M) reduced first-spike latency by 58.8 \pm 11.3\%, increased spike frequency by 35.4 \pm 4.7\%, and decreased action potential threshold from –39.5 \pm 1.8 to –42.8 \pm 1.2 mV. PMA also elicited a small but reproducible increase in action potential amplitude (5.2 \pm 0.9\%), but did not significantly change resting membrane potential or input resistance (Fig. 3). These results show that PMA increases superficial dorsal horn neuron excitability. To confirm that the effects of PMA on action potentials were a specific consequence of PKC activation, we examined the effects of a PKC inhibitor on action potentials. In response to addition of 1 \mu M Ro31-8220, a PKC inhibitor, to the perfusion system for 5 min, first-spike latency was increased by 138 \pm 45\% and spike frequency was decreased by 32.0 \pm 5.8\%. Ro31-8220 also produced a small but significant decrease in action potential amplitude (4.3 \pm 1.1\%) and a slight increase in action potential threshold (from –41 \pm 2.6 to –39 \pm 2.5 mV), but had no effect on resting membrane potential or input resistance (Fig. 3). Thus the effect of Ro31-8220 was the opposite effect of PMA on neuronal excitability for each property that was modulated by PMA. We further tested whether PKC inhibition blocks the effects of PMA. When Ro31-8220 (1 \mu M) was applied before and during the application of PMA, no effects of PMA were observed (Fig. 3). These data support our hypothesis that PKC activation potentiates neuronal excitability, and further suggest that there is tonic PKC modulation of neuronal excitability in cultured spinal cord dorsal horn neurons.

PKA activation enhances neuronal excitability

Activators of PKA have been found to decrease A-type currents in the brain and spinal cord (Hoffman and Johnston 1998; Hu et al. 2003). To ascertain the effects of PKA activation on neuronal excitability in superficial dorsal horn neurons, we tested the effects of forskolin (FSK), an adenylyl cyclase activator, on action potential properties. After application of 50 \mu M FSK with IBMX, an inhibitor of phosphodiesterases, first-spike latency was decreased by 56.9 \pm 9.6\% and spike frequency was increased by 38.5 \pm 9.6\%, action potential amplitude was increased by 10.5 \pm 2.1\%, action potential threshold was reduced from –38 \pm 1.8 to –43 \pm 2.4 mV, and resting membrane potential was increased from –57 \pm 5.0 to –52 \pm 6.3 mV, with no significant change in input resistance (Fig. 4). When 50 \mu M PKI, a specific inhibitor of PKA, was included in the patch pipette, FSK did not produce a significant change in action potentials or membrane properties, suggesting that FSK + IBMX increased excitability by activating PKA (Fig. 5). To further establish the role of PKA, we tested the effects of 8-Br-cAMP, a membrane-permeable analog of cAMP, on action potential properties. As can be seen from Fig. 4, application of 8-Br-cAMP (1 mM) had effects similar to those of FSK. These results suggest that PKA activation enhances excitability of neurons in the superficial dorsal horn.

Inhibition of ERK signaling decreases neuronal excitability

Data from our lab indicate that ERK activation in the dorsal horn has an important role in inflammatory pain plasticity (Karim et al. 2001) and that ERK inhibition enhances A-type currents in dorsal horn neurons (Hu et al. 2003). To investigate whether ERK activation modulates neuronal excitability in the superficial dorsal horn, we applied PD98059 and U0126, specific inhibitors of mitogen-activated/ERK kinase (MEK) (the upstream activator of ERK), to our cultures. After application of 20 \mu M PD98059 for 4–5 min, first-spike latency was increased by 35.3 \pm 5.6\%, spike frequency was decreased by 35.8 \pm 5.9\%, and action potential amplitude was reduced by 10.2 \pm 2.5\%. PD98059 had no significant effects on resting membrane properties.
membrane potential, action potential threshold, or input resistance (Fig. 6). Similarly, U0126 produced inhibitory effects on dorsal horn neurons. U0126 (500 nM) prolonged first-spike latency, reduced spike frequency, and decreased action potential amplitude (Fig. 6). These data indicate that decreasing ERK activity attenuates excitability of neurons in mouse superficial dorsal horn.

ERK inhibition prevents PKC and PKA excitatory effects in superficial dorsal horn neurons

The results discussed above show that ERK, PKC, and PKA have similar effects on excitability of superficial dorsal horn neurons. To determine whether these kinases share a single pathway, we perfused 20 μM PD98059 to neurons for 5 min, which caused a prolongation of first-spike latency and a decrease in spike frequency that reached a steady state. Under these conditions, neither PMA nor FSK significantly altered any action potential or passive membrane properties (Fig. 7). Thus inhibition of ERK abolishes PKC- and PKA-mediated increases in neuronal excitability.

ERK inhibition has no effect on Na⁺ or Ca²⁺ currents, but reduces sustained K⁺ currents in dorsal horn neurons

The effects of PMA and FSK on ion channels have been studied extensively. However, little is known about effects of MEK inhibitors on sodium, calcium, and sustained potassium channels. We tested whether changes in these currents could contribute to the inhibitory effects of PD98059 on action potentials. Under voltage clamp, neurons were held at −80 mV, and 6 steps of depolarization from −50 to +50 mV evoked fast activating, fast inactivating voltage-dependent Na⁺ currents. These currents were completely blocked by 500 nM TTX (data not shown). Application of 20 μM PD98059 had no effect on Na⁺ currents (Fig. 8). We then tested the effects of
PD98059 on voltage-gated Ca\(^{2+}\) currents. Ba\(^{2+}\) was used as the charge carrier. Application of 20 \(\mu\)M PD98059 had no effect on Ba\(^{2+}\) currents (Fig. 8). To test the effect of PD98059 on sustained K\(^{+}\) currents, we applied a prepulse protocol to \(-20\) mV to inactivate the A-type K\(^{+}\) current. Sustained currents were evoked by a depolarizing step to 40 mV from a \(-80\) mV holding potential after the prepulse. PD98059 decreased sustained currents by 16\% (Fig. 8).

**DISCUSSION**

The companion study reports that PKA, PKC, and ERK activation leads to a decrease in A-type K\(^{+}\) currents in superficial dorsal horn neurons. In this study, we show that PKC, PKA, and ERK increase excitability of neurons in spinal cord superficial dorsal horn. The changes in action potential firing properties induced by these kinases are consistent with A-type current inhibition. Thus activation of PKC or PKA decreased first-spike latency, increased spike frequency, and increased action potential amplitude. In contrast, inhibition of PKC or ERK prolonged first-spike latency, reduced spike frequency, and decreased action potential amplitude. In addition, inhibition of ERK prevented PKC- and PKA-mediated modulation of action potentials.

A-type channels are believed to be important in controlling the rate of action potential generation, delaying the onset of firing, and lengthening the interspike interval. Downregulation of A currents decreases first-spike latency and increases spike frequency in other brain regions (Yang et al. 2001). Firing rate and first-spike latency are two major parameters that determine the timing of neurotransmitter release. An important conse-

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**FIG. 7.** Inhibition of ERK signaling blocks FSK- and PMA-mediated modulation of action potential firing and membrane properties in dorsal horn neurons. **A** and **C:** representative examples of action potentials (generated by current injection protocols shown below voltage traces) recorded before and after 5-min bath-application of 20 \(\mu\)M PD98059 and in the presence of PMA + PD98059 or FSK + PD98059. **B** and **D:** summary of changes in action potential firing and membrane properties induced by PMA (**B**) and FSK (**D**) in presence of PD98059. Values are means ± SE; \(n = 6\) neurons each. *\(P < 0.05\), paired \(t\)-test, compared with PD98059.
The sequence of the A current is to delay first-spike firing in response to small depolarizations. Data presented in these studies show that activation of PKA, PKC, or ERK in superficial dorsal horn neurons leads to an inhibition of A currents and a consequent decrease in first-spike latency. Thus a small, normally sub-threshold excitatory postsynaptic potential (EPSP) could generate an action potential when these kinases are activated. In this way, inflammation, which leads to enhanced ERK activation in superficial dorsal horn neurons (Karim et al. 2001), could increase the signal-to-noise ratio for synapses onto dorsal horn neurons. This property could account for a component of central sensitization observed after inflammation. Whether such changes actually occur in spinal cord dorsal horn neurons in the context of inflammation is an open question.

In addition to A-type K⁺ channels, many other ion channels also contribute to regulation of action potentials. Na⁺ currents, Ca²⁺ currents, and sustained K⁺ currents are known to affect the initiation, duration, and repolarization of action potentials. PKC and PKA activation decrease the peak amplitude of TTX-sensitive Na⁺ currents and increase Na⁺ current inactivation in other brain areas (Cantrell et al. 1996, 1997, 2002; Franceschetti et al. 2000). Such changes induced by PKC and PKA in dorsal horn neurons would not lead to the observed increase in neuronal excitability. In dendrites of hippocampal

**FIG. 8.** MEK inhibitor PD98059 has no effect on Na⁺ or Ca²⁺ channels but decreases sustained K⁺ currents. A: representative examples of Na⁺ currents (induced by depolarizing step as shown below current traces) recorded before and after PD98059. B: current–voltage plot of peak amplitude of Na⁺ current vs. test voltage recorded before and during application of PD98059 (20 μM). C: representative examples of voltage-gated Ca²⁺ channel currents (induced by depolarizing step as shown below current traces) recorded before and after application of PD98059 (20 μM). Ba²⁺ was used as charge carrier. D: summary of percentage changes in Na⁺ and Ba²⁺ currents induced by PD98059. Values are means ± SE; n = 5–8 neurons each. PD98059 had no significant effect on Na⁺ or Ba²⁺ currents (t-test, compared with control condition in which currents were recorded for same time with no drug addition). E: representative examples of sustained currents (induced by prepulse protocol shown below current traces; interval between prepulse and test pulse, 3 ms) recorded before and after application of 20 μM PD98059. F: mean current–voltage plot of peak amplitude of sustained current vs. test voltage recorded before and during application of 20 μM PD98059. n = 6 neurons.
CA1 pyramidal neurons, activation of PKC increases the amplitude of action potentials by modulating A-type K\(^+\) currents and not through modulation of Na\(^+\) channels (Yuan et al. 2002). Our data demonstrate that MEK inhibitors have no effect on Na\(^+\) or Ca\(^{2+}\) currents, but these drugs do decrease sustained K\(^+\) currents. The decrease in sustained K\(^+\) currents caused by PD98059 would not result in a decrease in neuronal excitability. The excitatory effects of PKC and PKA on action potentials in dorsal horn neurons require ERK, and these data suggest that their effects are likely not caused by modulation of Na\(^+\) channels and Ca\(^{2+}\) channels. Previous studies have also shown that activators of PKA and PKC modulate other ion channels, such as persistent Na\(^+\) channels and the hyperpolarization-activated channels (Boland and Jackson 1999; Franceschetti et al. 2000; Maccaferri and McBain 1996; Matthias et al. 2002), and these effects may also contribute to PKC- and PKA-mediated increases in neuronal excitability. Future studies will endeavor to explore the effects of PKC and PKA on these channels.

The present studies were performed using superficial dorsal horn neurons from mouse grown for several days in cell culture. Although we have shown that the electrophysiological properties of these neurons are similar to those reported for superficial dorsal horn neurons in acute slice preparations, some caution must always be taken when using cell culture systems. For example, it is possible that some of the cells from which data were collected are from deeper laminae, making their utility for dissecting pain mechanisms suspect. Furthermore, it is important to consider that some of the neurons recorded from in the present study may be GABAergic or glycinergic inhibitory interneurons. Increases in excitability in these cells may have profoundly different effects on pain perception in an animal than would similar changes in excitability of glutamatergic excitatory neurons. Finally, the neurons used were prepared from very young mice, and it is possible that such changes are not seen in more mature animals, where the role of the kinases under investigation have been shown to be behaviorally important in pain models. These are all important questions that are difficult to address with an in vitro culture system. Studies recording from visually identified neurons in laminae I–II of acute spinal cord slices will be done to confirm these findings in an identified population of cells from older animals.

In conclusion, our data suggest that A-type currents significantly regulate neuronal excitability in dorsal horn neurons, and that A-type potassium currents are likely important in the modulatory effects of PKA, PKC, and ERK in these cells. These results may have implications in pain plasticity.

DISCLOSURES

R. W. Gereau was supported by National Institutes of Health Grants R01 MH-60230 and R01 NS-42595, the Paralyzed Veterans of America Spinal Cord Research Foundation, and the American Heart Association, Texas Affiliate.

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