Differential Neurotrophic Regulation of Sodium and Calcium Channels in an Adult Sympathetic Neuron

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Ford CP, Wong KV, Lu VB, Posse de Chaves E, Smith PA. Differential neurotrophic regulation of sodium and calcium channels in an adult sympathetic neuron. J Neurophysiol 99: 1319–1332, 2008. First published January 23, 2008; doi:10.1152/jn.00966.2007. Adult neuronal phenotype is maintained, at least in part, by the sensitivity of individual neurons to a specific selection of neurotrophic factors and the availability of such factors in the neuron’s environment. Nerve growth factor (NGF) increases the functional expression of Na⁺ channel currents (I\(_{\text{Na}}\)) and both N- and L-type Ca²⁺ currents (I\(_{\text{Ca,N}}\) and I\(_{\text{Ca,L}}\)) in adult bullfrog sympathetic ganglion (BFSG) B-neurons. The effects of NGF on I\(_{\text{Na}}\) involve the mitogen-activated protein kinase (MAPK) pathway. Prolonged exposure to the ganglionic neurotransmitter luteinizing hormone releasing hormone (LHRH) also increases I\(_{\text{Na}}\) but the transduction mechanism remains to be elucidated as does the transduction mechanism for NGF regulation of Na⁺ channels. We therefore exposed cultured BFSG B-neurons to chicken II LHRH (0.45 μM; 6–9 days) or to NGF (200 ng/ml; 9–10 days) and used whole cell recording, immunoblot analysis, and ras or rap-1 pulldown assays to study effects of various inhibitors and activators of transduction pathways. We found that 1) LHRH signals via ras-MAPK to increase I\(_{\text{Ca,N}}\), 2) this effect is mediated via protein kinase C-β (PKC-β-II), 3) protein kinase A (PKA) is necessary but not sufficient to effect transduction, 4) NGF signals via phosphatidylinositol 3-kinase (PI3K) to increase I\(_{\text{Ca,L}}\), and 5) long-term exposure to LHRH fails to affect I\(_{\text{Ca,L}}\). Thus downstream signaling from LHRH has access to the ras-MAPK pathway but not to the PI3K pathway. This allows for differential retrograde and anterograde neurotrophic regulation of sodium and calcium channels in an adult sympathetic neuron.

MATERIALS

Male or female bullfrogs (Rana catesbeiana) were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care and experimental protocols were approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. Although biochemical, electrophysiological, and pharmacological experiments were carried out throughout the year, relatively few experiments were done in May and June when animals were frequently unavailable from the supplier (Anilah, Quebec City, PQ, Canada). Isolation, dissociation, and culture of BFSG neurons in defined medium, serum-free culture were carried out as described in preliminary reports (Ford et al. 2003a). Gonadotrophin receptors can couple via Ras-MAPK (Naor et al. 1998; Sim et al. 1995). The present experiments were therefore undertaken to determine 1) whether LHRH-induced increase of I\(_{\text{Ca,L}}\) in BFSG proceeds via the MAPK pathway in a similar fashion to NGF; 2) how G-protein–coupled LHRH receptors signal to the MAPK pathway, 3) the mechanism whereby NGF produces long-term increases in I\(_{\text{Ca,N}}\) and 4) whether LHRH has access to this transduction mechanisms so that it can produce long-term increases in I\(_{\text{Na}}\) in the same way as NGF. Parts of this work were published in preliminary reports (Ford and Smith 2000; Lu et al. 2002; Wong et al. 2006).

METHODS

Various neurotrophic factors have therefore been studied as important determinants of ion channel expression in adult neurons and in cell lines (Chalazonitis et al. 1987; Fanger et al. 1997; Khorkova and Golowasch 2007; Levine et al. 1995; Pollock and Rane 1996; Pollock et al. 1990). Target-derived nerve growth factor (NGF) is responsible for the maintenance of tetrodotoxin (TTX)-sensitive and TTX-insensitive sodium currents (I\(_{\text{Na}}\)) and of N- and L-type calcium currents (I\(_{\text{Ca,N}}\) and I\(_{\text{Ca,L}}\)) in B-neurons of adult bullfrog sympathetic ganglia (BFSG) (Lei et al. 1997, 1998, 2001; Petrov et al. 2001). In serum-free, defined medium culture, NGF-induced increases in I\(_{\text{Ca}}\) are initiated via the mitogen-activated protein kinase (MAPK) pathway (Lei et al. 1998), whereas the transduction process underlying the NGF-induced increase in I\(_{\text{Na}}\) remains to be determined.

We have also demonstrated that luteinizing hormone-releasing hormone (LHRH), a neurotransmitter released from preganglionic C-fibers in BFSG (Jan et al. 1979, 1980), is capable of regulating functional expression of Ca²⁺ channels (Ford et al. 2003a). Gonadotrophin receptors can couple via Ras/MAPK (Naor et al. 1998; Sim et al. 1995). The present experiments were therefore undertaken to determine 1) whether LHRH-induced increase of I\(_{\text{Ca,L}}\) in BFSG proceeds via the MAPK pathway in a similar fashion to NGF; 2) how G-protein–coupled LHRH receptors signal to the MAPK pathway, 3) the mechanism whereby NGF produces long-term increases in I\(_{\text{Ca,N}}\) and 4) whether LHRH has access to this transduction mechanisms so that it can produce long-term increases in I\(_{\text{Na}}\) in the same way as NGF. Parts of this work were published in preliminary reports (Ford and Smith 2000; Lu et al. 2002; Wong et al. 2006).

INTRODUCTION

Differential morphological, biochemical, and electrical properties define the broad range of neuronal phenotypes found in the adult nervous system. Expression of ion channels on a given neuronal type is not static and may change during development or aging or as a result of injury or disease (Craner et al. 2002; Cummins and Waxman 1997; Jasser et al. 1993, 1994; Lhuillier and Dryer 2002; Martin-Caraballo and Dryer 2002). Various neurotrophic factors have therefore been studied as important determinants of ion channel expression in adult neurons and in cell lines (Chalazonitis et al. 1987; Fanger et al. 1997; Khorkova and Golowasch 2007; Levine et al. 1995; Pollock and Rane 1996; Pollock et al. 1990). Target-derived nerve growth factor (NGF) is responsible for the maintenance of tetrodotoxin (TTX)-sensitive and TTX-insensitive sodium currents (I\(_{\text{Na}}\)) and of N- and L-type calcium currents (I\(_{\text{Ca,N}}\) and I\(_{\text{Ca,L}}\)) in B-neurons of adult bullfrog sympathetic ganglia (BFSG) (Lei et al. 1997, 1998, 2001; Petrov et al. 2001). In serum-free, defined medium culture, NGF-induced increases in I\(_{\text{Ca}}\) are initiated via the mitogen-activated protein kinase (MAPK) pathway (Lei et al. 1998), whereas the transduction process underlying the NGF-induced increase in I\(_{\text{Na}}\) remains to be determined.

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Electrophysiology

Whole cell recordings were made from neurons cultured in the presence of chicken II LHRH (0.45 μM) for 6–9 days or NGF (200...
ng/ml) for 9–10 days with or without various inhibitors of transduction pathways. Effects of LHRH on \( I_{Na} \) and \( I_{Ca} \) were compared with their effects in neurons cultured in the presence of inhibitors alone. Recordings were made from medium to large-sized cells with input capacitance >30 pF, which were almost certainly B-neurons (Kureny et al. 1994). Current and voltage were recorded using an Axoclamp 2A amplifier in discontinuous, single-electrode voltage-clamp mode. \( I_{Na} \) was evoked by a series of depolarizing voltage commands from a holding potential of -85 mV. With low-resistance electrodes (2–5 MΩ), it was possible to use high switching frequencies (>30 kHz) with high clamp gains (>8 to <30 mV/Na). The fidelity of the clamp was confirmed by examining the voltage recording. Recordings from cells where the voltage trace was slow to rise or distorted were discarded. For recording \( I_{Na} \), the solution in the bath (external) contained (in mM): NaCl, 97.5; tetraethylammonium (TEA)-Br, 20; MnCl₂, 4; and Tris-Cl, 2.5 (pH 7.2) and the solution inside the pipette (internal) contained (in mM): CsCl, 103; NaCl, 9; TEA-Br, 5; Cs-HEPES, 2.5; Cs-EGTA, and 1 (pH 7.2). \( I_{Ca} \) was recorded using Ba²⁺ as a charge carrier \( (I_{Na}) \). Currents were activated by incremental depolarizing voltage commands from a holding potential of -90 mV. To limit the amplitude, to lessen the rate of decay, and thereby to facilitate the analysis of tail currents, neurons were stepped to -40 mV at the conclusion of each depolarizing voltage command. The external solution contained (in mM): N-methyl-t-glucamine(NMG) chloride, 117.5; NMG-Hepes, 2.5; and BaCl₂, 2.0 (pH 7.2). The internal solution consisted of (in mM): NMG-Cl, 76.5; Hepes, 2.5; Tris-BAPTA, 10; Tris-ATP, 5; and MgCl₂, 4 (pH 7.2). Generally, external solutions were 250 mOsmol/kg and internal solutions were 240 mOsmol/kg.

During recording, the culture dishes were superfused with external solution at a flow rate of 2 ml/min. Neuronal input capacitance \( (C_{in}) \) was calculated by integrating the capacitive transient that accompanied a 10-mV depolarizing command from the holding potential. Current densities were expressed in terms of current per unit capacitance (i.e., pA/pF). All data are presented as means ± SE and Student’s two-tailed t-test or ANOVA was used to assess statistical significance \( (P < 0.05) \).

Being highly hydrophobic, some inhibitors were dissolved in DMSO to make stock solutions. Stock solutions were dissolved in culture medium to make final desired concentrations. The final DMSO concentration used in all cases was ≤0.1%. This concentration of DMSO did not affect \( I_{Na} \) because currents from cells cultured in the presence of DMSO were not significantly different from controls (data not shown, \( n = 10 \)). Inhibitors were used at five- to tenfold their published \( K_{i} \) or IC₅₀ values for their cognate enzymes. Cells treated with inhibitor plus chicken II LHRH (0.45 μM) or 200 ng/ml NGF were pretreated for 45 min with the inhibitor before addition of LHRH or NGF. NGF, LHRH, and/or enzyme inhibitors were added to medium at the time of dissociation and cells cultured in their presence for 6–10 days as specified. Medium containing wortmannin and PD-98059 was changed every 8 h, to maintain effective concentration used in all cases was 10 nM and applied to neurons for 1 h/day, followed by several washes to remove any residual drug.

### Immunoblot analysis

This was done using modifications of methodology we previously developed to study mammalian sympathetic ganglia (Song and Posse de Chaves 2003). The VIIth, IXth, and Xth paravertebral ganglia were removed from both sides of two adult bullfrogs and the neurons dissociated with trypsin and collagenase as described earlier. The cell suspension was plated into 10 wells of a 24-well dish at a density of 1.2 ganglia/well. The dissociated cells were cultured in L-15 medium for 5–6 days. At day 6, some treatment groups were given L-15 supplemented with 50 μM PD-98059 for 24 h to reduce basal phosphorylation of extracellurally regulated kinase (ERK). In these experiments, incubation with 100 nM LHRH began after two 5-min washes in L-15 to remove PD-98059. Cells were incubated for 10 min, 1 h, or 6 h in the presence of the peptide. For 6-day incubations, the LHRH-containing medium was replaced every other day. NGF experiments involved treatment of different groups of cells with 200 ng/ml NGF for 15 min, 1 h, and 6 h or 6 days with medium exchanges every other day. BFSG neurons cultured in 24-well dishes were washed with ice-cold L-15 medium prior to harvesting. Akt experiments included four treatment groups: 100 nM LHRH treatment for 10 min; 200 ng/ml NGF treatment for 15 min; 1 μM wortmannin treatment for 6 h; and 1 μM wortmannin pretreatment for 6 h followed by 200 ng/ml NGF incubation for 10 min. Protein kinase A (PKA) experiments involved incubation with Sp-cAMPS, or its inactive form Rp-cAMPS, for 1 h to activate PKA prior to examining ERK phosphorylation. The neurons were washed with ice-cold buffer with 1 mM Na₂VO₄ and 1 mM NaF to inhibit phosphatase activity. Cells from two wells of the same treatment were harvested with modified Laemmli sample buffer [40 mM Tris-HCl, pH 6.8, 0.002% bromophenol blue, 10% glycerol, 1% sodium dodecyl sulfate (SDS) and 4% 2-mercaptoethanol] and boiled for 2 min. Proteins were separated by SDS-PAGE on 10% polyacrylamide containing 0.1% SDS. After electrophoresis, proteins were transferred to “Immobilin” polyvinylidene difluoride (PVDF, Bio-RAD; Hercules, CA) membranes overnight at 4°C in 25 mM Tris (192 mM glycine, 16% methanol buffer, 0.1% SDS, and pH 8.3). Membranes were blocked for 1 h in 0.1% TTBS (Tris-buffered saline with 0.1% Tween-20) with 5% nonfat milk at room temperature and incubated in the primary antibody overnight at 4°C. The primary antibodies used were: rabbit polyclonal anti-phospho TrkA (Tyr490) (1:1,000), anti-phospho p42/44 MAPK (Thr202/Tyr204) (ERK1/2) (1:1,000), anti-phospho Akt (S473) (1:1,000), anti-phosphoPKC βII Ser 660 (1:1,000), and anti-phosphoPKC γ Thr 514 (1:1,000) from Cell Signaling Technology (Beverly, MA); anti-pan-Trk polyclonal antibody Trk (C-14) (1:500), and polyclonal anti-ERK 1 (C-16) (1:1,000) from Santa Cruz Biotechnology (Santa Cruz, CA). Membranes underwent three washes in TBS, TTBS, and TBS followed by incubation with the secondary antibody (goat-anti-rabbit IgG; Pierce, Brockville, Ontario, Canada) (1:2,000) in blocking buffer for 1 h at room temperature. Immunoreactivity was detected using enhanced chemiluminescence (ECL Plus Detection System; Amersham Biosciences, Piscataway, NJ). Equal protein loading was checked by probing for the nonphosphorylated forms of ERK 1 and TrkA or tubulin.

Data from immunoblots and activation assays (see following text) were scanned and their density assessed quantitatively using “Unscan-it gel” software (Silk Scientific, Orem, UT). Changes in density were assessed relative to loading controls for three to four gels for each experiment. For presentation and comparison of data across multiple experiments, relative densities were normalized to those for the control situation in each series. Care was taken to avoid quantification of data from immunoblots that appeared to have saturated.

### Ras activation assay

The Ras activation assay was performed according to the method of Herrmann et al. (1995). After treatments, cells were rinsed with ice-cold phosphate-buffered saline (PBS; with protease inhibitor cocktail, NaF, and Na₂VO₄), then immediately harvested and lysed in 300 μL of BOS buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% Nonidet P-40, 10% glycerol, 10 mM NaF, 2.5 mM MgCl₂, 1 mM EDTA). Protein assay was performed on the lysates and protein concentration was normalized. Equal amounts of GST-RBD (gift of Dr. Jim Stone, Department of Biochemistry, University of Alberta)
construct precoupled to glutathione-agarose beads in BOS buffer were added to each concentration-normalized lysate group. Samples were incubated for 2 h at 4°C with gentle shaking to pull down all activated Ras. Samples were then rinsed three times followed by addition of 2 x sample buffer and boiling for 5 min to cleave glutathione-agarose beads from GST-RBD constructs with active Ras attached. Equal volumes of GST-RBD with bound active Ras in 2 x sample buffer for each experimental group were loaded for gel electrophoresis.

Anti-Ras antibody from Upstate Cell Signaling Solutions/Millipore (Billerica, MA) was used to detect active Ras across experimental groups and anti-GST antibody (Abcam, Cambridge, MA) was used to detect loading amounts.

**Rap-1 activation assay**

Procedures were followed according to EZ-Detect RAP1 activation kit (Pierce, Brockville, Ontario, Canada). Briefly, after treatments, cells were rinsed with ice-cold PBS (with protease inhibitor cocktail, NaF, and Na2VO4) then immediately harvested and lysed in 300 µL of lysis buffer. Protein assay was performed on the lysates and protein concentration was normalized. Equal amounts of GST-RalGDS-RBD construct were added to several SwellGel (immobilized glutathione disc) and each normalized lysate experimental groups was immediately added to each separate SwellGel-GST-RalGDS-RBD construct. Samples were incubated for 1 h at 4°C with gentle shaking to pull down activated Rap-1. Samples were then rinsed three times followed by addition of 2 x sample buffer and boiling for 5 min to cleave glutathione beads from GST-RalGDS-RBD construct with active Rap-1 attached. Equal volumes of GST-RalGDS-RBD with bound active Rap-1 in 2 x sample buffer for each experimental group were loaded for gel electrophoresis. Anti-Rap-1 antibody was used to detect active Rap-1 across experimental groups and anti-GST antibody was used to detect loading amounts.

**Drugs and chemicals**

The following drugs and reagents were from Biomol (Plymouth Meeting, PA): wortmannin, PP1, LY294002 [2-(4-morpholino)-8-phenyl-4H-1-benzo[4,5]imidazolo[1,2-a]pyridine-3-carboximide], PD-98059 (2’-amino-3’-methoxyflavone), H-89 (N-[2-((3R,4S,5R,7R)-4-[(3R,4S,5R,7R)-4-(2-carboxyethyl)-2-cyclopentene-1-carboxamido]-3-methoxy-4-oxo-2,3-dihydro-6H-pyridin-5-yl]-phenyl)3-[(3R,4S,5R,7R)-4-(2-carboxyethyl)-2-cyclopentene-1-carboxamido]-4-oxo-2,3-dihydro-6H-pyridin-5-yl]-phenyl)-5-isouquinolinesulfonamide-2HCl), chelerythrine (chelerythrine chloride), U-73122 [1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-5-isoquinoline-N-phenyl-4H-1-benzopyran-4-one], PD-98059 (2’-amino-3’-methoxyflavone), Sp- and Rp-cAMPS (reversibly, Sp- and Rp-adenosine-3’5’-cyclic monophosphorothioate triethylamine salt), PMA (phorbol 12-myristate-13-acetate), and 4α-phorbol. Chicken II luteinizing hormone releasing hormone (LHRH) and phorbol ester. All recordings are families of Ba2+ current (Iba) produced in response to 20-nV incremental steps from a holding potential of ~90 mV. Tail currents recorded at ~40 mV. A: currents recorded after 6 days in defined medium culture. B: currents recorded after 6 days in the presence of 0.45 µM chicken II LHRH (note larger amplitude compared with that in A); inset is voltage recording corresponding to the current trace. C: current density−voltage relationship for cells cultured with (n = 35) and without LHRH (n = 49); error bars = SE. D: family of Iba currents recorded after 6 days in the presence of the methyl ethyl ketone (MEK) inhibitor 2'-amino-3'-methoxyflavone (PD-98059, 10 µM). E: currents recorded after 6 days in the presence of 0.45 µM LHRH plus PD-98059 (note similarity in amplitude compared with D). F: currents recorded after 6 days in the presence of 4α-phorbol (80 nM). G: currents recorded following 1-h daily exposure to 80 nM phorbol 12-myristate-13-acetate (PMA) for 6 days (note increased amplitude compared with P). Calibration bar in A refers to all traces except that in B.

Iba does not reflect a change in the voltage dependence of activation or inactivation. It does, however, reflect a selective increase in IcαN in a transcription-dependent manner (Ford et al. 2003a). Exposure to LHRH may therefore induce synthesis or affect the trafficking of N-type Ca2+ channels.

There is variation in the rate of tail current delay in the traces presented in Fig. 1. In particular, the tails illustrated in Fig. 1B (in the presence of LHRH) are slower than those in the control (Fig. 1A). Such slowing may be indicative of inadequate voltage clamp in neurons that express extensive processes, although there was considerable variation in the rate of tail current decay in both control and LHRH-treated neurons. This might imply that some neurons extended processes under both conditions. We found that 43% (28/65) of control neurons and 32% (25/78) of neurons examined in LHRH exhibited between one and five primary neurites. Mean numbers of neurites were 2.1 ± 0.2 (n = 28 in controls) and 2.2 ± 0.2 (n = 25 in LHRH) (P > 0.7). Mean lengths of longest process were 89.6 ± 18.3 µm (n = 28, range = 10–500 µm) in control neurons and 104 ± 21.4 µm (n = 25, range = 20–500 µm) in LHRH-

**RESULTS**

**Mechanism of LHRH-induced increase of Ica**

Figure 1A illustrates a family of Iba currents recorded in a BFSG B-neuron after 6 days in defined medium culture. Figure 1B was recorded from another cell and illustrates the increase in Iba amplitude after 6-day culture with 0.45 µM LHRH. The bottom trace illustrates voltage recordings associated with this current trace. Figure 1C illustrates that the maximum current density, recorded at ~10 mV from a holding potential of ~90 mV, in 6-day cultured neurons was 113 ± 7 pA/pF (n = 49). This increased by 55% to 175 ± 16 pA/pF (n = 35; P < 0.02) in cells cultured for 6 days in the presence of 0.45 µM LHRH. We have shown previously that this LHRH-induced increase in

**FIG. 1.** Long-term regulation of total Ca2+ current (Ica) by luteinizing hormone releasing hormone (LHRH) and phorbol ester. All recordings are families of Ba2+ current (Iba) produced in response to 20-nV incremental steps from a holding potential of ~90 mV. Tail currents recorded at ~40 mV. A: currents recorded after 6 days in defined medium culture. B: currents recorded after 6 days in the presence of 0.45 µM chicken II LHRH (note larger amplitude compared with that in A); inset is voltage recording corresponding to the current trace. C: current density−voltage relationship for cells cultured with (n = 35) and without LHRH (n = 49); error bars = SE. D: family of Iba currents recorded after 6 days in the presence of the methyl ethyl ketone (MEK) inhibitor 2’-amino-3’-methoxyflavone (PD-98059, 10 µM). E: currents recorded after 6 days in the presence of 0.45 µM LHRH plus PD-98059 (note similarity in amplitude compared with D). F: currents recorded after 6 days in the presence of 4α-phorbol (80 nM). G: currents recorded following 1-h daily exposure to 80 nM phorbol 12-myristate-13-acetate (PMA) for 6 days (note increased amplitude compared with P). Calibration bar in A refers to all traces except that in B.

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treated neurons ($P > 0.6$). Thus the relatively slow tail currents seen in Fig. 1B do not imply that only LHRH-treated neurons exhibited processes. It may rather imply that minor clamping problems occasionally occurred in neurons across the whole population and this may have correlated with those that exhibited processes. Since the steady-state currents flowing during depolarizing voltage commands are smaller than the peak amplitudes of tail currents recorded at $-40 \text{ mV}$, clamp problems are unlikely to have affected the recorded $I_{\text{Ba}}$ amplitudes. This assumption is supported by the stability of the recorded membrane voltage even when large-amplitude currents were elicited (see inset to Fig. 1B). The failure of tail currents to return to the baseline level at the end of the recorded traces in Fig. 1 likely also reflects incomplete deactivation at $-40 \text{ mV}$.

The involvement of mitogen-activated protein kinase/extracellular-regulated kinase kinase (MEK) in LHRH-mediated changes in $I_{\text{Ca}}$ was examined using the inhibitor PD-98095 (10 $\mu$M) (Alessi et al. 1995). This substance, which prevents the NGF-induced increase in $I_{\text{Ba}}$ in BFSG neurons (Lei et al. 1998), also blocked the LHRH-induced increase in current density ($P < 0.05$) (Fig. 1, D and E; Table 1). This finding is consistent with the hypothesis that the effect of LHRH on $I_{\text{ca}}$ involves the activation of MEK, and thus presumably the activation of MAPK.

If this is so, it should be possible to demonstrate the activation of MAPK following 6-day exposure to LHRH (i.e., using the same exposure conditions as those used in the electrophysiological experiments). We therefore examined phosphorylation of extracellularly regulated kinase 1/2 (ERK1/2), members of the MAPK family, by immunoblot analysis (Fig. 2A). For this and all immunoblots illustrated in Figs. 2, 3, and 4 corresponding panels (such as Fig. 2B) illustrate quantification of results from at least three replicate experiments. As expected, 6 days of exposure to NGF promoted a dramatic increase in ERK1/2 phosphorylation (Fig. 2A, lane 2; summarized in Fig. 2B). The increase in ERK activation following 6 days of incubation of neurons with 100 nM LHRH (Fig. 2A, lane 3; summarized in Fig. 2B) was much more modest. Because ERK phosphorylation was prominent in untreated (control) neurons (Fig. 2A, lane 1), this complicated the detection of ERK activation by LHRH. Thus in all subsequent experiments, cultures were pretreated with the MEK inhibitor PD-98095 (50 $\mu$M for 24 h) to decrease basal ERK phosphorylation before addition of LHRH (Fig. 2C, lane 2 vs. lane 1). Under these conditions, a robust phosphorylation of ERK could be demonstrated (Fig. 2C, lane 3 vs. lane 2; summarized in Fig. 2D).

Cellular effects mediated by ERK depend strongly on the temporal pattern of ERK activation. For example, sustained activation of ERK is required for the induction of axonal growth (Huang and Reichardt 2003). It is therefore possible that LHRH may induce a transient activation of ERK that may signal the surface expression of new Ca$^{2+}$ channels via a transcription-dependent process (Ford et al. 2003a). We therefore further characterized the time course of ERK phosphorylation and found that it was maximal after 10 min of LHRH treatment and returned to basal activation levels after 6 h of exposure to the peptide (Fig. 2C, lanes 2–5; summarized Fig. 2D). On the other hand, NGF induced sustained ERK activation throughout the duration of the experiment (Fig. 2F, lanes 2–4; data summarized in Fig. 2G). Elevated ERK phosphorylation was not the result of loading a greater amount of protein since equivalent amounts of total ERK and total TrkA were demonstrated by blotting with the corresponding antibodies. No phosphorylation of ERK was detected when LHRH was applied to the neurons in the continued presence of PD-98095 (data not shown).

Ligand binding to G-protein–coupled receptors (GPCRs) can lead to transactivation of Trk and other growth factor receptors (Rajagopal et al. 2004). Since phospho ERK1/2 is a well-defined downstream effector of TrkA (Kaplan and Stephens 1994; Sofroniew et al. 2001), it is possible that the
observed effect of LHRH was mediated via transactivation of TrkA. This seemed unlikely because LHRH did not induce observed effect of LHRH was mediated via transactivation of TrkA (Fig. 2C, lanes 3–5; summarized in Fig. 2E). By contrast, NGF (200 ng/ml) caused a clear and sustained activation of TrkA (Fig. 2F, lanes 2–4; summarized in Fig. 2H). Moreover, treatment with NGF, but not with LHRH, led to activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which is also downstream of TrkA (Fig. 2I, lane 3 vs. lane 2; summarized in Fig. 2J).

In all the immunoblot experiments performed, antibodies against phosho-ERK as well as antibodies against total ERK detected a single band with molecular weight corresponding to ERK 2 in BFSG, although the characteristic doublet was observed in rat neuronal samples processed in parallel (not shown). Since both antibodies (anti total ERK and anti phosho ERK) were unable to detect the band corresponding to ERK 1, it is possible that frogs do not express ERK 1. However, it is also possible that limited cross-reactivity of mammalian-origin antibodies with frog proteins plays a role or that amphibian ERK 1 has the same molecular weight as that of amphibian ERK 2.

Ras or Rap-1?

At least two different low molecular weight G-proteins, ras or rap-1, can participate in activation of MAPK by extracellular ligands (Sofroniew et al. 2001). Indeed, rap-1 has been implicated in regulation of Ca\(^{2+}\) channels by neurotropins in PC12 cells (Black et al. 2003). We examined ras and rap-1 activation using pulldown assays to evaluate their possible role in the action of LHRH. Figure 3, A (lanes 4 and 5) and B illustrates activation of ras by both LHRH and NGF. Neither ras activated rap-1 (Fig. 3, C, lanes 4 and 5 and D). Lane 1 in Fig. 3, A and C represents positive controls illustrating Ras and Rap-1 activation by GTP-γ-S (see also Fig. 3, B and D).

How do LHRH receptors couple to MAPK?

In pituitary cell lines, GnRH (LHRH) receptor signaling to MAPK involves phospholipase C (PLC) and protein kinase C (PKC) (Naor et al. 2000; Reiss et al. 1997; Sim et al. 1995). Figure 4A (lane 2) illustrates activation of PKCβII by LHRH. Activation was demonstrated by using an antibody directed against the phosphorylated form of the enzyme. This yielded two bands at about 75 and 100 kDa, both of which were made more intense by LHRH. By contrast, LHRH failed to activate PKCy. Quantification of the data for four replicate experiments is shown in Fig. 4B.

In electrophysiological experiments, we used the PKC inhibitor chelerythrine (Herbert et al. 1990) to examine the role of PKC in LHRH-induced potentiation of \(I_{\text{Ba}}\). At 1 μM, chelerythrine prevented LHRH-mediated increases in current density because \(I_{\text{Ba}}\) recorded in the presence of chelerythrine plus LHRH was not significantly different from cells cultured for 6 days with chelerythrine alone (\(P > 0.05\)) (Table 1). These are the results that would be expected if LHRH signaling involves PKC.

The PLC inhibitor U-73122, together with its inactive structural analog U-73343, provides a specific way to investigate the role of PLC in signal transduction. Moreover, U-73122 has already been shown to inhibit agonist-induced PLC activation in BFSG neurons (Stemkowski et al. 2002). Inclusion of 20 μM U-73122 in the cultures prevented the increase in \(I_{\text{Ba}}\) produced by 0.45 μM LHRH (\(P > 0.05\), Table 1). However, a substantial increase in \(I_{\text{Ba}}\) current density still occurred when cells were maintained for 6 days in the presence of 0.45 μM...
Fig. 4. Activation of protein kinase C (PKC) and ERK by phorbol esters and Sp-adenosine-3',5'-cyclic monophosphorothioate triethylamine salt (Sp-cAMPS). A: immunoblots to illustrate activation of PKCβII but not PKCγ by LHRH and PMA. Note maximal activation of PKCβII is seen after 1 h with PMA and activation is attenuated after 24-h exposure or during intermittent exposure as was done in electrophysiological experiments. C: activation of ERK by LHRH, PMA, and Sp-cAMPS, but not by Rp-cAMPS or 4α-phorbol. E: inhibition of LHRH-induced ERK activation by Rp-cAMPS. B, D, and F: quantitative representation of data from experiments similar to those presented in A, C, and E. All data were measured in terms of percentage change in gel density relative to loading controls. For further ease of comparison, these ratios were normalized to the ratio observed in the control experimental situation. All experiments were done 3 or 4 times; the clear symbols, (circles, diamonds, or triangles) indicate results from individual experiments. The black horizontal bars represent mean values for each experimental situation.
LHRH and the inactive analogue, U-73343 (20 μM) compared with U-73343 alone (Table 1, P < 0.05). These findings implicate PLC in the action of LHRH.

In some pituitary cell lines, the actions of GnRH are mediated via PKA (Han and Conn 1999). Furthermore, signaling pathways involving PKA and/or cAMP are known to affect the ras-MAPK cascade (Impey et al. 1998; Naor et al. 2000). These findings prompted us to test the effect of the PKA inhibitor H-89 (Chijiiwa et al. 1990). Cells treated for 6 days with H-89 plus LHRH did not show the increase in current density that was seen with LHRH alone (P < 0.05; Table 1). This finding raised the possibility that PKA is also involved in the LHRH response.

\( G_c \)-coupled GPCRs can activate MAPK via their \( \beta \gamma \) subunits and the transactivation of growth factor receptors. This effect proceeds via the nonreceptor tyrosine kinase, c-src, and the downstream activation of the phosphatidylinositol 3 kinase-\( \gamma \) (PI3K-\( \gamma \)) (Koch et al. 1991; Lopez-Ilasaca et al. 1997). The role of src kinase in LHRH-mediated Ca\(^{2+} \) channel regulation was investigated using PP1, an inhibitor of the src kinases (Hanke et al. 1996). PP1 (1.5 μM) failed to prevent LHRH from increasing \( I_{Ba} \) density because there was a significant increase (P < 0.05) in current density with PP1 plus LHRH compared with the current density seen in PP1 alone (Table 1). This result is consistent with the observation that LHRH failed to promote TrKA phosphorylation (Fig. 2, C, E, and I).

The role of PI3K in LHRH-mediated Ca\(^{2+} \) channel regulation was examined with the PI3K inhibitor, wortmannin (Yano et al. 1993). Like PP1, 100 nM wortmannin failed to prevent the increase in \( I_{Ba} \) density by LHRH (Table 1; P < 0.05). This excludes PI3K and c-src from the LHRH-mediated increases in Ca\(^{2+} \) channel expression.

These effects of inhibitors suggest that LHRH signals, through both PKC and PKA, to cause activation of MAPK that, in turn, leads to an increase in \( I_{Ba} \) density. To test this possibility further, we directly stimulated PKC or PKA in an attempt to mimic the action of LHRH. BFGS B-cells treated intermittently with the phorbol ester PMA (80 nM), for 1 h/day over a 6-day period to activate PKC (Ryves et al. 1991), exhibited \( I_{Ba} \) densities (71 ± 6 pA/pF; n = 17) that were significantly greater than those of neurons treated with 4α-phorbol, the negative control for PMA (55 ± 4 pA/pF; n = 17; P < 0.05) (Fig. 1, F and G). This indicates that direct activation of PKC is sufficient to cause an increase in the level of Ca\(^{2+} \) channels expressed in BFGS.

A concern with the use of phorbol esters is that prolonged exposure may downregulate rather than activate PKC (Mathies et al. 1987). We hoped that the intermittent application protocol we developed for the electrophysiological experiments would promote sustained kinase activation for the whole 6-day experimental period. Figure 4, A (lanes 3–6) and B illustrates the effect of treatment for various time periods with PMA (80 nM). An exposure of 1 h promotes obvious phosphorylation of both the 75- and 100-kDa isoforms of PKC\( \beta \)II (Fig. 4, A, lanes 3 and 4 and B, top). By contrast, a continuous 24-h exposure or application according to the “intermittent” schedule used in our electrophysiological experiments (1 h/day for 6 days) failed to convincingly demonstrate activation of PKC\( \beta \)II (Fig. 4A, lanes 5 and 6). Our intermittent application protocol therefore did not achieve the sustained PKC activation we had anticipated. This may be of little consequence because the effect of LHRH on ERK phosphorylation is transient (Fig. 2, C and D). Thus the first 1-h exposure of neurons to PMA in the electrophysiological experiments and the transient activation of PKC\( \beta \)II likely initiated the genomic changes responsible for altered functional expression of \( I_{Ba} \). Subsequent applications of PMA likely did not activate PKC but this is irrelevant if the first stimulus initiated the signal for altered channel expression.

**PKA is “necessary but not sufficient” for LHRH activation of MAPK**

Unlike PKC, the direct activation of PKA with the cAMP analogue Sp-cAMPS was not sufficient to increase \( I_{Ba} \) density. Current densities recorded from cells treated with Sp-cAMPS (57 ± 4 pA/pF; n = 41) were no different from the current density recorded from cells treated with the nonselective Rp-cAMPS, a competitive inhibitor of the activation of PKA by cAMP (64 ± 3 pA/pF; n = 42; P > 0.05). This, taken with the results from the inhibition of PKA and PKC during stimulation with LHRH, suggests that both pathways are necessary in the regulation of Ca\(^{2+} \) channels by LHRH, yet only PKC on its own is sufficient to produce this effect.

To show that the lack of effect of extracellularly applied Sp-cAMPS on \( I_{Ba} \) was not due to its failure to penetrate neurons, we examined its ability to activate ERK by immunoblotting effects of Sp-cAMPS compared with Rp-cAMPS, PMA, and 4α-phorbol (Fig. 4, C and D). The effect of Sp-cAMPS was weaker than that of PMA and Rp-cAMPS was ineffective. This confirms the effectiveness of extracellularly applied Sp-cAMPS and the fact that it is less effective than PMA may relate to the suggestion that PKA is necessary but not sufficient to alter the functional expression of Ca\(^{2+} \) channels.

The requirement for PKA activation for the action of LHRH is illustrated further by the experiments shown in Fig. 4, E and F. This shows that the competitive cAMP antagonist Rp-cAMPS attenuates LHRH activation of ERK.

**Mechanism of NGF-induced increase in \( I_{Na} \)**

We have shown that long-term exposure of BFGS B-neurons to 200 ng/ml nerve growth factor (NGF) results in a doubling of Na\(^{+} \) current density. This effect is transcription dependent and does not reflect changes in activation or inactivation kinetics (Lei et al. 2001).

Although the PI3K inhibitor wortmannin failed to prevent LHRH or NGF-induced increases in \( I_{Ba} \) (see Table 1 and Lei et al. 1998), it was highly effective in inhibiting NGF-induced increases in total \( I_{Na} \). Relevant sample recordings are shown in Fig. 5, A, B, D, and E. Exposure to NGF for 10 days increased peak \( I_{Na} \) density at −5 mV from 299 ± 36 (n = 28) to 447 ± 53 pA/pF (n = 35; P < 0.03; Fig. 5C). By contrast, NGF failed to affect \( I_{Na} \) density in the presence of 1 μM wortmannin (peak \( I_{Na} \) density in wortmannin 344 ± 39; n = 32, in wortmannin + NGF 344 ± 49 pA/pF; n = 25; P > 0.85; Fig. 5F). The effect of NGF was also blocked by the more selective PI3K inhibitor LY294002 (10 μM). Peak \( I_{Na} \) in LY294002 was 329 ± 35 (n = 27) compared with 327 ± 34 pA/pF (n = 26; P < 0.95; Fig. 5G) in the presence of LY294002 + NGF. These results are consistent with the involvement of the PI3K pathway in the action of NGF on Na\(^{+} \) channel expression.
Does LHRH increase $I_{Na}$?

Peak, total $I_{Na}$ density was unaffected by LHRH. For 6-day cultures, maximum $I_{Na}$ in the presence of 0.45 μM LHRH was $239 \pm 35$ pA/pF ($n = 17$) compared with $230 \pm 16$ pA/pF for controls ($n = 20$, $P > 0.8$). Similarly, for 9-day cultures, current density in LHRH was $276 \pm 42$ ($n = 19$) compared with $260 \pm 35$ pA/pF for controls ($n = 19$; $P > 0.75$). For 6-day cultures, there was no shift in the voltage dependence of $g_{Na}$ activation with LHRH treatment from $-80$ to $+50$ mV ($n = 17$ for LHRH and 20 for control). Also, for 6-day cultures, there was no shift in the $h_{Na}$ curves with LHRH treatment ($g/g_{max}$) from $-105$ to $+30$ mV and stepping to $+10$ mV ($n = 17$ for LHRH and $n = 20$ for control; data not shown). Last, there was no change in the rate of onset of inactivation. For example, at 0 mV, $\tau$ for $g_{Na}$ inactivation after 6 days in LHRH was $1.8 \pm 0.1$ ms ($n = 17$) compared with $1.9 \pm 0.1$ ms in controls ($n = 20$, $P > 0.2$).

Since NGF produces effects through both the MAPK and PI3K pathways (Kaplan and Stephens 1994; Sofroniew et al. 2001), the present data suggest that the PI3K pathway is involved in regulation of $I_{Na}$ and previous data suggest the MAPK pathway is involved in NGF regulation of $I_{Ca}$ (Lei et al. 1998). Since LHRH regulates $Ca^{2+}$ and not $Na^{+}$ channels, it may be able to signal only through the MAPK pathway and not via the PI3K pathway. We tested this possibility by examining
the effect of LHRH on phosphorylation of Akt, one of the downstream effectors of PI3K (Sofroniew et al. 2001). Figure 2, I (lane 2) and J shows that 10-min exposure to 100 nM LHRH failed to increase Akt phosphorylation, whereas a robust increase was seen in the presence of 200 ng/ml NGF (Fig. 2, I, lane 3 and J). The effect of NGF was attenuated by 6-h prior exposure to the PI3K inhibitor wortmannin (1 μM, data not shown).

**DISCUSSION**

The main findings of this study are 1) LHRH signals via ras-MAPK to increase Ca\(^{2+}\) current density in an adult sympathetic neuron, 2) LHRH signaling to ras involves PKCβII, 3) PKA is necessary but not sufficient to effect the transduction process, 4) NGF signals via PI3K to increase Na\(^+\) channel currents, and 5) LHRH fails to affect functional expression of Na\(^+\) channel current. Since NGF has previously been reported to signal via MAPK to increase \(I_{Ca}\) density (Lei et al. 1998) it is suggested that downstream signaling from LHRH has selective access to MAPK and not to PI3K. These findings are summarized in Fig. 6.

**Role of ras-MAPK in the LHRH-induced increase in \(I_{Ca}\)**

The suggestion that the effect of LHRH is independent of src and the PI3K pathway (Koch et al. 1991; Lopez-Ilasaca et al. 1997) but may instead involve PLC and the ras-MAPK pathway is supported by the lack of effect of the src kinase inhibitor PP1 and the PI3K inhibitor wortmannin as well as the positive results with U-73122 and PD-98059 (Table 1). Corroborative results emerge from the immunoblot analyses; LHRH promotes phosphorylation of ERK1/2 and not Akt, whereas NGF phosphorylates and activates both enzymes (Fig. 2). Another important result from the immunoblot experiments is that LHRH fails to activate TrkA. This provides additional evidence against a role for transactivation of growth factor receptors in the action of LHRH. There is good evidence, however, for the operation of this mechanism for other G-protein-coupled receptors in other systems (Rajagopal et al. 2004). Finally, the ability of LHRH and NGF to activate ras rather than rap-1 suggests that the former is involved in gonadotropin actions to increase \(Ca^{2+}\) current (Figs. 3, A and C and 6). Although surrogate growth factor receptors activate rap-1 in PC12 cells (Black et al. 2003) this does not appear to happen with native TrkA receptors in a sympathetic neuron.

Although U-73122 and PD-98059 prevent NGF-induced increases in \(I_{Ba}\) in BFSG neurons (Lei et al. 1998), the pharmacological approach always raises concerns about inhibitor concentration and specificity (Smith 1995). This issue may be especially relevant to studies on amphibian tissues using a series of agents that have been defined in terms of affinity and specificity in mammalian systems. Although “molecular” approaches such as RNA interference (Holen and Mobbs 2004) or introduction of dominant negative or constitutively active forms of ras (Fitzgerald 2000) might be considered, the lack of information on the *Rana catesbeiana* genome and the fact that suitable reagents are not readily available makes this approach difficult or infeasible. Since this study is a continuation of an extensive series of physiological studies (Ford et al. 2003a; Jassar et al. 1993; Lei et al. 1997, 1998, 2001; Petrov et al. 2001) on *Rana catesbeiana*, it is not appropriate to change to a mammalian (Fitzgerald 2000; Fitzgerald and Dolphin 1997) or cell line system even though they are clearly more amenable to molecular biological approaches (Black et al. 2003).

Amphibian sympathetic ganglia contain both vasomotor C-neurons and B-neurons that project to mucous glands in the skin (Smith 1994). The present immunoblot experiments were done on cultures containing both neuron types, whereas the electrophysiological experiments were confined to B cells. It is likely that NGF and LHRH modulate \(I_{Na}\) and \(I_{Ca}\) in a similar fashion in C-cells because these express LHRH receptors

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**FIG. 6.** Scheme to illustrate proposed mechanisms for NGF and LHRH regulation of Na\(^{+}\) and Ca\(^{2+}\) channels.
Role of G-proteins and protein kinases in the effect of LHRH

The present experiments were based on the premise that the neurotrophic effects of LHRH are mediated by a heterotrimeric G-protein. It is possible, however, that some other signaling process was involved. For example, internalization of peptide-bound receptors may trigger transduction processes (Miller and Lefkowitz 2001) or a G-protein–independent receptor, such as tyrosine kinase, may have been directly activated. These possibilities seem unlikely because all documented effects of LHRH in neurons, endocrine glands, and cancer cells seem to progress via receptors that exert their effects via heterotrimeric G-proteins (Cheng and Leung 2000; Gründker et al. 2001; Kakar et al. 2002; Mc Ardle et al. 2002; Naor et al. 1998, 2000). Since it is generally accepted that LHRH signals via a GPCR to activate the ras-MAPK system in gonadotrophs (Han and Conn 1999; Reiss et al. 1997; Sim et al. 1995; Sundaresan et al. 1996), it is likely that a similar pathway exists in neurons.

LHRH (GnRH) receptors activate different heterotrimeric G-proteins/signal transduction pathways in different cell types (Conn et al. 1979; Kuphal et al. 1994). There are at least two mammalian GnRH receptor subtypes and coupling to $G_{q/11}$, $G_q$, and $G_i$ has been reported (Hawes et al. 1993; Janovick and Conn 1994; Stanislaus et al. 1997; Ullóo-Aguirre et al. 1998). In bullfrogs, there are three distinct GnRH receptor subtypes, all of which appear capable of coupling to $G_q$ (Wang et al. 2001). Within the sympathetic ganglia, LHRH activates PLC to increase inositol triphosphate turnover and intracellular Ca$^{2+}$ concentration (Paffinger et al. 1988) and to suppress M-type Na$^+$ current (Ford et al. 2003b, 2004), suggesting that the LHRH/GnRH receptor(s) in this tissue is/are also coupled to $G_{q/11}$. It is yet to be determined which of the three identified bullfrog GnRH receptors is/are involved in both this process and the neurotrophic effects described in the present work.

In some pituitary cell lines, the effect of GnRH on ras-MAPK is mediated via PKC (Reiss et al. 1997; Sundaresan et al. 1996). Direct stimulation of PKC with a phorbol ester is also known to activate MAPK (Reiss et al. 1997) and our electrophysiological results with U-73122 and chelerythrine suggest that the LHRH receptor signals via PLC and PKC in BFSG B-neurons (Table 1; Fig. 6). Furthermore, direct stimulation of PKC by phorbol ester is sufficient to cause an increase in $I_{Na}$ (Fig. 1, F and G), suggesting that PKC is both “necessary and sufficient” to regulate BFSG Ca$^{2+}$ channels. The use of isoform-specific antibodies implicate PKCβII and not PKCγ in the action of LHRH, although these findings do not exclude the participation of additional PKC isoforms. Because activation of PKC is known to affect inactivation of Na$^+$ conductance ($g_{Na}$) in a variety of neuronal systems (Franceschetti et al. 2000), one might predict that LHRH may have a similar effect in BFSG neurons, although this was not observed. It has been shown, however, that the membrane-bound εPKC is involved in altering $g_{Na}$, inactivation (Chen et al. 2005) and it is possible that this particular isoform, like PKCγ, is not activated by the downstream effectors of LHRH actions.

Pathways also exist for cAMP to signal to ERK1/2 in neurons and PC12 cells. One is via cAMP/PKA/B-raf and the other via cAMP/Epac/rap1/B-raf (de Rooij et al. 1998; Grewal et al. 2000; Kawasaki et al. 1998; Vossler et al. 1997). The pituitary adenylate cyclase-activating polypeptide (PACAP) receptor Pac1 stimulates both a cAMP pathway and the PLC pathway to activate rap-1 and ras. These effectors act in a complex synergistic manner to activate ERK1/2. Cyclic AMP/PKA and ras are both permissive for rap-1 activation of ERK1/2. They are thus “necessary but not sufficient” for the action of Pac1 (Bouschet et al. 2003). If a similar mechanism exists for the LHRH receptor in BFSG, this would explain the finding that H-89 blocked the effects of LHRH, although the cAMP analogue Sp-cAMPS failed to mimic the effects of LHRH, even though it was able to promote a weak activation of ERK1/2 (Fig. 4, C and D). A permissive role for ras is supported by the fact that wild-type ras (p21ras) is also “necessary but not sufficient” to mediate neurotrophin induction of Ca$^{2+}$ channels in PC12 cells (Pollock and Rane 1996).

There are some differences between the actions of NGF and LHRH on Ca$^{2+}$ channels. Although LHRH exclusively increases $I_{Ca,L}$ with no effect on activation or inactivation kinetics (Ford et al. 2003a), the effects of NGF are more complex and involve an increase in $I_{Ca,L}$, decreased inactivation of total $I_{Ca}$, and an increase in $I_{Ca,N}$ (Lei et al. 1997). Mechanisms additional to ras-MAPK may be involved in NGF effects on $I_{Ca,L}$ and inactivation processes. The differences also may relate to the lasting activation produced by NGF (Fig. 2, A, B, F, and H) compared with the transient ERK1/2 activation promoted by LHRH (Fig. 2, C and D). This transient activation is unlikely to reflect LHRH receptor internalization because an acute modulatory effect of LHRH on $I_{Ca,L}$ (Elmslie et al. 1990) was preserved even after 6 days in the continued presence of the peptide (for details see Ford et al. 2003a).

Role of PI3K in NGF-induced increase in $I_{Na}$

Experiments with LY294002 and wortmannin support the involvement of the PI3K pathway in NGF-induced increases in Na$^+$ current. Since downstream signaling from PI3K often proceeds through Akt (Sofroniew et al. 2001), the ability of NGF to phosphorylate this enzyme (Fig. 2, I and J) adds further support to the involvement of this pathway. A ras-independent increase in Na$^+$ density in PC12 cells was previously reported (Fanger et al. 1993, 1997; Hilborn et al. 1998). However, in the latter report (Fanger et al. 1997) it was suggested that the ras-independent pathway may involve members of the src nonreceptor tyrosine kinase family rather than PI3K. One possible reason for this difference was that our experiments were done with native TrkA on intact adult neurons, whereas the experiments of Fanger et al. (1997) were done on PC12 cells expressing surrogate platelet-derived growth factor (PDGF) beta receptors with mutations that eliminate activation of specific signaling molecules. Alternatively, it could be argued that our data, which depend on the supposed selectivity of inhibitors, are less reliable than those obtained using molecular and cell biological techniques.

About 15% of the total $I_{Na}$ in BFSG B-neurons is TTX resistant (Jassar et al. 1993) and is presumably a different gene product from the TTX-sensitive current (Goldin et al. 2000). We have shown previously that TTX-resistant $I_{Na}$ is also upregulated by NGF in BFSG neurons (Lei et al. 2001). It would therefore be instructive to examine the associated transduction mechanism: Does the PI3K regulate both genes in
parallel? Are the TTX-resistant and TTX-sensitive currents regulated by different transduction processes? Does the PI3K-mediated increase of total $I_{Na}$ reflect a posttranslational modification or an action on trafficking of all Na$^+$ channel types?

**Lack of effect of LHRH on Na$^+$ currents**

The lack of effect of LHRH on $I_{Na}$ is readily explained by the observation that the PI3K pathway involved in NGF-induced increases in $I_{Na}$ is not activated by LHRH (Fig. 2, I and J). Thus the PI3K pathway appears to control functional expression of Na$^+$ channels, whereas control of Ca$^{2+}$ currents is exerted via the MAPK pathway. NGF, which has access to both pathways, increases both currents, whereas LHRH, which can access only the MAPK pathway via ras, regulates only Ca$^{2+}$ currents.

Whereas functional upregulation of Ca$^{2+}$ and Na$^+$ channel function by NGF in BFSG persists as long as target-derived neurotrophin is available, the neurotrophic effect of LHRH is more labile because it depends on peptide release and thus neuronal activity in preganglionic nerve fibers. This may relate to the transient effect of LHRH compared with the sustained effect of NGF on ERK1/2 phosphorylation. Since neuromodulation release is favored by intense neuronal activity (Peng and Horn 1991), regulation of Ca$^{2+}$ channels is favored by intense neuronal activity. The release of neuropeptides from preganglionic fibers also causes long-term increases in tyrosine hydroxylase activity and noradrenaline synthesis (McKeon and Zigmond 1993). Due to the dependence of neurotransmitter release on Ca$^{2+}$ influx, increased Ca$^{2+}$ channel availability at sympathetic postganglionic terminals may augment sympathetic output to target tissues. By contrast, control of Na$^+$ channels is independent of ganglionic transmission and instead depends on the availability of target-derived NGF (Lei et al. 2001). This differential regulation of channel types is brought about by the ability of NGF to signal via MAPK and PI3K compared with the selective activation of ras-MAPK by LHRH.

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