

Extrinsic Regulation of T-Type Ca²⁺ Channel Expression in Chick Nodose Ganglion Neurons

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ABSTRACT: Functional expression of T-type Ca²⁺ channels is developmentally regulated in chick nodose neurons. In this study we have tested the hypothesis that extrinsic factors regulate the expression of T-type Ca²⁺ channels *in vitro*. Voltage-gated Ca²⁺ currents were measured using whole-cell patch clamp recordings in E7 nodose neurons cultured under various conditions. Culture of E7 nodose neurons for 48 h with a heart extract induced the expression of T-type Ca²⁺ channels without any significant effect on HVA currents. T-type Ca²⁺ channel expression was not stimulated by survival promoting factors such as BDNF. The stimulatory effect of heart extract was mediated by a heat-labile, trypsin-sensitive factor. Various hematopoietic cytokines including CNTF and LIF mimic the stimulatory effect of heart extract on T-type Ca²⁺ channel expression. The stimulatory effect of heart extract and CNTF requires at least 12 h continuous exposure to

reach maximal expression and is not altered by culture of nodose neurons with the protein synthesis inhibitor anisomycin, suggesting that T-type Ca²⁺ channel expression is regulated by a posttranslational mechanism. Disruption of the Golgi apparatus with brefeldin-A inhibits the stimulatory effect of heart extract and CNTF suggesting that protein trafficking regulates the functional expression of T-type Ca²⁺ channels. Heart extract- or CNTF-evoked stimulation of T-type Ca²⁺ channel expression is blocked by the Jak/STAT and MAP kinase blockers, AG490 and U0126, respectively. This study provides new insights into the electrical differentiation of placode-derived sensory neurons and the role of extrinsic factors in regulating the functional expression of Ca²⁺ channels. © 2007 Wiley Periodicals, Inc.

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Keywords: nodose neuron; development; Ca²⁺ channels; trophic factors

INTRODUCTION

Regulation of ion channel expression is an important landmark of neuronal differentiation, which allows neurons to control gene expression, neurotransmitter release, and integration of synaptic inputs into various patterns of repetitive firing. Ion channel expression appears to be regulated by both intrinsic and ex-

trinsic factors. For example, in *Xenopus* spinal neurons, expression of voltage-gated Ca²⁺ channels (VGCC) occurs in a cell-independent manner and precedes expression of other voltage-dependent conductances (O'Dowd et al., 1988; reviewed by Spitzer, 1991). Under certain conditions, extrinsic factors generated by cell–cell interactions regulate the functional expression of some ion channels in developing neurons. Thus, early interaction with target tissue and soluble target-derived neurotrophic factors regulates the functional expression of Ca²⁺-dependent K⁺ channels in avian sympathetic and spinal neurons (Dourado et al., 1994; Martin-Caraballo and Dryer 2002a,b).

Ca²⁺ influx through VGCC constitutes one main source of Ca²⁺ entry required for activity-dependent regulation of early embryonic development (Gu and

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Spitzer, 1995). On the basis of their biophysical and pharmacological properties, VGCC are broadly divided into low voltage- and high voltage-activated Ca^{2+} channels (LVA and HVA, respectively; reviewed by Catterall, 1998). LVA (or T-type) Ca^{2+} channels generate transient currents at relatively hyperpolarized potentials. Expression of HVA channels, in particular L- and N-type Ca^{2+} channels, appears to be widespread among different populations of neurons to regulate gene expression and synaptic transmission. In developing neurons, Ca^{2+} influx through T-type Ca^{2+} channels regulates various cellular processes including neurite outgrowth and electrical differentiation (Holliday and Spitzer, 1990; Gu and Spitzer, 1993; Umemiya and Berger, 1994; Martin-Caraballo and Greer, 2001; Chemin et al., 2002). In sensory neurons, T-type Ca^{2+} channels mediate the transmission of certain modalities of sensory information to the nervous system (Dubreuil et al., 2004; reviewed by Heppenstall and Lewin, 2006). Thus, age-dependent changes in T-type Ca^{2+} channel expression can have a significant effect on the functional properties of developing neurons. Expression of T-type Ca^{2+} channels is often limited to a number of neuronal populations, such as matured thalamic or sensory neurons or to particular developmental periods as seen in embryonic spinal motoneurons (McCobb et al., 1989; reviewed by Perez-Reyes, 2003). Although the physiological role of VGCC is well established, we have very little understanding about the regulation of Ca^{2+} channel expression during neuronal differentiation.

Chick nodose ganglion neurons are a useful model to study various aspects of neuronal development and differentiation (Lindsay et al., 1985a,b; Forgie et al., 1999; Ledda et al., 2002). In chick nodose neurons, HVA Ca^{2+} currents are first detected in E5 but not in E3 embryos. The normal pattern of HVA Ca^{2+} channel expression occurs *in vitro* independently of culture conditions, suggesting that epigenetic factors such as interaction with target tissue do not play a role in the regulation of channel expression (Larmet et al., 1992). Expression of T-type Ca^{2+} currents is developmentally regulated and occurs significantly later (Pachuau and Martin-Caraballo, 2006). Between E7 and E10, T-type Ca^{2+} currents were found in only a few nodose neurons but were present in a majority of neurons in older embryos (>E17). Since adult nodose neurons express both HVA and T-type Ca^{2+} channels, the question arises as to what factors regulate the functional expression of voltage-gated Ca^{2+} channels during normal development. In this study we have tested the hypothesis that extrinsic factors regulate the functional expression of T-type Ca^{2+}

channels in developing nodose neurons. Our present results indicate that functional expression of T-type but not HVA Ca^{2+} channels is regulated by a heat-labile, trypsin-sensitive cardiac factor and can be mimicked by exposure of nodose neurons to various hematopoietic cytokines including ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF). Heart extract- or CNTF-induced T-type Ca^{2+} channel expression does not require protein synthesis but is sensitive to disruption of protein trafficking and inhibition of Erk and Janus tyrosine kinases (Jak) signals.

METHODS

Dissociated Cell Cultures

Nodose ganglia were isolated from chick embryos at various developmental stages (E7 and E20). The ganglia were excised into a HEPES-based, Ca^{2+} - and Mg^{2+} -free solution, mildly trypsinized [0.05% trypsin for 12 min (E7); 15 min (E10); 18 min (E17); and 25 min (E20)], dissociated by trituration, and plated onto poly-D-lysine-coated glass coverslips. Basal culture medium consisted of Eagle's minimal essential medium supplemented with 10% heat-inactivated horse serum, 2 mM glutamine, 50 U/mL penicillin, and 50 $\mu\text{g}/\text{mL}$ streptomycin. Unless indicated otherwise, the culture medium was also supplemented with BDNF (50–100 ng/mL) to promote neuronal survival. Cell cultures were maintained in a 5% CO_2 incubator at 37°C for up to 48 h. To study the expression of ionic currents in acutely dissociated neurons, recordings were made 3–4 h after nodose ganglia dissociation. The potential influence of tissue extracts and various culture conditions on Ca^{2+} channel expression was studied in cells isolated at E7 and cultured for up to 48 h prior to whole cell recording. Immediately after isolation, nodose neurons have a round morphology (Harrison et al., 1994) but after a few hours in culture begin to grow one or two short neurites from the cell body.

In some experiments, nodose neurons were cultured with a heart or chick extract. Hearts from E7 or E20 chick embryos were excised, cleared of connective tissue, cut into small pieces to remove blood, and homogenized in ice-cold, serum-free phosphate buffered solution (PBS). To obtain whole chick extract, an E15 chick embryo was passed through a 10-mL syringe into a centrifuge tube containing 3 mL of serum-free medium prior to centrifugation (Ko et al., 2001). Tissue extracts were kept at 4°C for 2 h and then centrifuged at 15,000 rpm for 75 min. Protein concentration of the extracts was determined according to the Bradford assay using a commercially available protein assay reagent (BioRad, Hercules, CA). Aliquots of the supernatant were stored at -80°C until use. Extracts were added to the cell culture media at a concentration of 200–400 $\mu\text{g}/\text{mL}$. For inactivation of extract activity, heart extracts were treated with trypsin (0.1 mg/mL) for 2 h at 37°C, followed by 1 h treatment at 37°C with trypsin inhibitor

(1 mg/mL) to inactivate the trypsin effect. Controls consisted of nontreated heart extracts or heart extracts exposed to trypsin inhibitor for 1 h.

For neutralization experiments, samples of the heart extract were directly exposed to specific CNTF or LIF neutralizing antibodies at 4°C overnight with gentle shaking. Controls consisted of heart extract samples maintained under the same conditions but in the absence of antibody or treated with a nonimmune rabbit serum. Treated or control samples were added to nodose cell cultures for 24 h. Heart extract samples were immunodepleted of CNTF following incubation with a rabbit anti-chick CNTF antibody bound to Zymed protein-A sepharose beads (Invitrogen). Heart extract (500 μ L, 2 mg/mL protein concentration) was incubated for 4 h at 4°C with rabbit anti-chick CNTF (1:20 dilution). Treated extract was exposed to protein-A sepharose beads (50 μ L slurry) for 1 h at 4°C with gentle rocking. After incubation, beads were collected by centrifugation and the supernatant was applied to nodose cell cultures or stored in aliquots at -80°C for further use. Controls consisted of heart extract treated with nonimmune rabbit serum (1:20) and protein-A sepharose beads.

Electrophysiology

Dissociated nodose neurons were visualized using an Olympus X71 inverted microscope equipped with Hoffman optics. Recordings were performed at room temperature (22–24°C). Recording electrodes were made from thin wall borosilicate glass (3–4 M Ω) and filled with a solution consisting of (in mM) 120 CsCl, 2 MgCl₂, 10 HEPES-KOH, 10 EGTA, 1 ATP, and 0.1 GTP (pH 7.4 with CsOH). Normal external saline for measurements of Ca²⁺ currents contained (in mM) 145 tetraethylammonium chloride (TEACl), 10 CaCl₂, and 10 HEPES (pH 7.4 with CsOH). The composition of the extracellular Ca²⁺-free solution was the same except that CaCl₂ was replaced with an equimolar concentration of MgCl₂. To measure Ca²⁺ currents, a 200 ms-depolarizing step to various potentials was applied in normal external saline and following a 3 min incubation in Ca²⁺-free external saline, and net current amplitude was obtained by digital subtraction (control—Ca²⁺-free). This procedure also allows us to remove linear leak and capacitive currents. For quantitative analyses, we normalized for cell size by dividing current amplitudes by cell capacitance, determined by integration of the current transient evoked by a 10 mV voltage step from a holding potential of -60 mV (Martin-Caraballo and Dryer, 2002a). T-type Ca²⁺ current density was determined from the peak current amplitude generated by a voltage step to -20 mV from a holding potential of -100 mV, whereas HVA Ca²⁺ current density was determined from the peak current amplitude generated by a voltage step to +20 mV from a holding potential of -100 mV. Ca²⁺ channel antagonists were applied by a gravity-fed perfusion system. Voltage commands and data acquisition and analysis were performed with a MultiClamp 700A amplifier and Pclamp software (Axon Instruments, Foster City, CA). Pipette offset, whole cell capacitance, and

series resistance (usually <10 M Ω) were compensated automatically with the MultiClamp 700B Commander. Inadequate space clamp was minimal in acutely isolated nodose neurons because of their round shape devoid of any dendritic process. A few cultured neurons (~10% of all recorded cells) were not included in our analysis because of insufficient space clamp as evidenced by the lack of T-type Ca²⁺ channel inactivation.

The activation and inactivation time constants were obtained by fitting the rising or decay portion of the transient currents with one exponential function in the form $I(t) = A \exp(-t/\tau)$, where A is peak current and τ is the time constant. Steady-state activation curves were obtained from current-voltage (I/V) relationships using the equation $G = I/(V - V_r)$ where I is the current at a given voltage, V is the voltage command, and V_r is the reversal potential of calcium currents obtained by extrapolating the ascending portion of the IV curve. Conductance values were normalized to the maximum conductance at -20 mV and plotted as a function of voltage before being fitted with a Boltzman equation in the form $G/G_{max} = (1 + \exp(V_{1/2} - V)k)^{-1}$ where G is conductance at membrane voltage V , G_{max} is maximal conductance at -20 mV, $V_{1/2}$ is the half-activation voltage, and k is the slope factor. Steady-state inactivation curves were fitted with a Boltzman equation using normalized current values $I/I_{max} = (1 + \exp(V_{1/2} - V)k)^{-1}$ where I is current at membrane voltage V , I_{max} is maximal current with a -100 mV conditioning prepulse, $V_{1/2}$ and k have the same meaning as above. The nickel dose response curves were fitted with a Hill equation in the form $I(x) = [1 + (IC_{50}/x)^n]^{-1}$, where IC_{50} is the drug concentration at half maximal block, and n is the Hill coefficient. Throughout, all data values are presented as mean \pm SEM. Statistical analyses consisted of Student's unpaired t -test when single comparisons were made, and one-way ANOVA followed by *post hoc* analysis using Tukey's honest significant difference test for unequal n for the more typical experimental designs that entailed comparisons between multiple groups (Statistica software, Tulsa, OK). Throughout, $p \leq 0.05$ was regarded as significant. In every experiment, data were collected from a minimum of two platings (i.e., from multiple cultures).

Chemicals and Drugs

Anisomycin, trypsin, and trypsin inhibitor were from Sigma (St. Louis, MO). SB203580, U0126 and brefeldin-A were purchased from LC Labs (Woburn, MA). Anti-chick CNTF serum and chick CNTF were kindly provided by Dr. Rae Nishi (University of Vermont). Anti-human CNTF antibody, recombinant human CNTF, BDNF, and LIF were purchased from R&D (Minneapolis, MN). Anti-chick LIF antibody was a generous gift from Dr. Hiroyuki Horiuchi (Hiroshima University, Japan). LY294002 and AG490 were obtained from Calbiochem (La Jolla, CA). Culture medium and supplements including serum were from BioWhittaker (Walkersville, MA).

RESULTS

Our previous data indicate that the functional expression of T-type Ca^{2+} channels is developmentally regulated in chick nodose neurons (Pachau and Martin-Caraballo, 2006). This pattern of channel expression could reflect an intrinsic program of development or could be regulated by some extrinsic factors. To study the role of extrinsic factors in the regulation of T-type Ca^{2+} channel expression, nodose neurons were isolated at E7 when the expression of functional T-type Ca^{2+} channels is low and maintained for up to 48 h under several culture conditions. If an intrinsic program regulates T-type Ca^{2+} channel expression independently of cell survival, then we would expect channel expression to occur independently of culture conditions to some degree. Ca^{2+} currents were isolated by substitution of Na^+ ions with external tetraethylammonium (TEA) and by blocking outward K^+ currents with Cs^+ ions in the pipette solution. Voltage-dependent Ca^{2+} currents were generated in the whole-cell patch clamp configuration using a series of 200 ms long depolarizing steps. To compensate for changes in cell size that may occur under different culture conditions, whole-cell currents were normalized to cell capacitance (see Methods section, Martin-Caraballo and Dryer, 2002a,b). T-type Ca^{2+} current density was determined from peak T-type Ca^{2+} current evoked by a voltage step to -20 mV from a holding potential of -100 mV. HVA Ca^{2+} current density was determined from the peak current generated by a voltage step to $+20$ mV from a holding potential of -100 mV.

One group of nodose neurons was cultured in the presence of a heart-derived extract from E7 or E20 chick embryos or a whole chick extract for 48 h. Heart or whole chick extracts were chosen because they have the potential to stimulate survival and differentiation of nodose neurons during early embryonic development (Lindsay et al., 1985a; Xue et al., 1985). Another group of neurons was maintained for 48 h in normal culture media supplemented with 100 ng/mL of BDNF (brain-derived neurotrophic factor). The last group of nodose neurons was cultured in the presence of 50 μM SB 203580. The last two culture conditions were chosen because they have been shown to promote survival of nodose neurons *in vitro* according to previous studies (Davies et al., 1986; Buj-Bello et al., 1994, 1995; Horstmann et al., 1998). Although all these culture conditions supported the survival of over 60% of nodose neurons, they had different effects on T-type Ca^{2+} channel expression, suggesting that epigenetic factors and the

growing environment regulate the functional expression of T-type Ca^{2+} channels in nodose neurons *in vitro*.

Culture of E7 nodose neurons with a heart extract from E20 chick embryos (400 $\mu\text{g}/\text{mL}$) for 48 h resulted in increased expression of T-type Ca^{2+} currents in all recorded cells ($n = 23$), when compared with acutely isolated E7 neurons [$n = 36$, Fig. 1(A,B)]. Functional expression of T-type Ca^{2+} channels following 48 h treatment with E20 heart extract was evident from the appearance of a shoulder in the current-voltage relationship [Fig. 1(C), arrow]. Culture of nodose neurons with the E20 heart extract resulted in a significant increase in the mean T-type current density when compared to other culture conditions [Fig. 1(D)]. The stimulatory effect of heart extract on T-type Ca^{2+} channels was specific, and no significant effect was reported on HVA current densities [Fig. 1(E)], suggesting that heart extract can stimulate T-type Ca^{2+} channel expression *in vitro* without any noticeable effect on HVA Ca^{2+} currents. Analysis of T-type Ca^{2+} current densities of nodose neurons from multiple cultures indicates that the stimulatory effect of heart extract on T-type Ca^{2+} channel expression is the result of increased channel expression. Thus, 48 h exposure to heart extract resulted in a rightward shift in the distribution of current densities as compared with acutely isolated E7 neurons or neurons exposed to BDNF for 48 h [Fig. 2(A–C)].

Culture of E7 nodose neurons with heart extract from E7 embryos or whole chick extract did not increase T-type Ca^{2+} channel expression significantly, suggesting that the stimulatory effect of heart extract is age-dependent and target-specific [Fig. 1(D)]. The survival-promoting factor BDNF did not stimulate T-type Ca^{2+} channel expression after 48 h in culture [Fig. 1(D)]. T-type Ca^{2+} channel expression was also lacking in E7 nodose neurons cultured for 48 h with SB 203580. As previously reported, SB 203580 promotes neuronal survival in several populations of sensory neurons in the absence of trophic support by specifically blocking the stress-activated protein p38 (Horstmann et al., 1998; Maas et al., 1998). In the presence of 50 μM SB 203580, nodose neurons were large and bright and suitable for intracellular recordings after 48 h in culture. However, adding SB 203580 to the culture medium did not allow for T-type Ca^{2+} channel expression [Fig. 1(D)]. These results are consistent with the idea that T-type Ca^{2+} channel expression in nodose neurons was not a simple matter of time in culture. We should also point out that it is not possible to maintain nodose neurons in culture media without any trophic support for prolonged periods of time because of ongoing apoptotic

cell death that occurs in these neurons in the absence of neurotrophic factors (unpublished observations, Buj-Bello et al., 1994; Horton et al., 1998).

The results described earlier indicate that functional expression of T-type but not HVA Ca^{2+} channels is regulated by extrinsic factors present in the culture medium. Is the stimulatory effect of heart extract mediated by a protein? To investigate this possibility, the heart extract was inactivated by heating for 1 h at 65°C . Expression of T-type Ca^{2+} channels increased significantly after 48 h treatment with heart extract when compared with cells maintained *in vitro* in the presence of BDNF [control, Fig. 3(A)]. The stimulatory effect of heart extract on T-type Ca^{2+} channel expression was significantly reduced by heat inactivation, suggesting that a heat-labile factor mediates the stimulatory effect of cardiac tissue

on channel expression [Fig. 3(A)]. Culture of E7 nodose neurons with heat-inactivated heart extract did not affect cell capacitance (26.2 ± 2 , $n = 15$ vs. 29.2 ± 2.4 , $n = 14$, $p > 0.5$) or HVA current density (23.9 ± 4.2 , $n = 15$ vs. 16.6 ± 2.2 , $n = 14$, $p > 0.5$) when compared with cells exposed to nontreated heart extract. This was further confirmed by trypsin inactivation of the heart extract [Fig. 3(B)]. Exposure of the heart extract for 2 h to 0.1 mg/mL trypsin resulted in a significant decrease in T-type Ca^{2+} current density. No significant differences in T-type Ca^{2+} current densities were found in cell cultures exposed to heart extract alone or heart extract treated with trypsin inhibitor used to inactivate trypsin [Fig. 3(B)]. Culture of E7 nodose neurons with trypsinized heart extract did not affect cell capacitance (28.4 ± 2.4 , $n = 12$ vs. 28.9 ± 1.8 , $n = 15$, $p > 0.5$) or HVA current density (17.9 ± 3.7 , $n = 12$ vs. 16 ± 1.6 , $n = 15$, $p > 0.5$) when compared with cells exposed to normal heart extract. Thus, the present results indicate that the stimulatory effect of heart extract on T-type Ca^{2+} channel expression is mediated by a heat-labile, trypsin-sensitive factor.

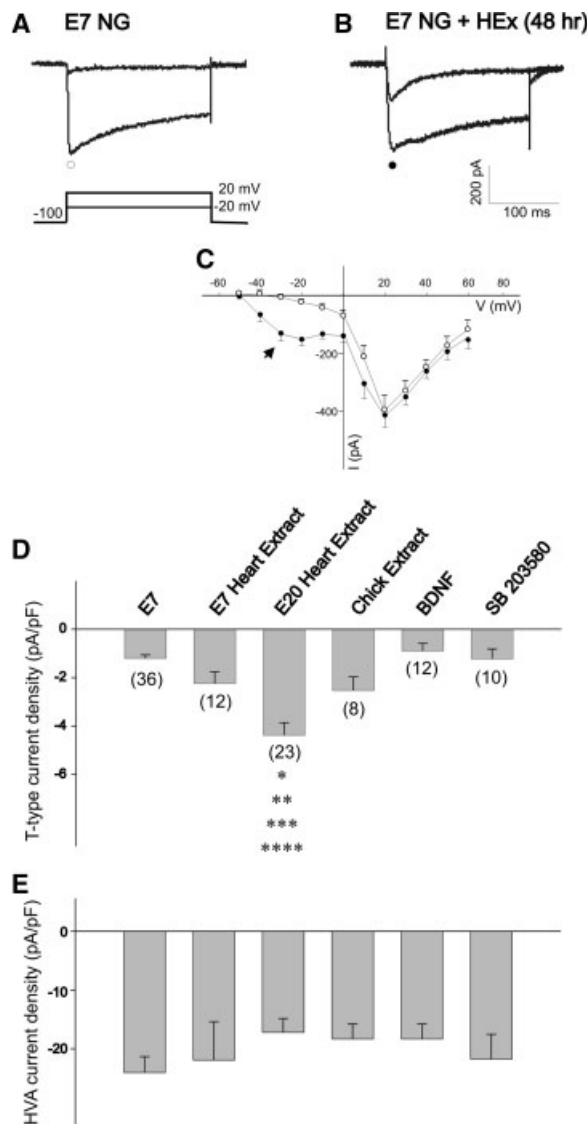


Figure 1 Effect of heart and whole chick extracts, and survival-promoting factors on T-type Ca^{2+} channel expression *in vitro*. (A, B) Representative traces of inward Ca^{2+} currents in acutely isolated E7 nodose neurons and after 48 h in culture with heart extract derived from E20 chick embryos. Ca^{2+} currents were generated by 200 ms depolarizing pulses to -20 and $+20$ mV from a holding potential of -100 mV. (C) Current-voltage (I-V) relationship for the peak currents shown in A (O) and B (●), respectively. The arrow represents the LVA component. (D) Mean T-type Ca^{2+} current densities after 48 h treatment with heart extract from E7 and E20 chick hearts, whole chick extract, BDNF, and SB 203580. Note robust stimulation of T-type Ca^{2+} current densities with E20 heart extract but not E7 heart extract, whole chick extract, BDNF, and SB 203580. T-type Ca^{2+} current density was calculated from the peak current amplitude generated by a voltage step to -20 mV from a holding potential of -100 mV. (E) Culture of E7 nodose neurons with tissue extracts and survival promoting factors does not alter mean HVA Ca^{2+} current densities. HVA Ca^{2+} current density was determined from the peak current amplitude evoked by voltage step to $+20$ mV from a holding potential of -100 mV. Nodose neurons were isolated at E7 and cultured for 48 h with the following factors: heart extract ($400 \mu\text{g/mL}$), whole chick extract ($400 \mu\text{g/mL}$), BDNF (100 ng/mL), and SB 203580 ($50 \mu\text{M}$). In this and subsequent figures, error bars represent SEM and the number of cells recorded is provided above each bar. * denotes $p \leq 0.05$ versus E7; **, $p \leq 0.05$ versus E7 heart extract; ***, $p \leq 0.05$ versus BDNF; and ****, $p \leq 0.05$ versus SB203580.

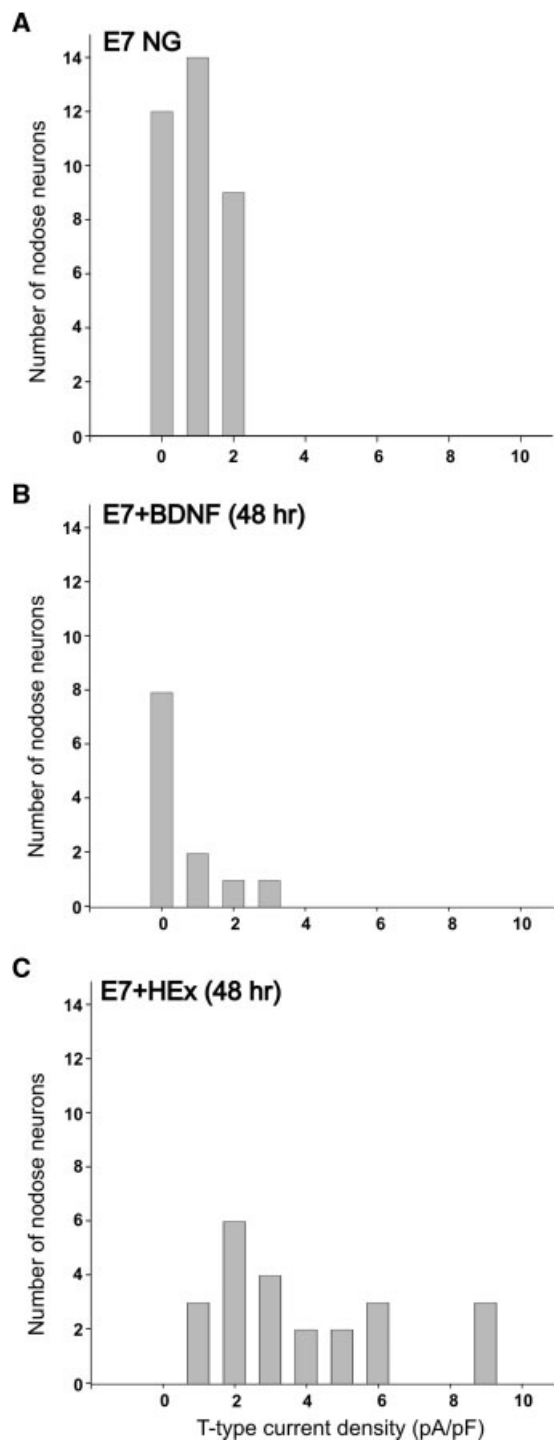


Figure 2 Histograms of T-type Ca^{2+} current densities in acutely isolated E7 nodose neurons (A) and E7 nodose neurons cultured for 48 h in the presence of BDNF (B) or heart extract (HEx, 400 $\mu\text{g}/\text{mL}$, C). Ca^{2+} currents were generated by 200 ms depolarizing pulses to -20 mV from a holding potential of -100 mV. Note the rightward shift in the number of nodose neurons expressing high current densities following 48 h exposure to heart extract.

Are the biophysical properties of T-type Ca^{2+} channels generated *in vitro* different from those channels expressed by nodose neurons undergoing normal development? To investigate whether exposure of E7 nodose neurons to heart extract evokes the expression of T-type Ca^{2+} channels with different properties from those found in matured neurons

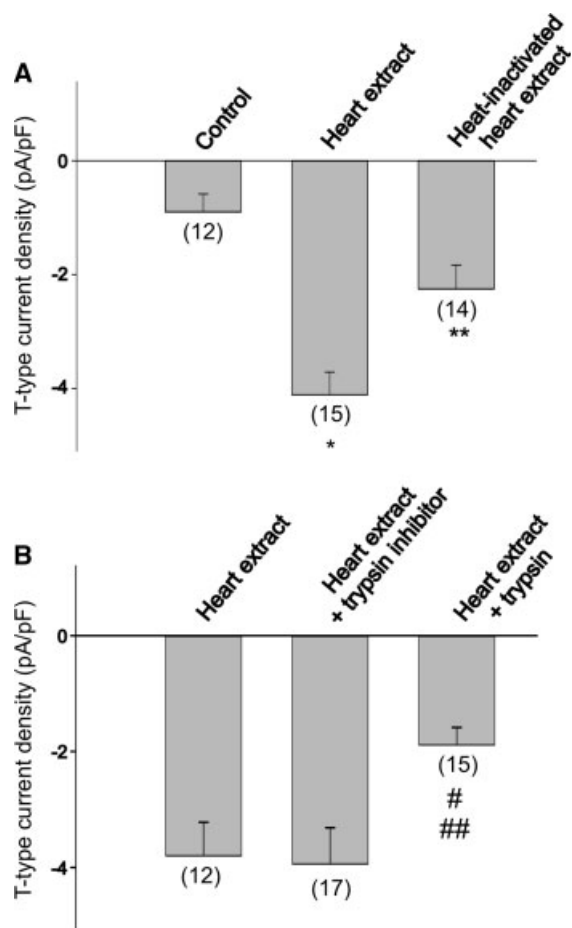


Figure 3 Effect of heat-inactivated or trypsinized heart extract on T-type Ca^{2+} current densities. (A) Treatment of nodose neurons with heat-inactivated heart extract causes a significant decrease in T-type Ca^{2+} current densities. (B) Trypsinization of heart extract causes a significant reduction in T-type Ca^{2+} current densities. In these experiments, nodose neurons were dissociated at E7 and maintained in culture for 48 h in the presence of normal or treated heart extracts (400 $\mu\text{g}/\text{mL}$). To promote neuronal survival, the culture medium was also supplemented with BDNF (50 ng/mL). Mean current densities were obtained from currents generated by a step pulse to -20 mV from a holding potential of -100 mV. * denotes $p \leq 0.05$ versus control; ** and # denote $p \leq 0.05$ versus heart extract (nontreated); and ## denotes $p \leq 0.05$ versus heart extract treated with the trypsin inhibitor alone.

(E20) we conducted a biophysical analysis of heart extract-evoked currents. Activation time constant and inactivation decay time were analyzed following a voltage step to -20 mV from a -100 mV holding potential. In E7 nodose neurons cultured for 48 h with heart extract, activation time constant and inactivation decay time were similar to that reported in E20 neurons (2.9 ± 0.4 and 28.5 ± 3 ms ($n = 12$) versus 2.1 ± 0.8 and 23.4 ± 3.7 ms, respectively; Pachua and Martin-Caraballo, submitted for publication). The steady-state activation of T-type Ca^{2+} channels in heart extract-treated neurons was determined by plotting the relative conductance against depolarizing voltage steps and fitting that relationship with a Boltzmann equation [Fig. 4(B)]. Fitting of activation curve yielded $V_{1/2} = -36.9 \pm 1.9$ mV (step potential resulting in half maximal activation of normalized conductance) and $k = 4.0 \pm 1.1$ mV (steepness of the curve). These values were not significantly different from those reported in E20 neurons ($V_{1/2} = -37 \pm 1$ mV, $k = 3.6 \pm 1$ mV, respectively; $p > 0.05$). The steady-state inactivation of T-type Ca^{2+} channels was studied using a 200 ms-test pulse to -20 mV preceded by a series of 5s-conditioning prepulses between -100 and -30 mV [Fig. 4(A)]. The relative amplitude of the peak current was plotted as a function of the prepulse potential and fitted by a Boltzmann function [Fig. 4(B)]. Inactivation was completely removed at potentials more negative than -80 mV, whereas at potentials more positive than -50 mV inactivation became complete. The mean half-inactivation potential ($V_{1/2}$) and the slope factor were -67.5 ± 1.3 and 4.3 ± 0.2 mV, respectively, which was not significantly different from the values obtained in E20 nodose neurons ($V_{1/2} = -66.9 \pm 1.3$ mV, $k = 4.3 \pm 0.3$ mV, respectively; $p > 0.05$). In matured nodose neurons, T-type Ca^{2+} currents are generated by $\alpha 1\text{H}$ subunits that are highly sensitive to nickel block (Pachua and Martin-Caraballo, submitted for publication). Therefore, we investigated whether heart extract-evoked T-type Ca^{2+} currents were also sensitive to nickel ions. As illustrated in Fig. 4(C), in nodose neurons cultured in the presence of heart extract, nickel ions produced a concentration-dependent inhibition of T-type Ca^{2+} currents with an $\text{IC}_{50} = 25 \mu\text{M}$ ($n = 4$). This value is close to that obtained in acutely isolated E20 neurons ($\text{IC}_{50} = 17 \mu\text{M}$, Pachua and Martin-Caraballo, submitted for publication). These data suggest that the biophysical and pharmacological properties of T-type Ca^{2+} channels expressed in nodose neurons after heart extract stimulation do not differ significantly from those in neurons undergoing normal development.

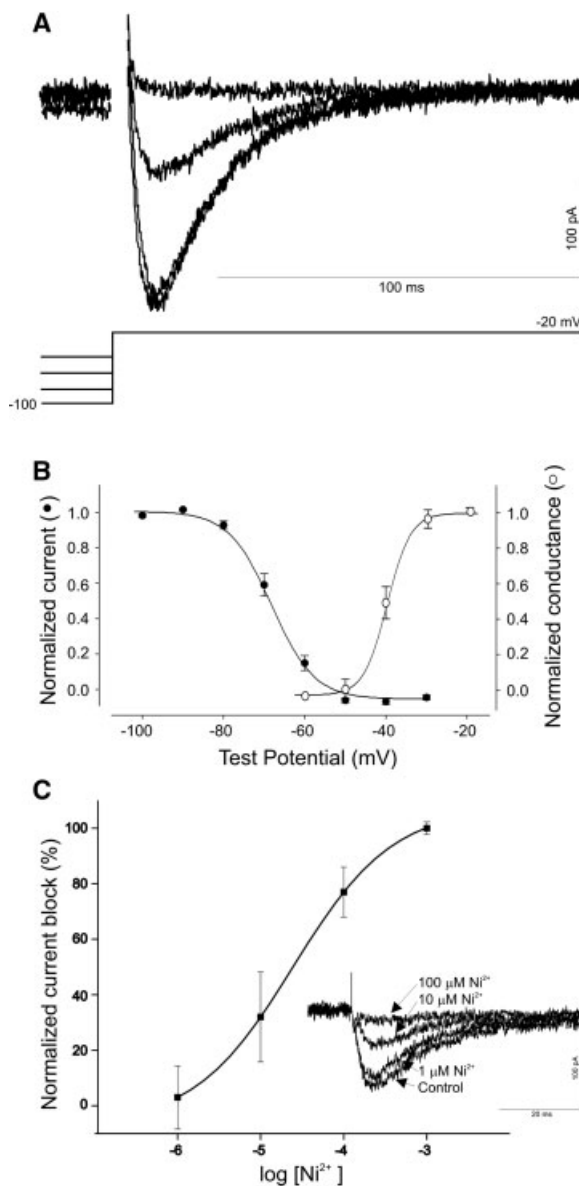
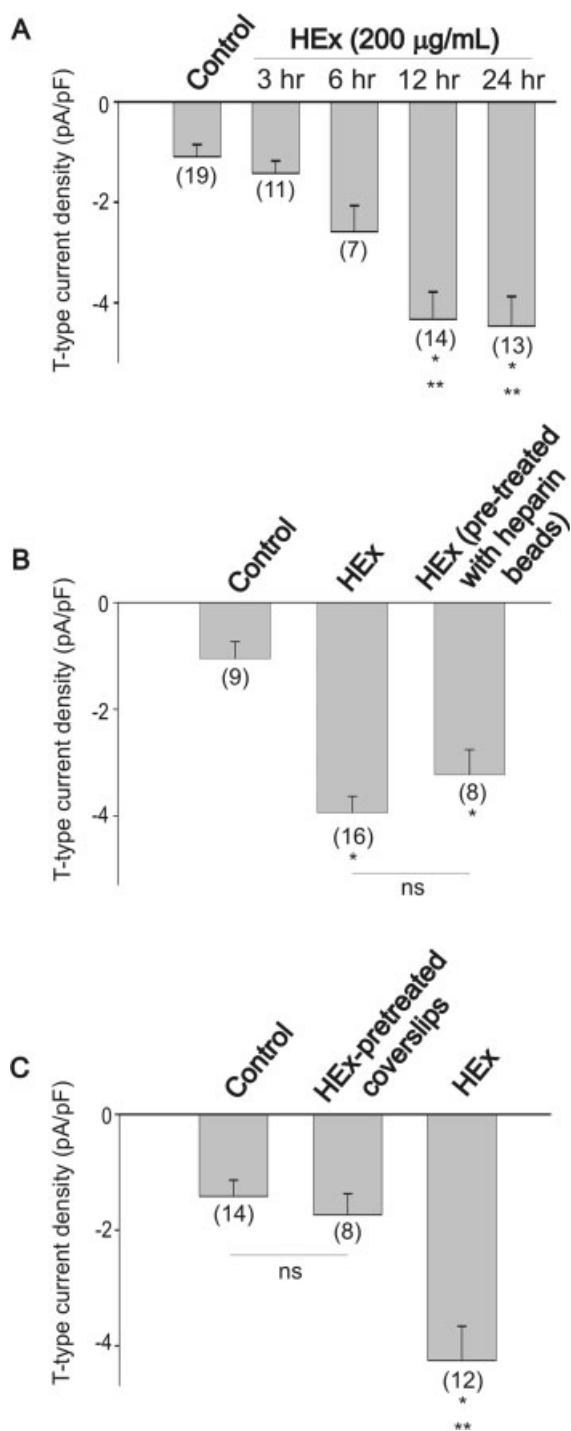


Figure 4 Biophysical and pharmacological properties of heart extract-evoked T-type Ca^{2+} channels. (A) Typical sample traces showing steady-state inactivation of T-type Ca^{2+} channels evoked in E7 neurons after 24 h treatment with heart extract ($200 \mu\text{g/mL}$). Membrane potential was held at voltages ranging from -100 to -30 mV for 5 s before applying a 200 ms depolarizing step to -20 mV (lower trace in A represents the voltage protocol used). Traces during the prepulse have been removed for clarity. (B) Voltage dependence of activation and steady-state inactivation curves. The solid lines represent the best fit obtained with a Boltzmann equation for steady-state activation and inactivation values ($n = 12$). (C) Effect of nickel ions on heart extract-evoked T-type Ca^{2+} currents. Normalized current block was plotted as a function of nickel concentration and fitted with the Hill equation $I(x) = [(1 + (\text{IC}_{50})/x)^n]^{-1}$.

Although our initial experiments were conducted in cell cultures maintained for 48 h in the presence of heart extract, stimulation of T-type Ca^{2+} channel expression appears to involve a relatively fast-acting mechanism. Expression of T-type Ca^{2+} channels was low in E7 nodose neurons exposed to heart extract for 3 h when compared with cells maintained *in vitro* for the same amount of time without any trophic support,



suggesting that the stimulatory effect of heart extract on T-type Ca^{2+} channel expression does not involve a rapid allosteric modification of channels already present in the membrane [Fig. 5(A)]. T-type Ca^{2+} channel expression became evident after 6 h continuous exposure to heart extract. Exposure of E7 nodose neurons for 12 h to heart extract induced maximal expression of T-type Ca^{2+} channels [Fig. 5(A)]. Treatment of nodose neurons for longer periods of time (24 h) did not result in any further increase in channel expression. Thus, these results suggest that the stimulatory effect of heart extract on T-type Ca^{2+} channel expression involves a relatively fast-acting mechanism.

What is the nature of the stimulatory factor found in heart extract? Several cardiac-derived factors including acidic and basic fibroblast growth factors (aFGF and bFGF) are known to contain heparin-binding sites (Casscells et al., 1990; Wellstein et al., 1992; Motoike and Unsicker, 1999). If a heparin-binding molecule mediates the stimulatory effect of

Figure 5 Effect of exposure time, heart extract treatment with heparin beads or treatment of culture substrate with heart extract on T-type Ca^{2+} channel expression. (A) Time course of heart extract on T-type Ca^{2+} current density. Maximal expression of T-type Ca^{2+} channels occurred after 12 h continuous exposure to heart extract (200 µg/mL). In these experiments nodose neurons were dissociated at E7 and maintained for up to 24 h in culture medium supplemented with BDNF (50 ng/mL). Control neurons were maintained *in vitro* for 3 h in the absence of heart extract. Exposure time represents the interval between HEx application (~1 h after cell plating) and electrophysiological recordings. (B) Pretreatment of heart extract with heparin-conjugated beads does not alter T-type Ca^{2+} channel expression. Heart extract was exposed to heparin-conjugated beads overnight at 4°C. Control represents E7 nodose neurons cultured for 24 h in the presence of BDNF alone. (C) Exposure of coverslips to heart extract does not evoke any significant increase in T-type Ca^{2+} channel expression. Poly-D-lysine coated coverslips were exposed to culture medium alone (control) or culture medium containing 200 µg/mL heart extract for 6 h in a cell culture incubator. After several washes, nodose neurons were added to pre-treated coverslips and maintained for 24 h in culture medium supplemented with BDNF (50 ng/mL). Notice that culture of nodose neurons in heart extract-pretreated coverslips does not result in any significant increase in channel expression compared with control neurons. Continuous exposure of nodose neurons to heart extract for 24 h is still able to evoke a significant increase in T-type Ca^{2+} channel expression. * denotes $p \leq 0.05$ versus control; ** denotes $p \leq 0.05$ versus 3 h exposure time (A) or heart extract-pretreated coverslips (C).

heart extract, then we would expect that exposure of heart extract to heparin-conjugated beads should prevent T-type Ca^{2+} channel expression. To test this hypothesis, aliquots of heart extract were exposed to heparin-conjugated beads for 2 h at 4°C. Control aliquots of heart extract were maintained for the same amount of time but without heparin-conjugated beads at 4°C. Incubation of heart extract with heparin-conjugated beads did not have any significant effect on T-type Ca^{2+} current density [Fig. 5(B)], suggesting that the stimulatory effect of heart extract is not caused by a heparin-binding factor. The stimulatory effect of heart extract could be mediated by a factor that binds to the poly-D-lysine substrate used in our cell cultures to promote attachment of nodose neurons to the coverslip (Lander et al., 1982). To investigate this possibility, poly-D-lysine coated coverslips were exposed to heart extract for at least 6 h. After washing several times with fresh culture medium, nodose neurons were plated onto heart extract pretreated coverslips and T-type Ca^{2+} channel expression was determined 24 h later by whole cell recordings. Control neurons were cultured onto poly-D-lysine coated coverslip alone (with culture medium containing BDNF). There were no significant differences in the T-type Ca^{2+} current densities generated in nodose neurons cultured on nontreated or heart extract pretreated coverslips [Fig. 5(C)].

Previous studies indicate that cardiac tissue produces several hematopoietic cytokines including CNTF or LIF, and interleukins-6 and 11 (Yamamori et al., 1989; Wang and Halverson, 1998; Ancy et al., 2002). Thus, the question arises whether hematopoietic cytokines mimic the stimulatory effect of heart extract on T-type Ca^{2+} channel expression in chick nodose neurons. To test this possibility we first investigated whether CNTF and LIF mimic the stimulatory effect of heart extract on T-type Ca^{2+} channel expression. Chick nodose neurons were isolated at E7 and cultured for 24–48 h in the presence of chick CNTF (also known as growth promoting activity or GPA), recombinant human CNTF, or LIF. Although both mammalian and chick CNTF activate the same GPI-linked receptor in chick ciliary ganglion neurons in order to promote neuronal survival (Heller et al., 1993, 1995), they have some striking differences (Leung et al., 1992; Finn and Nishi, 1996). Thus, there is only a 50% homology in the amino acid sequence of chick and mammalian CNTF (Leung et al., 1992; Finn and Nishi, 1996). Moreover, chick CNTF can be released in a soluble form, whereas mammalian CNTF lacks a crucial export sequence (Leung et al., 1992). As previously demonstrated, neurons cultured for 24 h with BDNF (50 ng/mL) alone did

not express significant levels of T-type Ca^{2+} channels. After 24 h in culture, CNTF and LIF induced a significant increase in T-type Ca^{2+} current density that was not statistically different from the stimulatory effect generated by heart extract [Fig. 6(A)]. Culture of nodose neurons with heart extract and chick CNTF combined did not result in any significant change in the current density generated by each factor acting separately, suggesting that the stimulatory effect of heart extract and CNTF was not additive.

Our earlier experiments suggest that the stimulatory effect of heart extract on T-type Ca^{2+} channel expression involves a relatively fast-acting mechanism. Does CNTF act by a similar mechanism? Treatment of E7 nodose neurons with CNTF for 3 h did not reveal any significant increase in T-type Ca^{2+} current density when compared with nodose neurons cultured without CNTF, suggesting that CNTF-evoked expression of T-type Ca^{2+} channels does not involve a rapid allosteric modification of channels that may be already present in the membrane [Fig. 6(B)]. Similar to the effect of heart extract on T-type Ca^{2+} channel expression, a noticeable increase in current density occurred after 6 h treatment with CNTF and reached a maximum after 12 h exposure [Fig. 6(B)]. Are the CNTF-evoked changes in T-type Ca^{2+} channel expression caused by synthesis of new channel proteins or does it involve a posttranslational mechanism? To answer this question, the functional expression of T-type Ca^{2+} channels was determined in E7 nodose neurons cultured for 12 h in the presence of the translational inhibitor anisomycin (0.1 mg/mL). This concentration of anisomycin has been proven effective in blocking protein synthesis in cultured chick neurons (Subramony et al., 1996; Martin-Caraballo and Dryer, 2002b). Culture of E7 nodose neurons with CNTF in the presence of anisomycin did not cause any significant change in T-type Ca^{2+} current density when compared with neurons treated with CNTF alone [Fig. 7(A)]. To determine if a protein synthesis-independent mechanism is also involved in the stimulatory effect of heart extract, nodose neurons were pretreated with anisomycin in the presence of heart extract. Culture of E7 nodose neurons for 12 h in the continuous presence of anisomycin did not prevent the stimulatory effect of heart extract [Fig. 7(B)]. These data suggest that both the heart extract- and CNTF-evoked expression of T-type Ca^{2+} channels involves a posttranslational mechanism. We should point out, however, that anisomycin treatment of nodose cell cultures caused a significant reduction in cell size likely by disrupting cell growth ($\text{HEX} = 24.9 \pm 1.8$ pF, $n = 19$; $\text{Hex} + \text{anisomycin} = 7.2 \pm 0.7^*$ pF, $n = 12$, $*p \leq 0.05$ vs. HEX ; $\text{CNTF} =$

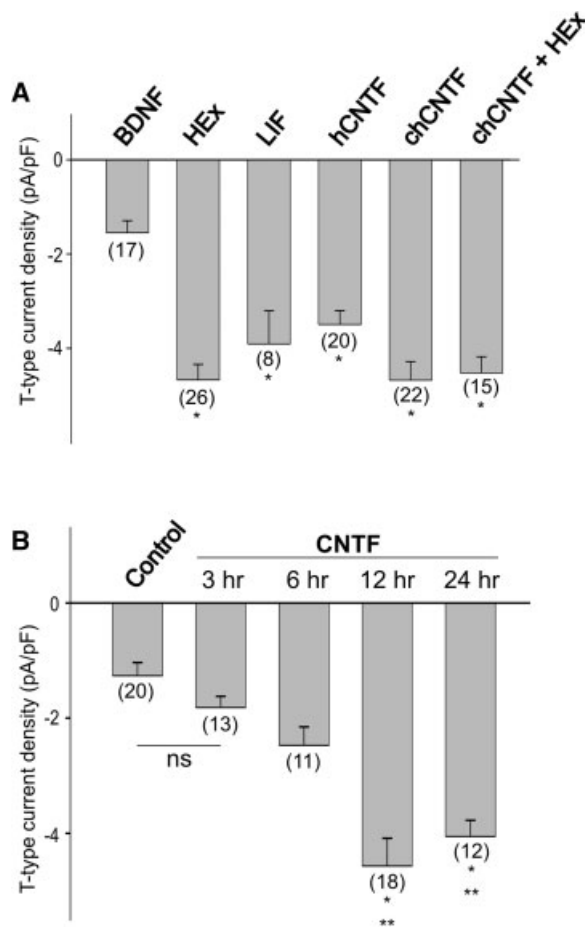


Figure 6 Effect of hematopoietic cytokines on the expression of T-type Ca^{2+} channels in nodose neurons *in vitro*. (A) Summary of the effects of recombinant human CNTF (hCNTF), chick CNTF (chCNTF, also known as growth promoting factor), and LIF on the functional expression of T-type Ca^{2+} channels in cultured nodose neurons. Nodose neurons were isolated at E7 and cultured for 24 h in the presence of 200 $\mu\text{g}/\text{mL}$ heart extract or 50 ng/mL hCNTF, chCNTF, or LIF. The culture medium was also supplemented with 50 ng/mL BDNF. Note stimulation of T-type Ca^{2+} channel expression with hCNTF, chCNTF, and LIF was similar to that evoked with 200 $\mu\text{g}/\text{mL}$ heart extract. Incubation with BDNF fails to increase T-type Ca^{2+} channel expression. * denotes $p \leq 0.05$ vs. BDNF. (B) Time course of CNTF effect on T-type Ca^{2+} current density. Maximal expression of T-type Ca^{2+} channels occurred after 12 h continuous exposure to CNTF (50 ng/mL). In these experiments nodose neurons were dissociated at E7 and maintained for up to 24 h in culture medium supplemented with BDNF (50 ng/mL). Control neurons were maintained *in vitro* for 3 h in the absence of CNTF. Exposure time represents the interval between CNTF application (~ 1 h after cell plating) and the beginning of our electrophysiological recordings. * denotes $p \leq 0.05$ versus control; ** denotes $p \leq 0.05$ versus 3 h exposure time.

24.2 ± 1.1 pF, $n = 25$; CNTF + anisomycin = 12.0 ± 0.6 pF, $n = 11$; ** $p \leq 0.05$ vs. CNTF).

In ciliary neurons, the stimulatory effect of target-derived neurotrophic factors on large-conductance, Ca^{2+} -dependent K^{+} channels is mediated by rapid recruitment of channel proteins from an intracellular pool into the membrane (Chae et al., 2005a). If the CNTF-evoked effect on T-type Ca^{2+} channels requires trafficking and insertion of channel proteins, then this effect would be blocked by drugs that alter the targeting and translocation of plasma proteins. To test this possibility we used brefeldin-A (1 $\mu\text{g}/\text{mL}$), a compound that causes disassembly of the Golgi appa-

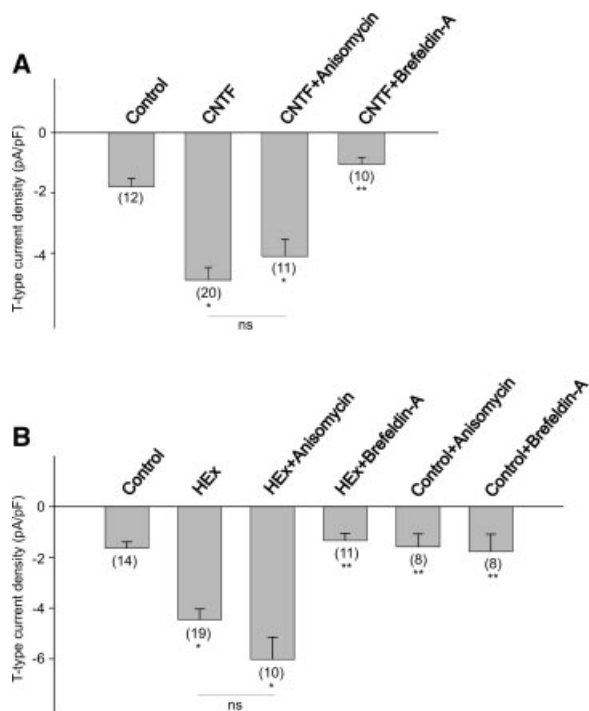


Figure 7 Effect of protein synthesis blockade or disruption of Golgi apparatus on the CNTF and heart extract-evoked expression of T-type Ca^{2+} channels. Inhibition of protein synthesis with anisomycin does not alter the stimulatory effect of CNTF (A) or heart extract (B) on T-type Ca^{2+} channel expression. In these experiments, nodose neurons were dissociated at E7 and maintained in culture for 12 h in the presence of CNTF or heart extract with or without anisomycin (0.1 mg/mL). Inhibition of the Golgi apparatus with brefeldin-A blocked T-type Ca^{2+} channel expression evoked by CNTF (50 ng/mL, A) or heart extract (200 $\mu\text{g}/\text{mL}$, B). Nodose neurons were dissociated at E7 and maintained in culture for 12 h in the presence of CNTF or heart extract with or without brefeldin-A (1 $\mu\text{g}/\text{mL}$). * denotes $p \leq 0.05$ versus control (A and B); ** denotes $p \leq 0.05$ versus (A) CNTF or (B) heart extract.

ratus and therefore prevents translocation of plasma proteins (Chae et al., 2005b). Treatment of E7 nodose neurons with brefeldin-A for 1 h prior to application of CNTF caused a complete inhibition of T-type Ca^{2+} channel expression [Fig. 7(A)]. Similarly, the stimulatory effect of heart extract on T-type Ca^{2+} channel expression was also blocked by culture of nodose neurons in the presence of brefeldin-A [Fig. 7(B)]. Anisomycin or brefeldin-A treatment of control cultures did not have any effect on T-type Ca^{2+} channel expression as indicated by the lack of effect on basal currents [Fig. 7(B), control + anisomycin, control + brefeldin-A]. Cell size was also significantly reduced after brefeldin-A treatment of nodose cell cultures in the presence of heart extract (HEX = 24.9 ± 1.8 pF, $n = 19$; Hex + brefeldin-A = $10.4 \pm 0.9^*$ pF, $n = 11$; $p < 0.05$ vs. HEX) or CNTF (CNTF = 24.2 ± 1.1 pF, $n = 25$; CNTF + brefeldin-A = $9.8 \pm 0.8^{**}$ pF, $n = 10$; $p < 0.05$ vs. CNTF).

Hematopoietic cytokines including CNTF and LIF can generate changes in neuronal function through transduction cascades that include the MAP kinase Erk and Jak/STAT (Dziennis and Habecker, 2003; Jiao et al., 2003; reviewed by Heinrich et al., 2003). Therefore, we decided to test whether inhibitors of the Erk and Jak/STAT signaling pathways prevent the stimulatory effect of heart extract and CNTF on T-type Ca^{2+} channel expression. E7 nodose cell cultures were pretreated with $50 \mu\text{M}$ of U0126, a concentration proven to effectively inhibit Erk phosphorylation in neurons (Schonhoff et al., 2001; Chae and Dryer, 2005). Culture of nodose neurons with U0126 prevented the expression of T-type Ca^{2+} channels evoked with CNTF [Fig. 8(A)]. A similar effect was also observed following exposure of nodose neurons to the inhibitor of the Jak/STAT signaling pathway AG490 (at $50 \mu\text{M}$, Reis et al., 2002) [Fig. 8(A)]. These data suggest that the stimulatory effect of CNTF on T-type Ca^{2+} channel expression is regulated by activation of both the Erk and Jak/STAT signaling pathways. Does the stimulatory effect of heart extract on T-type Ca^{2+} channel expression also require activation of the Erk and Jak/STAT signaling pathways? Indeed, pretreatment of E7 nodose neurons with U0126 ($50 \mu\text{M}$) or AG 490 ($50 \mu\text{M}$) also reduced the stimulatory effect of heart extract on T-type Ca^{2+} channel expression [Fig. 8(B)]. Heart extract or CNTF-evoked currents were not affected by inhibition of PI3 kinase signaling with $50 \mu\text{M}$ LY 294002 [Fig. 8(A,B)], a concentration that effectively inhibits PI3 kinase downstream signaling (Lhuillier and Dryer, 2002). These data suggest that functional expression of T-type Ca^{2+} channels in nodose neurons does not require activation of the PI3/Akt

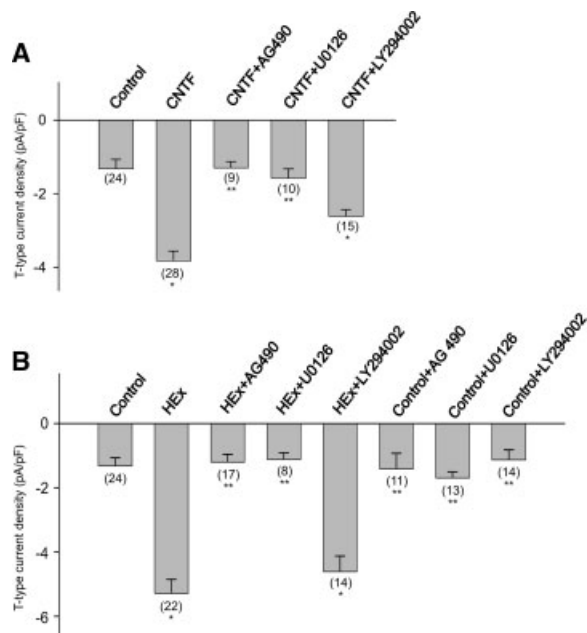


Figure 8 Effect of Erk and Jak/STAT signaling inhibitors on the CNTF- and heart extract-evoked stimulation of T-type Ca^{2+} channel expression. In these experiments, nodose neurons were isolated at E7 and maintained in culture for 12 h in the presence of CNTF (50 ng/mL, A) or heart extract (200 $\mu\text{g}/\text{mL}$, B). The culture medium was also supplemented with BDNF (50 ng/mL) to promote neuronal survival. Controls consisted of BDNF-treated cultures. Culture of nodose neurons with AG490 (50 μM) and U0126 (50 μM), but not LY294002 (50 μM), caused a significant reduction in T-type Ca^{2+} current densities. * denotes $p \leq 0.05$ versus control (A and B); ** denotes $p \leq 0.05$ versus (A) CNTF or (B) heart extract.

kinase-signaling pathway. We should point out that all these drug treatments did not have any effect on basal control currents as represented in Figure 8(B) (control + AG490, control + U0126, control + LY294002). Moreover, these inhibitors did not have any acute effect on T-type Ca^{2+} channels already present in the membrane as recordings from acutely isolated E20 nodose neurons demonstrated (results not shown).

The experiments described earlier suggest that both CNTF and LIF mimic the stimulatory effect of heart extract on T-type Ca^{2+} channel expression. If CNTF and LIF mediate the stimulatory effect of heart extract on T-type Ca^{2+} channel expression, then removal of these factors from the heart extract should prevent channel expression. To test this hypothesis, aliquots of heart extract were exposed overnight to specific antisera against the chick or

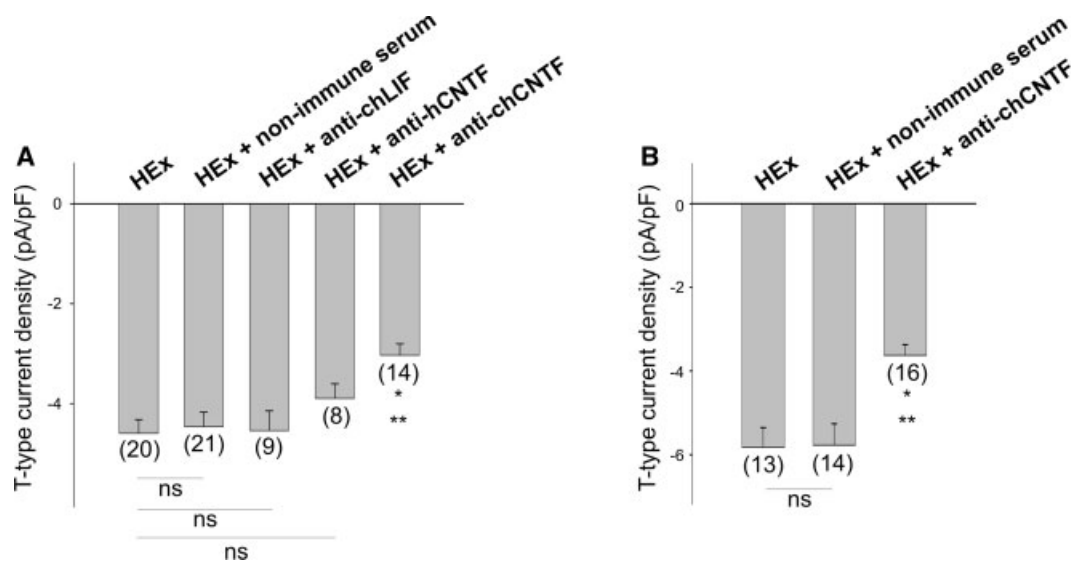


Figure 9 Effect of anti-chick CNTF and LIF antisera on T-type Ca^{2+} channel expression. (A) The stimulatory effect of heart extract on T-type Ca^{2+} channel expression was significantly reduced by pretreatment of heart extract with a rabbit anti-chick CNTF antibody. Pretreatment of heart extract with a nonimmune serum does not cause any significant change in the stimulatory effect of heart extract on T-type Ca^{2+} channel expression. The stimulatory effect of heart extract on T-type Ca^{2+} channel expression was not affected by pretreatment of the heart extract with a neutralizing antiserum against human CNTF or chick LIF. (B) Immunodepletion causes a significant reduction in the stimulatory effect of heart extract on T-type Ca^{2+} channel expression. Heart extract was immunodepleted of chick CNTF by incubation with a rabbit anti-chick CNTF antibody bound to protein-A sepharose beads. Pretreatment of heart extract with a nonimmune rabbit serum and protein-A sepharose beads, does not cause any significant change in the stimulatory effect of heart extract alone on T-type Ca^{2+} channel expression. * denotes $p \leq 0.05$ versus heart extract alone; ** denotes $p \leq 0.05$ versus heart extract exposed to nonimmune serum.

mammalian isoforms of CNTF. We also treated the heart extract with a neutralizing antibody against the chick isoform of LIF (Yamashita et al., 2006). Control aliquots were exposed to nonimmune rabbit serum. Nodose neurons were then cultured for 24 h and the functional expression of T-type Ca^{2+} channel channels was determined by whole-cell recordings. As described earlier, exposure of E7 nodose neurons to nontreated heart extract (200 $\mu\text{g}/\text{mL}$) resulted in a significant increase in T-type Ca^{2+} current density [Fig. 9(A)]. Exposure of heart extract to nonimmune serum did not alter the stimulatory effect of the extract *in vitro*. Treatment of the heart extract with a neutralizing antibody against human CNTF or chick LIF also evoked a significant increase in T-type Ca^{2+} current density that was not significantly different from that evoked by heart extract alone [Fig. 9(A)]. However, T-type Ca^{2+} current density was reduced by 35% in nodose neurons cultured with a heart extract that was previously treated with an anti-chick CNTF antibody. To

maximize the effect of the anti-chick CNTF antibody, heart extract samples were immunodepleted of chick CNTF using protein-A sepharose beads (see Methods section). Treatment of heart extract samples with nonimmune rabbit serum and protein-A sepharose beads evoked a significant increase in T-type Ca^{2+} current density that was not significantly different from that evoked by heart extract alone [Fig. 9(B)]. Immunodepletion of the heart extract with the anti-chick CNTF antibody resulted in a significant reduction of T-type Ca^{2+} current density by 40%. Cell size was not affected by immunodepletion of the heart extract with the rabbit anti-chick CNTF (HEX = 28 ± 2.5 pF, $n = 13$; Hex + nonimmune rabbit serum = 31 ± 2 pF, $n = 14$; Hex + rabbit anti-chick CNTF = 33 ± 2.8 pF, $n = 16$; $p > 0.05$ vs. HEX). Although the inhibitory effect of the chick CNTF antibody was not complete, these results indicate that the stimulatory effect of heart extract on T-type Ca^{2+} channel expression is partially mediated by CNTF or a closely related molecule.

DISCUSSION

In this study we have examined the influence of various growth conditions on the functional expression of T-type Ca^{2+} channels in chick nodose neurons developing *in vitro*. Three main conclusions can be drawn from these experiments. First, functional expression of T-type Ca^{2+} channels *in vitro* can be evoked by exposure of nodose neurons to a cardiac tissue extract. Second, hematopoietic cytokines including CNTF and LIF mimic the stimulatory effect of cardiac tissue, whereas the stimulatory effect of heart extract is partially blocked by an anti-chick CNTF antibody. Third, the stimulatory effect of CNTF and heart extract on T-type Ca^{2+} channel expression occurs by a posttranslational mechanism that does not require protein synthesis, and appears to involve activation of Erk and Jak/STAT signaling pathways.

Regulation of T-Type Ca^{2+} Channel Expression by Heart Extract and Hematopoietic Cytokines in Chick Nodose Neurons

Although the physiological role of T-type Ca^{2+} channels has been well established, we have very little understanding about the regulation of Ca^{2+} channel expression during neuronal differentiation. To our knowledge, this is the first study demonstrating a possible role of epigenetic factors in the regulation of T-type Ca^{2+} channel expression. Our present findings indicate that functional expression of T-type Ca^{2+} channels in developing chick nodose neurons is upregulated by a heat-labile, trypsin-sensitive factor derived from cardiac tissue. Thus, 48 h-exposure of nodose neurons to an extract derived from E20 chick hearts stimulates T-type Ca^{2+} channel expression. The lack of effect of cardiac tissue extracts on HVA Ca^{2+} currents is consistent with previous work showing that HVA Ca^{2+} channel expression occurs independently of cell-cell interactions and can proceed *in vitro* in the absence of trophic support (Larmet et al., 1992). The specificity of the heart extract-mediated effect on T-type Ca^{2+} channel expression was further confirmed by the use of a whole chick extract, which constitutes an abundant source of trophic factors capable of supporting survival and differentiation of various neuronal populations. Differently from the effect obtained with the E20 heart extract, exposure of nodose neurons to a whole chick extract does not cause a significant stimulation of channel expression. The different effects of E20 heart extract and whole

chick extract on T-type Ca^{2+} channel expression could be explained by the presence of a differentiation factor (or factors) in the heart extract that could become dilute in the whole chick extract. The sensitivity of this factor to heat inactivation and trypsinization would suggest that a protein mediates the stimulatory effect of heart extract on T-type Ca^{2+} channel expression. However, the stimulating effect of heart extract on T-type Ca^{2+} channel expression is not replicated by BDNF, the most potent survival-promoting factor for nodose neurons tested so far (Lindsay et al., 1985a; Davies et al., 1986). These data suggest that some other factor is responsible for the stimulating effect of heart extract on T-type Ca^{2+} channel expression in nodose neurons.

A cytokine-like factor appears to mediate the stimulatory effect of heart extract *in vitro*. First, the stimulatory effect of the heart extract on T-type Ca^{2+} channel expression can be mimicked by several hematopoietic cytokines including CNTF and LIF. Second, both the stimulatory effect of heart extract and CNTF on T-type Ca^{2+} channels do not require protein synthesis but are sensitive to disruption of protein trafficking and inhibition of Erk and Jak signaling. Furthermore, coapplication of heart extract and chick CNTF does not result in any further increase in channel expression, supporting the conclusion that the same factor or a closely-related factor mediates the stimulatory effect of heart extract on channel expression. Third, exposure of heart extract to an anti-chick CNTF antibody caused a partial inhibition of the stimulatory effect of the extract on T-type Ca^{2+} channel expression. Although both chick and mammalian CNTF are effective in increasing T-type Ca^{2+} channel expression *in vitro*, only the chick CNTF antibody (but not the mammalian CNTF neutralizing antibody) was effective in reducing the stimulatory effect of the heart extract on channel expression, providing further support for chick CNTF as a cardiac derived factor capable of regulating channel expression in nodose neurons. We should point out, however, that the chick CNTF antibody has only a partial effect on the stimulatory effect of heart extract. One possibility is that the stimulatory effect of heart extract is mediated by a cytokine-like factor that is not fully recognizable by this antibody. Another possibility is that multiple factors mediate the stimulatory effect of the heart extract. This effect is not mediated by LIF or heparin-binding factors (such as bFGF or aFGF), which may also be present in the heart extract (Casscells et al., 1990; Wellstein et al., 1992; Motoike and Unsicker, 1999; Ancey et al., 2002). Previous studies have demonstrated that target-derived, cytokine-like factors promote various aspects of neuronal differentiation.

For example, a cholinergic differentiation factor has been identified in cardiac tissue, which promotes neurotransmitter expression in rat sympathetic neurons (Rao et al., 1990). This factor has been identified as LIF (Yamamori et al., 1989). Several cytokines including LIF, CNTF, and cardiotrophin-1 also mimic the neurotransmitter phenotype switch normally induced in sympathetic neurons following innervation of rat sweat glands (Saadat et al., 1989; Habecker et al., 1997). However, neutralizing antibodies against these factors do not prevent the neurotransmitter switch caused by sweat gland extract, suggesting that some other factor is involved in this effect.

CNTF expression in embryonic chick cardiac tissue is developmentally regulated and reaches a peak by E11 (Wang and Halvorsen, 1998). Our present results also indicate that the presence of a stimulating factor in cardiac tissue is developmentally regulated since we did not see any effect with an extract derived from E7 embryos. These results are consistent with the idea that nodose neurons are able to respond to a cardiac stimulating factor already by E7 but perhaps access to this factor is restricted by its own expression in the target. One possibility is that expression of this differentiation factor is coupled to innervation by nodose neurons. In this regard, it has been demonstrated that sympathetic innervation of sweat glands is absolutely required for expression of a footpad-derived cholinergic differentiating factor (Habecker et al., 1995). In the chick, limited innervation of target tissue and limited expression of this cardiac stimulating factor at early stages of development could explain why T-type Ca^{2+} channel expression occurs relatively late compared with expression of HVA Ca^{2+} channels, which are already functional by E7.

It is becoming increasingly evident that target-derived epigenetic factors regulate neuronal differentiation in developing neurons. In ciliary ganglion neurons, a 50 kDa-factor present in embryonic eye tissue promotes expression of nicotinic acetylcholine receptors (Halvorsen et al., 1991). Previous findings in chick autonomic and spinal motoneurons indicate that expression of Ca^{2+} -dependent K^+ channels is regulated by inductive interactions with target tissue via soluble target-derived neurotrophic factors (Dourado et al., 1994; Subramony et al., 1996; Martin-Caraballo and Dryer, 2002b). Similarly, a soluble cardiac-derived factor evokes the induction of a cholinergic phenotype in sympathetic neurons (Rao et al., 1990), whereas innervation of specific target tissue regulates neurotransmitter expression in nodose neurons (reviewed by Katz et al., 1987). Thus, it is

plausible that a similar mechanism regulates the functional expression of T-type Ca^{2+} channels following interaction of nodose neurons with cardiac tissue during normal development.

Mechanisms of T-Type Ca^{2+} Channel Expression Evoked by Cardiac Tissue Extract and CNTF

Heart extract- or CNTF-evoked T-type Ca^{2+} channel expression does not require protein synthesis, as it cannot be blocked by the ribosomal inhibitor anisomycin. Posttranslational regulation of T-type Ca^{2+} channel expression is further supported by our previous work showing that $\alpha 1\text{H}$ transcripts are already present at E7, although a significant increase in the functional expression of T-type Ca^{2+} channels occurs only in older embryos during normal development (Pachuau and Martin-Caraballo, submitted for publication). Whether expression of $\alpha 1\text{H}$ transcripts at E7 correlates with protein expression awaits the development of a species specific antibody against this chick subunit. Thus, the question arises as to what posttranscriptional mechanism regulates the functional expression of T-type Ca^{2+} channels in chick nodose neurons. Our present results do not support the possibility that rapid channel phosphorylation is involved in the functional expression of T-type Ca^{2+} channels since exposure of nodose neurons to CNTF or heart extract for up to 3 h did not stimulate channel expression. One possibility is that CNTF or cardiac-derived factors regulate the recruitment and insertion of an intracellular pool of T-type Ca^{2+} channels into the plasma membrane. A similar mechanism regulates the functional expression of K^+ channels in chick ciliary ganglion neurons (Lhuillier and Dryer, 2000; Chae et al., 2005a). In these cells, the functional expression of large conductance, Ca^{2+} -dependent K^+ channels is mediated by $\text{TGF}\beta 1$ secreted from striated muscle target cells in the iris (Cameron et al., 1998). The $\text{TGF}\beta 1$ effect in ciliary cells involves a posttranslational mechanism that entails insertion of preexisting channels into the plasma membrane (Subramony et al., 1996; Chae et al., 2005a). Consistent with a recruitment mechanism involved in the regulation of T-type Ca^{2+} channel expression in nodose neurons, we observed that disruption of the Golgi apparatus with brefeldin-A blocked the stimulatory effect of HEx and CNTF on channel expression. However, further investigation is required to determine whether heart-extract- or CNTF-evoked currents require trafficking of the main $\alpha 1$ pore forming

subunit or some other auxiliary subunit to the plasma membrane.

Hematopoietic cytokines stimulate neuronal survival and differentiation through activation of heteromeric receptor complexes composed of a ligand-specific glycosyl-phosphatidylinositol (GPI)-anchored receptor and the signaling receptor complex gp130/LIFR β (reviewed by Inoue et al., 1996). Ligand-induced dimerization of the receptor complex leads to phosphorylation of Jak. Once activated, Jak phosphorylate various tyrosine residues on the cytoplasmic tail of gp130, which then become a docking site for several cytoplasmic proteins containing a src homology 2 (SH2) domain. Ultimately, receptor stimulation causes activation of various intracellular signaling molecules including MAP kinases and STAT transcription factors (Dziennis and Habecker, 2003; Jiao et al., 2003; Rhee et al., 2004; reviewed by Heinrich et al., 2003). Cytokine activation of the PI3 kinase-signaling pathway may also occur in some cases (Boulton et al., 1994; Alonzi et al., 2001). Our present results demonstrate that inhibition of Erk blocks the stimulatory effect of cardiac tissue extract and CNTF on T-type Ca²⁺ channel expression, whereas LY294002, which inhibits PI3 kinase signaling, does not have any effect. We were surprised by these results since previous findings in chick ciliary neurons have implicated both Erk and PI3 kinase activation in the regulation of channel protein trafficking (Lhuillier and Dryer, 2000, 2002; Chae et al., 2005a). However, our present results indicate that functional expression of T-type Ca²⁺ channels *in vitro* also requires Jak signaling since inhibition of Jak signaling with AG490 (Meydan et al., 1996) caused a significant inhibition of T-type Ca²⁺ channel expression in cultured nodose neurons. Cytokine-evoked activation of Jak kinases is often associated with long-term changes in gene expression through dimerization of STAT transcription factors followed by nuclear translocation (Symes et al., 1997). Therefore, it is unclear by which mechanism STAT regulates the functional expression of T-type Ca²⁺ channels induced by CNTF or heart extract. One possibility is that Jak activation leads to phosphorylation of other substrate proteins (Boulton et al., 1994), which may regulate protein trafficking to the membrane. In this regard, it has been shown that once activated Jak can act as an adaptor protein for several signaling molecules including Raf and Btk tyrosine kinases (Xia et al., 1996; Takahashi-Tezuka et al., 1997). These data are consistent with a model in which both Erk and Jak signaling are involved in the CNTF or cardiac tissue regulation of T-type Ca²⁺ channel expression in developing chick nodose neurons.

REFERENCES

- Alonzi T, Middleton G, Wyatt S, Buchman V, Betz UA, Muller W, Musiani P, et al. 2001. Role of STAT3 and PI 3-kinase/Akt in mediating the survival actions of cytokines on sensory neurons. *Mol Cell Neurosci* 18:270–282.
- Ancey C, Corbi P, Froger J, Delwail A, Wijdenes J, Gascan H, Potreau D, et al. 2002. Secretion of IL-6, IL-11 and LIF by human cardiomyocytes in primary culture. *Cytokine* 18:199–205.
- Boulton TG, Stahl N, Yancopoulos GD. 1994. Ciliary neurotrophic factor/leukemia inhibitory factor/interleukin 6/oncostatin M family of cytokines induces tyrosine phosphorylation of a common set of proteins overlapping those induced by other cytokines and growth factors. *J Biol Chem* 269:11648–11655.
- Buj-Bello A, Buchman VL, Horton A, Rosenthal A, Davies AM. 1995. GDNF is an age-specific survival factor for sensory and autonomic neurons. *Neuron* 15:821–828.
- Buj-Bello A, Pinon LG, Davies AM. 1994. The survival of nodose-dependent but not BDNF-dependent cranial sensory neurons is promoted by several different neurotrophins early in their development. *Development* 120:1573–1580.
- Cameron J, Lhuillier L, Subramony P, Dryer SE. 1998. Developmental regulation of neuronal K⁺ channels by target-derived TGF β *in vivo* and *in vitro*. *Neuron* 21:1045–1053.
- Casscells W, Speir E, Sasse J, Klagsbrun M, Allen P, Lee M, Calvo B, et al. 1990. Isolation, characterization, and localization of heparin-binding growth factors in the heart. *J Clin Invest* 85:433–441.
- Catterall WA. 1998. Structure and function of neuronal Ca²⁺ channels and their role in neurotransmitter release. *Cell Calcium* 24:307–323.
- Chae KS, Dryer SE. 2005. Regulation of neuronal K(Ca) channels by β -neuregulin-1 does not require activation of Ras-MEK-extracellular signal-regulated kinase signaling cascades. *Neuroscience* 135:1013–1016.
- Chae KS, Martin-Caraballo M, Anderson M, Dryer SE. 2005a. Akt activation is necessary for growth factor-induced trafficking of functional K(Ca) channels in developing parasympathetic neurons. *J Neurophysiol* 93:1174–1182.
- Chae KS, Oh KS, Dryer SE. 2005b. Growth factors mobilize multiple pools of KCa channels in developing parasympathetic neurons: Role of ADP-ribosylation factors and related proteins. *J Neurophysiol* 94:1597–1605.
- Chemin J, Nargeot J, Lory P. 2002. Neuronal T-type α 1H calcium channels induce neuritogenesis and expression of high-voltage-activated calcium channels in the NG108-15 cell line. *J Neurosci* 22:6856–6862.
- Davies AM, Thoenen H, Barde YA. 1986. The response of chick sensory neurons to brain-derived neurotrophic factor. *J Neurosci* 6:1897–1904.
- Dourado MM, Brumwell C, Wisgirda ME, Jacob MH, Dryer SE. 1994. Target tissues and innervation regulate

- the characteristics of K⁺ currents in chick ciliary ganglion neurons developing in situ. *J Neurosci* 14 (5, Part 2):3156–3165.
- Dubreuil AS, Boukhaddaoui H, Desmadryl G, Martinez-Salgado C, Moshourab R, Lewin GR, Carroll P, et al. 2004. Role of T-type calcium current in identified D-hair mechanoreceptor neurons studied in vitro. *J Neurosci* 24:8480–8484.
- Dziennis S, Habecker BA. 2003. Cytokine suppression of dopamine- β -hydroxylase by extracellular signal-regulated kinase-dependent and -independent pathways. *J Biol Chem* 278:15897–15904.
- Finn TP, Nishi R. 1996. Expression of a chicken ciliary neurotrophic factor in targets of ciliary ganglion neurons during and after the cell-death phase. *J Comp Neurol* 366:559–571.
- Forgie A, Doxakis E, Buj-Bello A, Wyatt S, Davies AM. 1999. Differences and developmental changes in the responsiveness of PNS neurons to GDNF and neurturin. *Mol Cell Neurosci* 13:430–440.
- Gu X, Spitzer NC. 1993. Low-threshold Ca²⁺ current and its role in spontaneous elevations of intracellular Ca²⁺ in developing *Xenopus* neurons. *J Neurosci* 13:4936–4948.
- Gu X, Spitzer NC. 1995. Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca²⁺ transients. *Nature* 375:784–787.
- Habecker BA, Symes AJ, Stahl N, Francis NJ, Economides A, Fink JS, Yancopoulos GD, et al. 1997. A sweat gland-derived differentiation activity acts through known cytokine signaling pathways. *J Biol Chem* 272:30421–30428.
- Habecker BA, Tresser SJ, Rao MS, Landis SC. 1995. Production of sweat gland cholinergic differentiation factor depends on innervation. *Dev Biol* 167:307–316.
- Halvorsen SW, Schmid HA, McEachern AE, Berg DK. 1991. Regulation of acetylcholine receptors on chick ciliary ganglion neurons by components from the synaptic target tissue. *J Neurosci* 11:2177–2186.
- Harrison TA, Stadt HA, Kirby ML. 1994. Developmental characteristics of the chick nodose ganglion. *Dev Neurosci* 16:67–73.
- Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F. 2003. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J* 374 (Part 1):1–20.
- Heller S, Finn TP, Huber J, Nishi R, Geissen M, Puschel AW, Rohrer H. 1995. Analysis of function and expression of the chick GPA receptor (GPAR α) suggests multiple roles in neuronal development. *Development* 121:2681–2693.
- Heller S, Huber J, Finn TP, Nishi R, Rohrer H. 1993. GPA and CNTF produce similar effects in sympathetic neurons but differ in receptor binding. *Neuroreport* 5:357–360.
- Heppenstall PA, Lewin GR. 2006. A role for T-type Ca²⁺ channels in mechanosensation. *Cell Calcium* 40:165–174.
- Holliday J, Spitzer NC. 1990. Spontaneous calcium influx and its roles in differentiation of spinal neurons in culture. *Dev Biol* 141:13–23.
- Horstmann S, Kahle PJ, Borasio GD. 1998. Inhibitors of p38 mitogen-activated protein kinase promote neuronal survival in vitro. *J Neurosci Res* 52:483–490.
- Horton AR, Barlett PF, Pennica D, Davies AM. 1998. Cytokines promote the survival of mouse cranial sensory neurons at different developmental stages. *Eur J Neurosci* 10:673–679.
- Inoue M, Nakayama C, Noguchi H. 1996. Activating mechanism of CNTF and related cytokines. *Mol Neurobiol* 12:195–209.
- Jiao J, Kaur N, Lu B, Reeves SA, Halvorsen SW. 2003. Initiation and maintenance of CNTF-Jak/STAT signaling in neurons is blocked by protein tyrosine phosphatase inhibitors. *Brain Res Mol Brain Res* 116:135–146.
- Katz DM, Adler JE, Black IB. 1987. Catecholaminergic primary sensory neurons: Autonomic targets and mechanisms of transmitter regulation. *Fed Proc* 46:24–29.
- Ko GY, Ko ML, Dryer SE. 2001. Developmental expression of retinal cone cGMP-gated channels: Evidence for rapid turnover and trophic regulation. *J Neurosci* 21:221–229.
- Lander AD, Fujii DK, Gospodarowicz D, Reichardt LF. 1982. Characterization of a factor that promotes neurite outgrowth: Evidence linking activity to a heparan sulfate proteoglycan. *J Cell Biol* 94:574–585.
- Larmet Y, Dolphin AC, Davies AM. 1992. Intracellular calcium regulates the survival of early sensory neurons before they become dependent on neurotrophic factors. *Neuron* 9:563–574.
- Ledda F, Paratcha G, Ibanez CF. 2002. Target-derived GFR α 1 as an attractive guidance signal for developing sensory and sympathetic axons via activation of Cdk5. *Neuron* 36:387–401.
- Leung DW, Parent AS, Cachianes G, Esch F, Coulombe JN, Nikolics K, Eckenstein FP, et al. 1992. Cloning, expression during development, and evidence for release of a trophic factor for ciliary ganglion neurons. *Neuron* 8:1045–1053.
- Lindsay RM, Barde YA, Davies AM, Rohrer H. 1985a. Differences and similarities in the neurotrophic growth factor requirements of sensory neurons derived from neural crest and neural placode. *J Cell Sci Suppl* 3:115–129.
- Lindsay RM, Thoenen H, Barde YA. 1985b. Placode and neural crest-derived sensory neurons are responsive at early developmental stages to brain-derived neurotrophic factor. *Dev Biol* 112:319–328.
- Lhuillier L, Dryer SE. 2000. Developmental regulation of neuronal KCa channels by TGF β 1: Transcriptional and posttranscriptional effects mediated by Erk MAP kinase. *J Neurosci* 20:5616–5622.
- Lhuillier L, Dryer SE. 2002. Developmental regulation of neuronal K(Ca) channels by TGF β 1: An essential role for PI3 kinase signaling and membrane insertion. *J Neurophysiol* 88:954–964.
- Maas JW Jr, Horstmann S, Borasio GD, Anneser JM, Shooter EM, Kahle PJ. 1998. Apoptosis of central and peripheral neurons can be prevented with cyclin-dependent kinase/mitogen-activated protein kinase inhibitors. *J Neurochem* 70:1401–1410.

- Martin-Caraballo M, Dryer SE. 2002a. Activity- and target-dependent regulation of large-conductance Ca^{2+} -activated K^{+} channels in developing chick lumbar motoneurons. *J Neurosci* 22:73–81.
- Martin-Caraballo M, Dryer SE. 2002b. Glial cell line-derived neurotrophic factor and target-dependent regulation of large-conductance KCa channels in developing chick lumbar motoneurons. *J Neurosci* 22:10201–10208.
- Martin-Caraballo M, Greer JJ. 2001. Voltage-sensitive calcium currents and their role in regulating phrenic motoneuron electrical excitability during the perinatal period. *J Neurobiol* 46:231–248.
- McCobb DP, Best PM, Beam KG. 1989. Development alters the expression of calcium currents in chick limb motoneurons. *Neuron* 2:1633–1643.
- Meydan N, Grunberger T, Dadi H, Shahar M, Arpaia E, Lapidot Z, Leeder JS, et al. 1996. Inhibition of acute lymphoblastic leukaemia by a Jak-2 inhibitor. *Nature* 379:645–648.
- Motoike T, Unsicker K. 1999. Identification of a potent neurotrophic substance for ciliary ganglionic neurons in fetal calf serum as insulin-like growth factor II. *J Neurosci Res* 56:386–396.
- O'Dowd DK, Ribera AB, Spitzer NC. 1988. Development of voltage-dependent calcium, sodium, and potassium currents in *Xenopus* spinal neurons. *J Neurosci* 8:792–805.
- Pachau J, Martin-Caraballo M. 2006. Regulation of low-voltage-activated calcium channels by cell-cell interactions and trophic factors in chick nodose ganglion neurons. *Abstr Soc Neurosci* 29.4.
- Perez-Reyes E. 2003. Molecular physiology of low-voltage-activated T-type calcium channels. *Physiol Rev* 83:117–161.
- Rao MS, Landis SC, Patterson PH. 1990. The cholinergic neuronal differentiation factor from heart cell conditioned medium is different from the cholinergic factors in sciatic nerve and spinal cord. *Dev Biol* 139:65–74.
- Reis RA, Cabral da Silva MC, Loureiro dos Santos NE, Bampton E, Taylor JS, de Mello FG, Linden R. 2002. Sympathetic neuronal survival induced by retinal trophic factors. *J Neurobiol* 50:13–23.
- Rhee KD, Goureau O, Chen S, Yang XJ. 2004. Cytokine-induced activation of signal transducer and activator of transcription in photoreceptor precursors regulates rod differentiation in the developing mouse retina. *J Neurosci* 24:9779–9788.
- Saadat S, Sendtner M, Rohrer H. 1989. Ciliary neurotrophic factor induces cholinergic differentiation of rat sympathetic neurons in culture. *J Cell Biol* 108:1807–1816.
- Schonhoff CM, Bulseco DA, Branchio DM, Parada LF, Ross AH. 2001. The Ras-ERK pathway is required for the induction of neuronal nitric oxide synthase in differentiating PC12 cells. *J Neurochem* 78:631–639.
- Spitzer NC. 1991. A developmental handshake: Neuronal control of ionic currents and their control of neuronal differentiation. *J Neurobiol* 22:659–673.
- Subramony P, Raucher S, Dryer L, Dryer SE. 1996. Post-translational regulation of $\text{Ca}(2+)$ -activated K^{+} currents by a target-derived factor in developing parasympathetic neurons. *Neuron* 17:115–124.
- Symes A, Gearan T, Eby J, Fink JS. 1997. Integration of Jak-Stat and AP-1 signaling pathways at the vasoactive intestinal peptide cytokine response element regulates ciliary neurotrophic factor-dependent transcription. *J Biol Chem* 272:9648–9654.
- Takahashi-Tezuka M, Hibi M, Fujitani Y, Fukada T, Yamaguchi T, Hirano T. 1997. Tec tyrosine kinase links the cytokine receptors to PI-3 kinase probably through JAK. *Oncogene* 14:2273–2282.
- Umemiya M, Berger AJ. 1994. Properties and function of low- and high-voltage-activated Ca^{2+} channels in hypoglossal motoneurons. *J Neurosci* 14:5652–5660.
- Wang X, Halvorsen SW. 1998. Reciprocal regulation of ciliary neurotrophic factor receptors and acetylcholine receptors during synaptogenesis in embryonic chick atria. *J Neurosci* 18:7372–7380.
- Wellstein A, Fang WJ, Khatri A, Lu Y, Swain SS, Dickson RB, Sasse J, et al. 1992. A heparin-binding growth factor secreted from breast cancer cells homologous to a developmentally regulated cytokine. *J Biol Chem* 267:2582–2587.
- Yamamori T, Fukada K, Aebersold R, Korsching S, Fann MJ, Patterson PH. 1989. The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. *Science* 246:1412–1416.
- Yamashita Y, Tategaki A, Ogawa M, Horiuchi H, Nishida K, Akita S, Matsuda H, et al. 2006. Effect of novel monoclonal antibodies on LIF-induced signaling in chicken blastodermal cells. *Dev Comp Immunol* 30:513–522.
- Xia K, Mukhopadhyay NK, Inhorn RC, Barber DL, Rose PE, Lee RS, Narsimhan RP, et al. 1996. The cytokine-activated tyrosine kinase JAK2 activates Raf-1 in a p21ras-dependent manner. *Proc Natl Acad Sci USA* 93:11681–11686.
- Xue ZG, Smith J, Le Douarin NM. 1985. Differentiation of catecholaminergic cells in cultures of embryonic avian sensory ganglia. *Proc Natl Acad Sci USA* 82:8800–8804.