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

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Running Header:- Neurotrophic Regulation of Na⁺ and Ca²⁺ channels

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ABSTRACT

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 neurons’ environment. Nerve growth factor (NGF) increases the functional expression of Na⁺ channel currents (I_{Na}) and both N- and L-type Ca²⁺ currents (I_{Ca,N} and I_{Ca,L}) in adult bullfrog sympathetic ganglion (BFSG) B-neurons. The effects of NGF on I_{Ca} involve the MAPkinase pathway. Prolonged exposure to the ganglionic neurotransmitter, LHRH (luteinizing hormone releasing hormone) also increases I_{Ca,N} 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 d) or to NGF (200 ng/ml; 9-10d) 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-MAP Kinase to increase I_{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 (PI 3kinase) to increase I_{Na}, and 5) long-term exposure to LHRH fails to affect I_{Na}. Thus, downstream signaling from LHRH has access to the ras-MAP kinase pathway but not to the PI 3kinase pathway. This allows for differential retrograde and anterograde neurotrophic regulation of sodium and calcium channels in an adult sympathetic neuron.
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 (Jassar et al. 1993; Jassar et al. 1994; Cummins and Waxman 1997; Lhuillier and Dryer 2002; Craner et al. 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; Levine et al. 1995; Pollock et al. 1990; Pollock and Rane 1996; Fanger et al. 1997; Khorkova and Golowasch 2007). Target-derived nerve growth factor (NGF) is responsible for the maintenance of TTX-sensitive and TTX-insensitive sodium currents (INa) and of N- and L-type calcium currents (ICa,N and ICa,L) in B-neurons of adult bullfrog sympathetic ganglia (BFSG) (Lei et al. 1997; Lei et al. 1998; Lei et al. 2001; Petrov et al. 2001). In serum-free, defined medium culture, NGF-induced increases in ICa are initiated via the MAP kinase pathway (Lei et al. 1998) whereas the transduction process underlying the NGF-induced increase in INa remains to be determined.

We have also demonstrated that LHRH (luteinizing hormone releasing hormone), a neurotransmitter released from preganglionic C-fibers in BFSG (Jan et al. 1979; Jan et al. 1980), is capable of regulating functional expression of Ca²⁺ channels (Ford et al. 2003a). Gonadotrophin receptors can couple via Gq/11, Gs or Gi to various downstream effectors including ras-MAP kinase (Sim et al. 1995; Naor et al. 1998b). The present experiments were therefore undertaken to determine 1) whether LHRH-induced increase of ICa in BFSG proceeds via the MAP kinase pathway in a similar fashion to NGF, 2) how G-protein coupled LHRH receptors signal to the MAP kinase pathway, 3) the mechanism whereby NGF produces long-term increases in INa and 4) whether LHRH has access to this transduction mechanisms so that it can produce long-term
increases in $I_{Na}$ in the same way as NGF. Preliminary reports of parts of this work have appeared (Ford and Smith 2000; Lu et al. 2002; Wong et al. 2006).

MATERIALS AND METHODS

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 (Anilab, Québec City, PQ, Canada). Isolation, dissociation and culture of BFSG neurons in defined medium-serum free-culture was carried out as described by Lei et al. (1998). Neurons were dissociated by incubation with trypsin (Sigma) and type 1A collagenase (Sigma) for 42-45 min, at 37°C. Final dissociation was accomplished by tituration with a Pasteur pipette. Cells were suspended in 3 ml of serum-free, modified L-15 medium (73% L-15, Gibco; pH 7.2), 10 mM glucose, 1 mM CaCl$_2$, 100 units/ml penicillin, 100 µg/ml streptomycin, 10 µM cytosine arabinoside. The dissociated cells were then redistributed into 2.5 ml of medium in each of 20 35-mm polystyrene tissue culture dishes. Dishes were placed in a light proof, humidified chamber and maintained at room temperature (22°C) for 6-10 days as specified.

*Electrophysiology*

Whole-cell recordings were made from neurons cultured in the presence of chicken II LHRH (0.45 µM) for 6-9 d or NGF (200 ng/ml) for 9-10 d with or without various inhibitors of transduction pathways. Effects of LHRH on $I_{Ca}$ and $I_{Na}$ 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 (Kurenny et al. 1994). Current and voltage were recorded using an Axoclamp 2A amplifier in discontinuous, single-electrode voltage-clamp mode. $I_{\text{Na}}$ 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 $I_{\text{Na}}$, the solution in the bath (external) contained (mM): NaCl, 97.5; TEA-Br, 20; MnCl₂, 4; Tris-Cl, 2.5 (pH7.2) and the solution inside the pipette (internal) contained (mM): CsCl, 103; NaCl, 9; TEA-Br, 5; Cs-HEPES, 2.5; Cs-EGTA, 1 (pH7.2). $I_{\text{Ca}}$ was recorded using Ba²⁺ as a charge carrier ($I_{\text{Ba}}$). 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 (mM): N-methyl-D-glucamine (NMG) chloride, 117.5; NMG-Hepes, 2.5; BaCl₂, 2.0; (pH 7.2). Internal solution consisted of (mM): NMG-Cl, 76.5; Hesper, 2.5; Tris-BAPTA, 10; Tris-ATP, 5; 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 ($C_{\text{in}}$) was calculated by integrating the capacitative transient which 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 ±S.E.M and Student's two-tailed t-test or analysis of variance 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 \(I_{Ba}\) as currents from cells cultured in the presence of DMSO were not significantly different from controls (data not shown, \(n=10\)). Inhibitors were used at 5-10 times their published \(K_D\) or \(IC_{50}\) values for their cognate enzymes. Cells treated with inhibitor plus chicken II LHRH (0.45 \(\mu\)M) 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 6-10d as specified. Medium containing wortmannin and PD98059 was changed every 8 hours, in order to maintain effective concentrations, since both of these chemicals are subject to hydrolysis in aqueous solutions. In an attempt to maintain activation yet avoid down-regulation of PKC, phorbol 12-myristate-13-acetate (PMA) and the negative control, 4\(\alpha\)-phorbol were used at 80 nM and applied to neurons for 1hr/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 2 adult bullfrogs and the neurons dissociated with trypsin and collagenase as described above. 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 \(\mu\)M PD98059 for 24 hours to reduce basal phosphorylation of ERK. In these experiments, incubation with 100 nM LHRH began after two 5min washes in L-15 to remove PD98059. Cells were incubated for 10 min,
1 h, or 6 h in the presence of the peptide. For 6 d incubations, the LHRH containing medium was replaced every other day. NGF experiments involved treatment of different groups of cells with 200ng/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 4 treatment groups: 100 nM LHRH treatment for 10 min; 200ng/ml NGF treatment for 15 min; 1 µM wortmannin treatment for 6hrs; and 1 µM wortmannin pretreatment for 6hrs followed by 200 ng/ml NGF incubation for 10 min. PKA experiments involved incubation with Sp-cAMPS or its inactive form Rp-cAMPS for 1 hr to activate PKA prior to examining ERK phosphorylation. PKC experiments involved incubation with PMA or its inactive form, 4α-phorbol, for 1 hr to activate PKC 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 nM 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, USA) 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 1h in 0.1% TTBS (Tris Buffered Saline with 0.1% Tween-20) with 5% non-fat 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) (1:1000), anti-phospho p42/44 MAPK (Thr202/Tyr204) (ERK 1/2) (1:1000), anti-phospho Akt (S473) (1:1000), anti-phosphoPKC β11 Ser 660 (1:1000), and anti-phosphoPKC γ Trp 514 (1:1000) from Cell Signaling Technology (Beverly, MA, USA); anti-pan-Trk polyclonal antibody Trk (C-14) (1:500), and polyclonal anti-ERK 1 (C-16) (1:1000) from Santa Cruz Biotechnology Inc., Santa Cruz, CA,
USA). Membranes underwent three washes in TBS, TTBS, and TBS followed by incubation with the secondary antibody (Goat anti Rabbit IGg; Pierce, Brockville, Ont., Canada) (1:2000) in blocking buffer for 1 hr at room temperature. Immunoreactivity was detected using enhanced chemo luminescence (‘ECL Plus Detection System’ Amersham Biosciences, Piscataway, NJ, USA). Equal protein loading was checked by probing for the non-phosphorylated forms of ERK 1 and TrkAor tubulin.

Data from immunoblots and activation assays (see below) were scanned and their density assessed quantitatively using ‘Un-scan-it gel’ software (Silk Scientific, Orem, UT). Changes in density were assessed relative to loading controls for 3-4 gels for each experiment. For presentation and comparison of data across a multiple experiments, relative densities were normalized to that 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**

This was performed according to the method of (Herrmann et al. 1995). After treatments, cells were rinsed with ice cold phosphate buffered saline (with protease inhibitor cocktail, NaF, and Na$_2$VO$_4$) then immediately harvested and lysed in 300 µL of BOS buffer (50 nM 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, Dept Biochemistry, University of Alberta) construct pre-coupled to glutathione-agarose beads in BOS buffer was added to each concentration normalized lysate group. Samples were incubated for 2hrs at 4°C with gentle shaking to pull down all activated Ras. Samples were then rinsed 3 times followed by addition of 2X 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 2X sample buffer for each
experimental group were loaded for gel electrophoresis.

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

*Rap1 activation assay*

Procedures were followed according to EZ-Detect RAP1 activation kit (Pierce Brockville,
Ont., Canada). Briefly, after treatments, cells were rinsed with ice cold phosphate buffered saline
(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 1hr at
4°C with gentle shaking to pull down activated Rap-1. Samples were then rinsed 3 times followed
by addition of 2X 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 2X 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, LY 294002 (2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), PD-98059 (2’-Amino-3’-methoxyflavone), H-89 (N-[2-(p-Bromocinnamylamina)ethyl]-5-isoquinolinesulfonamide-2HCl), Chelerythrine (chelerythrine chloride), U-73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione, U-73343 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-pyrrole-2,5-dione, Sp- and Rp-cAMPS (-Adenosine-3',5'-cyclic monophosphorothioate triethylamine salt), PMA (Phorbol 12-myristate-13-acetate) and 4α-phorbol. Chicken II Luteinizing Hormone Releasing Hormone (LHRH) and Nerve Growth Factor (NGF) were from Alomone Labs (Jerusalem, Israel). All other reagents were from Sigma-Aldrich (Oakville, Ont., Canada) or Fisher Scientific (Edmonton, AB, Canada).

RESULTS

Mechanism of LHRH-induced Increase of I_{Ca}

Fig 1A illustrates a family of I_{Ba}’s recorded in a BFSG B-neuron after 6 days in defined-medium culture. Fig 1B was recorded from another cell and illustrates the increase in I_{Ba} amplitude after 6d culture with 0.45 μM LHRH. The lower trace illustrates voltage recordings associated with this current trace. Fig 1C illustrates that the maximum current density, recorded at -10mV from a holding potential of -90mV, in 6d 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 6d in the presence of 0.45 μM LHRH. We have shown previously that this LHRH-induced increase in I_{Ba} does not reflect a change in the voltage-dependence of activation or inactivation. It does however reflect a selective increase in I_{Ca,N}
in a transcription-dependent manner (Ford et al. 2003a). Exposure to LHRH may therefore induce synthesis or affect the trafficking of N-type Ca\(^{2+}\) 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. There was however 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 1 and 5 primary neurites. Mean number of neurites = 2.1± 0.2 (n=28 in controls) and 2.2± 0.2 (n = 25 in LHRH) p>0.7. The mean length of longest process were 89.6±18.3 \(\mu\)m (n=28, range = 10-500 \(\mu\)m) in control neurons and 104±21.4 \(\mu\)m (n=25, range = 20-500 \(\mu\)m) in LHRH – 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 -40mV, clamp problems are unlikely to have affected the recorded \(I_{Ba}\) 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 -40mV.

{Table 1 and Figure 1 near here}
The involvement of MEK in LHRH-mediated changes $I_{Ca}$ was examined using the inhibitor, PD98095 (10µM) (Alessi et al. 1995). This substance, which prevents the NGF-induced increase in $I_{Ba}$ in BFSG neurons (Lei et al. 1998), also blocked the LHRH induced increase in current density (p<0.05) (Fig 1D and E; Table 1). This finding is consistent with the hypothesis that the effect of LHRH on $I_{Ca}$ 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 6d exposure to LHRH. (i.e. using the same exposure conditions as was 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 - 4 corresponding panels (such as Fig 2B) illustrate quantification of results from at least three replicate experiments. As expected, 6 d exposure to NGF promoted a dramatic increase in ERK1/2 phosphorylation (Fig. 2A lane 2 and summary in Fig 2B). The increase in ERK activation following 6d of incubation of neurons with 100nM LHRH (Fig 2A lane 3 and summary 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, PD98059 (50µM for 24 hours) to decrease basal ERK phosphorylation before addition of LHRH (Fig 2C lane 2 versus lane 1). Under these conditions, a robust phosphorylation of ERK could be demonstrated (Fig 2C lane 3 versus 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 6h 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 PD98059 (data not shown).

{Figures 2, 3 and 4 near here}

Ligand binding to G-protein coupled receptors (GPCR) 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 TrkA phosphorylation (Fig. 2C, lanes 3-5 summarized in Fig 2E). By contrast, NGF (200ng/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 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 phospho-ERKs as well as antibodies against total ERK detected a single band with molecular weight corresponding to ERK2 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 phospho 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 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 MAP kinase 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. Fig 3A (*lanes 4 and 5*) and Fig 3B illustrate activation of ras by both LHRH and NGF. Neither substance activated rap-1 (Fig 3C *lanes 4 and 5* and Fig 3D). *Lane 1* in Figs 3A and 3C are positive controls illustrating Ras and Rap-1 activation by GTP-\(\gamma\)-S (see also Figs 3B and 3D).

*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) (Reiss et al. 1997; Sim et al. 1995; Naor et al. 2000). Figure 4A (*lane 2*) illustrates activation of PKC\(\beta\)II by LHRH. Activation was demonstrated by using an antibody directed against the phosphorylated form of the enzyme. This yielded two bands at \(~75\) and \(~100\)kDa both of which were made more intense by LHRH. By contrast LHRH failed to activate PKC\(\gamma\). Quantification of the data for four replicate experiments is shown in Fig 4B.

In electrophysiological experiments, we used the PKC inhibitor, chelerythrin (Herbert et al. 1990) to examine the role of PKC in LHRH-induced potentiation of \(I_{Ba}\). At 1 \(\mu\)M, chelerythrine prevented LHRH-mediated increases in current density, as \(I_{Ba}\) 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 U73122, together with its inactive structural analog U73343, provides a specific way to investigate the role of PLC in signal transduction. Moreover, U73122 has already been shown to inhibit agonist-induced PLC activation in BFSG neurons (Stemkowski et al. 2002). Inclusion of 20 µM U73122 in the cultures prevented the increase in $I_{Ba}$ produced by 0.45 µM LHRH; (p>0.05, Table 1). However, a substantial increase in Ca$^{2+}$ current density still occurred when cells were maintained for 6d in the presence of 0.45 µM LHRH and the inactive analogue, U73343 (20 µM) compared to U73343 in 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 (Naor et al. 2000; Impey et al. 1998) These findings prompted us to test the effect of the PKA inhibitor, H-89 (Chijiwa et al. 1990). Cells treated for 6 d 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.

$G_{i}$-coupled GPCRs can activate MAPK via their $\beta\gamma$ subunits and the transactivation of growth factor receptors. This effect proceeds via the non-receptor tyrosine kinase, c-src and the downstream activation of the phosphatidylinositol 3 kinase-γ (PI3K-γ) (Koch et al. 1991; Lopez-Illasaca 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 p56$^{lck}$ and p59$^{fyn}$ (Hanke et al. 1996). PP1 (1.5µM) failed to prevent LHRH from increasing $I_{Ba}$ density, as there was a significant increase (P<0.05) in current density with PP1 plus LHRH compared to the current density seen in PP1 alone.
(Table 1). This result is consistent with the observation that LHRH failed to promote TrkA phosphorylation (Fig 2C, 2E and 2I).

The role of PI3 kinase in LHRH-mediated Ca$^{2+}$ channel regulation was examined with the PI3 kinase inhibitor, wortmannin (Yano et al. 1993). Like PP1, 100nM wortmannin failed to prevent the increase in $I_{Ba}$ density by LHRH (Table 1; $P<0.05$). This excludes PI3 kinase 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 which, 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 (80nM), for 1 hr/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 neurons treated with 4α-phorbol, the negative control for PMA (55±4 pA/pF; n=17; $P<0.05$) (Fig. 1F 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 (Matthies et al. 1987). We hoped that the intermittent application protocol we developed for the electrophysiological experiments would promote sustained kinase activation for the whole 6d experimental period. Figures 4A (lanes 3-6) and 4B illustrate the effect of treatment for various time periods with PMA (80nM). 1hr exposure promotes obvious phosphorylation of both the 75 and 100KDa isoforms of PKCβII (Fig 4A lanes 3 and 4 and Fig 4B, upper panel). By contrast, a continuous 24h exposure or application according to the ‘intermittant’ schedule used in our electrophysiological experiments (1hr/day for 6d) failed to convincing demonstrate activation of PKCβII (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 as the effect of LHRH on ERK phosphorylation is transient (Figs 2C and D). Thus, the first 1 hour exposure of neurons to PMA in the electrophysiological experiments and the transient activation of PKCβII likely initiated the genomic changes responsible for altered functional expression of I_{Ca}. 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 than the current density recorded from cells treated with the enantiomer 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 were compared with Rp-cAMPS, PMA and 4α phorbol (Fig 4C 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 4E 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 BFSG 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).

![Figure 5 near here]

Although the PI 3kinase 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 5A, B, D and E. Exposure to NGF for 10 days increased peak $I_{Na}$ density at -5mV 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 PI 3kinase inhibitor, LY294002 (10µM). Peak $I_{Na}$ in LY294002 was 329±35 (n=27) compared to 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 PI 3kinase 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 6d cultures, maximum $I_{Na}$ in the presence of 0.45 µM LHRH was 239±35 pA/pF (n=17) compared with 230±16 pA/pF for controls (n=20, P>0.8). Similarly, for 9d cultures, current density in LHRH was 276±42 (n=19) compared to 260±35 pA/pF for controls (n=19; P>0.75). For 6d cultures, there was no shift in the voltage-dependence of g$_{Na}$ activation with LHRH treatment from -80mV -> +50mV (n=17 for LHRH and
20 for control). Also, for 6 d cultures, there was no shift in the \( h_n \) curves with LHRH treatment (\( g/g_{\text{max}} \) from -105mV -> +30mV and stepping to +10mV (n=17 for LHRH and n=20 for control, data not shown). Lastly, there was no change in the rate of onset of inactivation. For example at 0mV, \( \tau \) for \( g_{\text{Na}} \) inactivation after 6d in LHRH was 1.8±0.1 ms (n=17) compared to 1.9±0.1 ms in controls (n=20 p>0.2).

Since NGF produces effects through both the MAPK and PI 3kinase pathways (Kaplan and Stephens 1994; Sofroniew et al. 2001), the present data suggest that the PI 3kinase pathway is involved in regulation of \( I_{\text{Na}} \) and previous data suggest the MAPK pathway is involved in NGF regulation of \( I_{\text{Ca}} \) (Lei et al. 1998). Since LHRH regulates \( \text{Ca}^{2+} \) and not \( \text{Na}^+ \) channels, it may only be able to signal 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 PI 3kinase (Sofroniew et al. 2001). Figs 2I (lane 2) and 2J show that 10min exposure to 100 nM LHRH failed to increase Akt phosphorylation whereas a robust increase was seen in the presence of 200ng/ml NGF (Fig 2I, lane 3 and Fig 2J). The effect of NGF was attenuated by 6 hours prior exposure to the PI 3kinase inhibitor, wortmannin (1 \( \mu \)M, data not shown).

**DISCUSSION**

The main findings of this study are 1) LHRH signals via ras-MAPK to increase \( \text{Ca}^{2+} \) current density in an adult sympathetic neuron 2) LHRH signaling to ras involves PKC\( \beta I \) 3) PKA is necessary but not sufficient to effect the transduction process 4) NGF signals via PI 3kinase to increase \( \text{Na}^+ \) channel currents 5) LHRH fails to affect functional expression of \( \text{Na}^+ \) channel current. Since NGF has previously been reported to signal via MAPK to increase \( I_{\text{Ca}} \) density (Lei et al. 1998) it is suggested that downstream signaling from LHRH has selective access to MAPK and not to PI 3kinase. 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, PPI and the PI3K inhibitor wortmannin as well as the positive results with U73122 and PD98059 (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 however good evidence for the operation of this mechanism for other G-protein coupled receptors in other systems (Rajagopal et al. 2004). Lastly, 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 3A, 3C 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 U73122 and PD98059 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 which 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 (Jassar et al. 1993; Lei et al. 1997; Lei et al. 1998; Lei et al. 2001; Petrov et al. 2001; Ford et al. 2003a) on *Rana catesbeina*, it is not appropriate to change to a mammalian (Fitzgerald and Dolphin 1997; Fitzgerald 2000) 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, as these express LHRH receptors (Jones 1987) and since they are vasomotor sympathetic neurons they likely also express TrkA.

*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 as 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 (Grundker et al. 2001; Kakar et al. 2002; McArdle et al. 2002; Cheng and Leung 2000; Naor et al. 1998a; Naor et al. 2000). Since it is generally accepted that LHRH signals via a GPCR to activate the ras-MAPK system in gonadotrophs (Sim et al. 1995;
Sundaresan et al. 1996) (Reiss et al. 1997; Han and Conn 1999), 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\(_s\) and G\(_i\) has been reported (Stanislaus et al. 1997; Janovick and Conn 1994; Ulloa-Aguirre et al. 1998; Hawes et al. 1993). 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 trisphosphate turnover and intracellular Ca\(^{2+}\) concentration (Pfaffinger et al. 1988) and to suppress M-type K\(^+\) current (Ford et al. 2003b; Ford et al. 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 U73122 and chelerythrine suggest that the LHRH receptor signals via PLC and PKC in BFSG B-neurons (Table 1; Fig 6). Furthermore, direct simulation of PKC by phorbol ester is sufficient to cause an increase in I\(_{Ba}\) (Figs 1F and 1G), suggesting that PKC is both ‘necessary and sufficient’ to regulate BFSG Ca\(^{2+}\) channels. The use of isoform specific antibodies implicate PKC\(_{\beta II}\) and not PKC\(_{\gamma}\) in the action of LHRH. 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. This was not observed. It has been shown however that the membrane bound ePKC
is involved in altering $g_{\text{Na}}$ inactivation (Chen et al. 2005) and it is possible that this particular isoform, like PKC$\gamma$, 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; Kawasaki et al. 1998; Vossler et al. 1997; Grewal et al. 2000). 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, but 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 4C and D). A permissive role for ras is supported by the fact that wild type ras (p21$^{\text{ras}}$) 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. Whilst LHRH exclusively increases $I_{\text{Ca,N}}$ with no effect on activation or inactivation kinetics (Ford et al. 2003a), the effects of NGF are more complex and involve an increase in $I_{\text{Ca,L}}$, decreased inactivation of total $I_{\text{Ca}}$ as well as an increase in $I_{\text{Ca,N}}$ (Lei et al. 1997). Mechanisms additional to ras-MAPK may be involved in NGF effects on $I_{\text{Ca,L}}$ and inactivation processes. The differences also may relate to the lasting activation produced by NGF (Figs 2A,B,F and H) compared to the transient ERK1/2 activation promoted by LHRH (Figs 2C and D). This transient activation is unlikely to reflect LHRH receptor internalization, because an acute modulatory effect of LHRH on $I_{\text{Ca}}$ (Elmslie et al. 1990) was preserved even after 6 d in the continued presence of the peptide (for details see Ford et al., 2003a).


Role of PI 3kinase in NGF-induced Increase in \( I_{Na} \)

Experiments with LY294002 and wortmannin support the involvement of the PI 3kinase pathway in NGF-induced increases in \( Na^+ \) current. Since downstream signaling from PI3kinase often proceeds through Akt (Sofroniew et al. 2001), the ability of NGF to phosphorylate this enzyme (Figs 2I and J), adds further support to the involvement of this pathway. A ras-independent increase in \( Na^+ \) density in PC12 cells has been previously reported (Hilborn et al. 1998; Fanger et al. 1993; Fanger et al. 1997). However, in the latter report (Fanger et al. 1997) it was suggested that the ras-independent pathway may involve members of the src non-receptor tyrosine kinase family rather than PI 3kinase. 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 associated transduction mechanism; does the PI 3kinase regulate both genes in parallel? Are the TTX-resistant and TTX-sensitive currents regulated by different transduction processes? Does the PI 3kinase mediated increase of total \( I_{Na} \), reflect a post-translational 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\(_{\text{Na}}\) is readily explained by the observation that the PI 3kinase pathway that is involved in NGF-induced increases in I\(_{\text{Na}}\) is not activated by LHRH (Fig 2I and J). Thus, the PI 3kinase 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 only access the MAPK pathway via ras, only regulates Ca\(^{2+}\) currents.

Whereas functional up-regulation 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, as it depends on peptide release and hence neuronal activity in preganglionic nerve fibers. This may relate to the transient effect of LHRH compared to the sustained effect of NGF on ERK1/2 phosphorylation. Since neuropeptide release is favored by intense neuronal activity (Peng and Horn 1991), regulation of Ca\(^{2+}\) channels by LHRH may couple pre-ganglionic activity to alterations in the electrical properties of post-ganglionic cells. The release of neuropeptides from pre-ganglionic 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 outflow 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 PI 3kinase compared to the selective activation of ras MAPK by LHRH.
ACKNOWLEDGEMENTS

Supported by Canadian Institutes of Health Research Grant # MOP 57798 and the Heart and Stroke Foundation of Canada. CPF received studentship support from the Alberta Heritage Foundation for Medical Research (AHFMR) and Neuroscience Canada. VBL was an AHFMR summer student. We thank Dr. Mee Sook Song, Dr. Lucila Saavedra and Ms. Valaria Mancinelli for help with SDS-PAGE and immunoblot procedures and Dr. W.F. Dryden for useful discussions and Dr. Kwai Alier for his comments on an early version of the manuscript.
Table 1. Effects of enzyme inhibitors on LHRH-induced I_Ba potentiation

<table>
<thead>
<tr>
<th>Enzyme Inhibitor</th>
<th>Control I_Ba Density (pA/pF)</th>
<th>I_Ba Density in LHRH (pA/pF)</th>
<th>I_Ba Density in Inhibitor (pA/pF)</th>
<th>I_Ba Density in LHRH + Inhibitor (pA/pF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD 98059 (10µM)</td>
<td>113±7 (49)</td>
<td>175±16(35) a</td>
<td>112±10(10)</td>
<td>129±9(20)</td>
</tr>
<tr>
<td>Chelerythrine (1µM)</td>
<td>113±7 (49)</td>
<td>175±16(35) a</td>
<td>74±4 (28)</td>
<td>91±9 (25)</td>
</tr>
<tr>
<td>U73122 (20µM)</td>
<td>67.±7 (23)*</td>
<td>117±16.4 (19)*a</td>
<td>63±5 (25)</td>
<td>67±5 (26)</td>
</tr>
<tr>
<td>H-89 (1µM)</td>
<td>113±7 (49)</td>
<td>175±16(35) a</td>
<td>123±10 (21)</td>
<td>96±5 (27)</td>
</tr>
<tr>
<td>PP1 (1.5µM)</td>
<td>113±7 (49)</td>
<td>175±16(35) a</td>
<td>44±3 (23)</td>
<td>94±10 (23)b</td>
</tr>
<tr>
<td>Wortmannin (100nM)</td>
<td>113±7 (49)</td>
<td>175±16(35) a</td>
<td>48±6 (18)</td>
<td>72±8 (17)b</td>
</tr>
</tbody>
</table>

Data are expressed as means±SEM with n-values in parentheses; statistical tests done with one-way ANOVA. Current densities are maximum values recorded at -10mV. *Control values for U73122 were obtained with the inactive analogue, U73343. a P<0.05 compared to ‘blank’ control; b P<0.05 compared to inhibitor alone. All other data for the blank and positive controls were pooled and obtained from cells in serum-free defined medium. Inhibitors: PD-98059, inhibitor of MEK. Chelerythrine, inhibitor of PKC. U73122, inhibitor of PLC. H-89, inhibitor of PKA. PP1, inhibitor of src kinases. Wortmannin, inhibitor of PI 3kinase.
**Figure 1. Long-term regulation of total $I_{Ca}$ by LHRH and phorbol ester.** All recordings are families of $I_{Ba}$ produced in response to 20mV incremental steps from a holding potential of -90mV. Tail currents recorded at -40mV. **A.** Currents recorded after 6d in defined-medium culture. **B.** currents recorded after 6d in the presence of 0.45 µM Chicken II LHRH (note larger amplitude compared to 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 = SEM. **D.** Family of $I_{Ba}$’s recorded after 6d in the presence of the MEK inhibitor PD 98059 (10 µM). **E.** Currents recorded after 6d in the presence of 0.45µM LHRH plus PD 98059 (note similarity in amplitude compared to D). **F.** Currents recorded after 6d in the presence of 4α-phorbol (80 nM). **G.** Currents recorded following 1hr daily exposure to 80nM PMA for 6d (note increased amplitude compared to F). Calibration bar in A refers to all traces except that in B.

**Figure 2. Activation of Erk by LHRH and lack of effect on Akt and Rap-1.** Proteins from neuronal extracts were separated by SDS PAGE and identified by immunoblot analysis with the antibodies indicated in Materials and Methods. Protein loading was evaluated by blotting for total ERK and/or total TrkA. The figure shows representative blots as well as quantification of data from 3 or 4 replicate experiments. The clear symbols, (circles, diamonds or triangles) indicate results from individual experiments. The black horizontal bars represent mean values for each experimental. All data were measured 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. **A.** Effect of long term (6 days) exposure to NGF (200ng/ml) or LHRH (100nM). **B.** Quantification of data from 3 experiments similar to that illustrated in A. **C.** Neurons were incubated without or with 50µM PD98059 for 24 h. At the end of the incubation, cultures as indicated were given 100nM Chicken II LHRH for times as specified. **D and E.**
Quantification of data from 4 experiments similar to that illustrated in C to illustrate LHRH activation of ERK but not TrkA. F. Neurons were treated with or without PD98059 as in C and exposed to NGF for times as indicated. G and H. Quantification of data from 4 experiments similar to that illustrated in F to illustrate NGF activation of ERK but not TrkA. I and J. Effect of NGF and lack of effect of LHRH on AkT activation.

**Figure 3. Activation of ras but not rap-1 by LHRH and NGF.** A. Neurons were cultured for 5-6 days then were treated with 100 nM LHRH for 10min, 200ng/mL NGF for 15min, 10mM GTPγS for 30min, or 100nM GDP for 30min. Cell lysates were then added to GST-RBD precoupled to glutathione-agarose beads to pulldown activated Ras then the supernatants were loaded and separated by SDS-PAGE. GST immunoreactivity was used as a loading control. B. Quantification of data such as that shown in A for three replicate experiments. C. Neurons were subjected to the same treatments as in A, then cell lysates were added to GST-RalGDS-RBD Swellgel® to pulldown activated Rap1 and the supernatants were loaded and separated by SDS-PAGE. GST immunoreactivity was used as a loading control. D. Quantification of data such as that shown in C for three replicate experiments.

**Figure 4 Activation of PKC and ERK by Phorbol Esters and 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 24h 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 that presented in A, C and E. All data were measured 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.

**Figure 5 Long-term regulation of $I_{Na}$ by NGF.** All recordings are families of $I_{Na}$ produced in response to 10mV incremental steps from a holding potential of -85mV. **A.** Currents recorded after 10d in defined medium culture. **B.** currents recorded after 10d in the presence of 200ng/ml NGF (note larger amplitude compared to A), lower trace is voltage recording from neuron illustrated in upper trace. **C.** Current-density-voltage relationship for cells cultured with (n=35) and without NGF (n=28), error bars on all graphs = SEM. **D.** Family of $I_{Na}$’s recorded after 10d in the continued presence of the PI3kinase inhibitor, wortmannin (1μM). **E.** Currents recorded after 10 d in the presence of 200ng/ml NGF plus wortmannin (note similarity in amplitude compared to D). **F.** Current-density-voltage relationships for cells cultured with wortmannin with (n=25) and without NGF (n=32). **G.** Current-density-voltage relationships for cells cultured with the PI3kinase inhibitor LY294002 with (n=26) and without NGF (n=27). Calibration bars in **B.** refer to all current traces.

**Figure 6 Scheme to illustrate proposed mechanisms for NGF and LHRH regulation of $Na^+$ and $Ca^{2+}$ Channels**
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Figure 1

A  6d Culture

D  PD 98059

F  4α Phorbol

B  6d LHRH

E  PD 98059 + LHRH

G  PMA

C  

\[ I_{\text{m}} \text{Density (pA/pF)} \]

\[ \text{Command Potential (mV)} \]

-90mV -40mV
Figure 5

A 10d Culture

B 10d NGF

C

D 10d Wortmannin

E 10d Wortmannin + NGF

F

G