Neurotrophic Regulation of Calcium Channels by the Peptide Neurotransmitter Luteinizing Hormone Releasing Hormone

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We exploited the simple organization of bullfrog paravertebral sympathetic ganglia (BFSG) to test whether the neurotransmitter peptide luteinizing hormone releasing hormone (LHRH), which generates the late slow EPSP, could also exert long-term neurotrophic control of ion channel expression. Whole-cell recordings from B-cells in BFSG showed that removal of all of the sources of ganglionic LHRH for 10 d by cutting preganglionic C-fibers *in vivo* caused a 28% reduction in Ca^{2+} current density. When BFSG B-neurons were dissociated from adult bullfrogs and maintained in a defined-medium, neuron-enriched, low-density, serum-free culture, the I_{Ca} density was increased by 49% after 6–7 d in the presence of $0.45 \mu\text{M}$ LHRH. This increase was not associated with alterations in the voltage dependence of Ca^{2+} current activation or inactivation and reflected a selective increase in N-type Ca^{2+} channel current. The increase in I_{Ca} density induced by LHRH was blocked by the transcription inhibitor actinomycin D. These results suggest that chronic exposure to a neurotransmitter that acts through G-protein-coupled receptors exerts long-term control of ion channel expression in a fully differentiated, adult sympathetic neuron *in vitro* or *in vivo*.

Key words: gonadotropin hormone releasing hormone; Ras; MAP kinase; protein kinase C; sympathetic; autonomic; G-protein-coupled receptor; GPCR; G_q

Introduction

The short-term neuromodulatory effects of neuropeptides that act through G-protein-coupled receptors (GPCRs) are very well documented (Ikeda and Dunlap, 1999). Although neuropeptides can also alter the expression of various neuronal proteins over periods of days or weeks (McKeon and Zigmond, 1993; Mohny and Zigmond, 1998; Drahushuk et al., 2002; Hamelink et al., 2002), less is known about the cellular mechanisms and physiological consequences of these slower, neurotrophic actions. We exploited the unique properties of bullfrog sympathetic ganglia (BFSG) to examine possible neurotrophic effects of the ganglionic neurotransmitter luteinizing hormone releasing hormone (LHRH).

The larger B-cells of BFSG, which project to mucous glands in the skin, receive synaptic input from preganglionic B-fibers, whereas the smaller C-cells, which project primarily to blood vessels (Horn et al., 1988), receive synaptic input from preganglionic C-fibers (Horn and Stofer, 1988; Jobling and Horn, 1996). Although LHRH is released exclusively from preganglionic C-fibers (Jan et al., 1979, 1980a,b; Jones, 1987a; Troskie et al., 1997), it induces a late-slow EPSP in both C- and B-neurons (Adams et al., 1982; Jones, 1987c). This is because it diffuses from preganglionic C-fibers to G-protein-coupled LHRH receptors on

B-cells (Jan et al., 1979, 1980a,b; Jobling and Horn, 1996). There is tonic release of LHRH from C-fibers *in vivo*, and this may mediate an ongoing physiological interaction between the vasomotor C-fiber and the exocrine B-cell system (Ivanoff and Smith, 1995; Ford et al., 2000).

LHRH also acts through a GPCR to stimulate mitogen-activated protein kinase (MAPK) [p42/44MAPK; ERK1/2 (extracellular signal-regulated kinase 1/2)] activity in mammalian gonadotropes and in GH_3 cells transfected with rat gonadotropin releasing hormone (GnRH) receptor (Sim et al., 1995; Sundaresan et al., 1996; Reiss et al., 1997; Han and Conn, 1999). Because Ca^{2+} currents in BFSG are increased by several days exposure to nerve growth factor (NGF) (Lei et al., 1997) in a Ras- and MAPK-dependent manner (Lei et al., 1998), we tested whether LHRH is also capable of long-term, neurotrophic regulation of Ca^{2+} channels in BFSG.

Because individual sympathetic neurons from adult bullfrogs remain viable for up to 2 weeks in a serum-free, low-density, defined-medium culture, this system provides an opportunity to study potential neurotrophic actions of neurotransmitters without the complicating effects of exogenously applied or endogenous neurotrophins (Lei et al., 1997). We found that 6–7 d exposure of cultured BFSG B-neurons to LHRH promoted a significant increase in N-type Ca^{2+} channels current ($I_{\text{Ca,N}}$). This effect was prevented by the transcription inhibitor actinomycin D. Moreover, removal of all of the sources of ganglionic LHRH *in vivo* resulted in a decrease in $I_{\text{Ca,N}}$. Thus, in addition to its action as a slow neurotransmitter (Jan et al., 1979), LHRH exerts long-term, neurotrophic control of Ca^{2+} channels in a sympathetic ganglion. Some of the results have appeared in abstract form (Ford and Smith, 1999; Ford and Smith, 2000).

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Materials and Methods

Animals 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 Welfare Committee of the University of Alberta.

Tissue culture. Isolation, dissociation, and culture of BFSG neurons were performed under aseptic conditions as described by Lei et al. (1997, 1998). Neurons were dissociated by incubation with trypsin (Sigma, St. Louis, MO) and type 1A collagenase (Sigma) for 42–45 min at 37°C. Final dissociation was accomplished by trituration with a 1 ml Pasteur pipette. Cells were suspended in 3 ml of serum-free, modified L-15 medium [73% L-15 (Invitrogen, San Diego, CA), 10 mM glucose, 1 mM CaCl_2 , 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 10 μM cytosine arabinoside, pH 7.2]. The dissociated cells were then redistributed into 2.5 ml of medium in each of 20 35 mm polystyrene tissue culture dishes (Nunc, Naperville, IL). Dishes were placed in a light-proof, humidified chamber and maintained at room temperature (22°C) for 6–7 d, unless otherwise stated.

In vivo experiments. The tonic influence of LHRH *in vivo* was prevented by cutting preganglionic C-fibers, which are the sole source of LHRH in BFSG (Jan et al., 1979, 1980a,b; Troskie et al., 1997). Bullfrogs were anesthetized by injecting a 0.01% solution of tricaine methane sulfonate (MS-222) into the dorsal lymph sac (≤ 1 ml). Under aseptic conditions, a rostral–caudal incision was made through the ventral skin and body wall. Internal organs were held aside, and the rami communicantes leading from both spinal nerves VII and VIII were cut on one side of the animal. This selectively destroys preganglionic C-fibers, which contain LHRH, without affecting B-fibers. Thus, normal cholinergic transmission from preganglionic B-fibers to B-cells is maintained. A 1–2 mm section of nerve was removed to prevent reinnervation. The C-fibers on the contralateral side were left intact, and this side served as a control for the cut side. The incision through the body wall and skin was sutured, and the animal was allowed to recover. Ten days postsurgery, the bullfrog was killed by pithing, and ganglia VIII–X were removed separately from each side. B-neurons from both the cut C-fiber side and contralateral side were enzymatically dissociated and plated in two sets of polystyrene tissue culture dishes (see above) for electrophysiological investigation.

Electrophysiology. Discontinuous, single-electrode, voltage-clamp methods (Axoclamp 2A; Axon Instruments, Foster City, CA) were used to record whole-cell Ca^{2+} channel currents (Sala, 1991). As described previously, Ba^{2+} was used as the charge carrier to monitor Ca^{2+} channel activity (I_{Ba}) (Lei et al., 1997). Data were acquired with a TL-1 DMA interface acquisition system operating under software control (pClamp 5.0; Axon Instruments) and filtered to -3 dB at 3 kHz. For recordings, medium was exchanged with external solution containing (in mM): 117.5 *N*-methyl-D-glucamine (NMG) chloride, 2.5 NMG-HEPES, and 2.0 BaCl_2 , pH 7.2. The flow rate was set so that complete exchange of medium for external Ba^{2+} solution was accomplished in 1–2 min. Fire-polished electrodes that had 4–6 M Ω DC resistance (as measured in external solution) were used. With this combination of discontinuous clamp and low-resistance electrodes, series resistance errors are negligible as long as the electrode voltage settles at the end of each cycle (Sala, 1991). Electrode settling was monitored on a separate oscilloscope. Using these methods, we could obtain switching frequencies of 35–45 kHz. Electrodes were filled with internal solution consisting of (in mM): 76.5 NMG-chloride, 2.5 HEPES, 10 BAPTA (brought to pH 7.2 with Tris), 5 Tris-ATP, and 4 MgCl_2 , pH 7.2. In cases in which the acute effects of LHRH on I_{Ba} were examined, 0.4 mM GTP was added to the internal solution.

Whole-cell recordings were first made in bridge-balance, current-clamp mode, before switching to single-electrode, discontinuous voltage-clamp mode for the study of I_{Ba} . The holding potential (V_h) was -90 mV, unless otherwise stated. Input capacitance was used as a measure of cell size. Cells with input capacitance (C_{in}) of >30 pF were selected for recordings. Because C_{in} of BFSG C-cells is <20 pF, it is assumed that all of the recordings were made from B-cells. C_{in} was calculated by integrating the capacitive transient induced by a 10 mV depolarizing step from -90 mV. Peak current measurements were subtracted using a P/4

protocol. Illustrated traces were filtered digitally at 1 kHz *post hoc* and presented using MicroCal (Northampton, MA) Origin 6.1 or 7 software.

Electrophysiological recordings were made from acutely dissociated BFSG B-neurons (acute), or from neurons cultured for 6–7 d in either the absence (6 d culture) or presence of 0.45 μM LHRH (6 d LHRH). This culture time was selected, because the effect of LHRH on I_{Ba} was significant at that time (see Fig. 2). In experiments in which the acute effects of LHRH or noradrenaline (NA) on I_{Ba} were examined, agonists were bath applied via a perfusion system (2 ml/min), which allowed complete exchange of solutions within 1–2 min (as measured by maximal I_{Ba} suppression). Unless otherwise stated, all of the values reported are peak current density (peak I_{Ba} ; recorded at -10 mV; normalized to cell size; C_{in}).

Chemicals. LHRH (chicken-II GnRH) was purchased from Peninsula Labs (Belmont, CA). Leibovitz's L-15 medium and penicillin–streptomycin antibiotics were from Invitrogen. Actinomycin D was from Biomol (Plymouth Meeting, PA). ω -Conotoxin-GVIA (ω -CgTX) was from Alomone Labs (Jerusalem, Israel). Nifedipine was from Tocris (Ballwin, MO). All of the other chemicals were from Sigma. Culture medium was changed daily to maintain effective concentrations of LHRH. Unless otherwise stated, all of the data are presented as means $\pm$ SEMs. Groups of data were considered significantly different when $p < 0.05$ using Student's unpaired, two-tailed *t* test or one-way ANOVA with Student–Newman–Keuls test for *post hoc* comparison.

Results

Neurotrophic effects of LHRH on Ca^{2+} channels

Figure 1 illustrates typical recordings of I_{Ba} from an acutely dissociated BFSG B-cell, a B-cell maintained in culture for 6 d, and a third B-cell maintained in culture for 6 d in the presence of LHRH (0.45 μM). Cells were held at -90 mV, and currents were evoked by a series of incremental 10 mV depolarizing steps; tail currents were recorded at -40 mV to slow their kinetics and facilitate their analysis. Because Ca^{2+} currents in BFSG neurons can be as large as 20 nA, we favor the use of discontinuous voltage clamp, which allows the cells to be clamped to the recorded membrane voltage (Jones, 1987b; Sala, 1991). Moreover, a record of membrane voltage, as opposed to command voltage, is available (Fig. 1C), so that gross inadequacies in voltage control can be avoided. This method worked well for acutely isolated cells. However, in cultured cells that had started to produce neurites, slowly activating currents were sometimes seen at certain voltages. These may reflect poor voltage control of distal Ca^{2+} channels situated on neuronal processes (Fig. 1C). Apart from aberrations of this type, most cultured and acutely isolated cells exhibited a steady increase in current with increasing voltage commands. Data were not collected from the few cells that exhibited all-or-none currents (unclamped Ca^{2+} spikes).

Six days of culture itself was associated with an increase in I_{Ba} compared with that recorded from acutely dissociated cells. An additional, substantial increase in I_{Ba} amplitude was seen in cells that had been cultured for 6 d with 0.45 μM LHRH. These effects are illustrated in Figure 1 and are further quantified in Figure 2. Acutely dissociated cells exhibited 3.9 ± 0.3 nA peak I_{Ba} at -10 mV ($n = 31$) (Fig. 2A), whereas cells maintained in serum-free, defined-medium culture for 6 d, exhibited a larger current of 9.1 ± 0.4 nA ($n = 49$) ($p < 0.05$) (Fig. 2A). This finding confirms the previous observation that a transient increase in peak current occurs during the first 3–6 d of culture (Lei et al., 1997). After BFSG B-cells have been maintained in culture for 15 d, the total I_{Ba} current seen was similar to that of acutely dissociated cells (Lei et al., 1997). Inclusion of 0.45 μM LHRH in the culture medium significantly increased the peak total I_{Ba} of 6 d cultured cells to 11.7 ± 0.7 nA ($n = 35$). This was significantly greater than the current seen in cells maintained in culture in the absence of LHRH ($p < 0.05$) (Fig. 2A).

Figure 1. Typical Ca^{2+} channel currents (I_{Ba}) recorded from an acutely dissociated BFGS B-cell (A), a B-cell cultured for 6 d in control media without LHRH (B), and a B-cell cultured for 6 d in control media with LHRH (C). Neurons were kept at a holding potential of -90 mV. Families of Ba^{2+} currents were evoked by a series of depolarizing voltage commands (pulse length, 30 msec; 10 mV per step); tail currents were recorded at -40 mV. Voltage recording shown is associated with I_{Ba} of the cell cultured for 6 d in control media.

As previously noted (Lei et al., 1997), B-cells maintained in culture for >2 d began to sprout processes. This coincided with an increase in input capacitance (C_{in}). After 6 d in culture, there was a greater than twofold increase in C_{in} ($p < 0.05$) (Fig. 2B). Inclusion of $0.45 \mu\text{M}$ LHRH did not appear to affect the growth and production of neurites or the growth of somata, because no significant difference in cell capacitance was measured ($p > 0.05$) (Fig. 2B).

The effect of LHRH on peak current density (peak I_{Ba} ; normalized to cell size; C_{in}) was examined. Current density takes into account the variability of cell size, because the size of the neuron may be proportional to the size of its current. Culturing B-cells for 6 d had no effect on I_{Ba} peak current density ($p > 0.05$) (Fig. 2C). The increase in peak I_{Ba} (Fig. 2A) was balanced by the increase in cell size, so that there was no appreciable change in density. However, inclusion of $0.45 \mu\text{M}$ LHRH in the culture medium increased the peak current density ($175.4 \pm 16.4 \text{ pA/pF}$; $n = 35$) by 49% compared with cells cultured in the absence of LHRH ($117.7 \pm 6.8 \text{ pA/pF}$; $n = 49$; $p < 0.05$) and by 75% compared with acutely dissociated cells ($100.6 \pm 7.3 \text{ pA/pF}$; $n = 31$; $p < 0.05$) (Fig. 2C).

Figure 2. Quantified effect of LHRH on I_{Ba} . A–C, Peak (total) current, current density, and input capacitance (C_{in}) recorded from acutely dissociated cells and from cells maintained in culture for 6 d in the presence or absence of LHRH. Asterisks indicate significance at the level of $p < 0.05$ between columns. D–F, Current–density voltage plots for I_{Ba} from acutely dissociated, control-cultured, and LHRH-cultured neurons. Three different holding potentials were used (V_h , -90 , -60 , and -40 mV). For all of the data points shown, $n > 20$. Error bars indicating SEM are not visible when error is less than the symbol used to designate the data point.

LHRH does not alter voltage dependence of activation or inactivation of Ca^{2+} channels

Figure 2, D–F, illustrates the I_{Ba} density–voltage relationships for acutely dissociated cells and for cells cultured for 6 d in the absence or presence of $0.45 \mu\text{M}$ LHRH. Neurons were held at V_h of -90 , -60 , and -40 mV. The peak current for all three groups occurred at -10 mV, which implies that the manipulations had little if any effect on the voltage dependence of activation [the voltage dependence of Ba^{2+} conductance (g_{Ba}) activation]. This was confirmed by analyzing the voltage dependence of current activation obtained from tail current amplitudes (Fig. 3A). No differences were observed between the normalized activation curves for acutely dissociated neurons, control neurons cultured for 6 d, and LHRH-treated, 6 d cultured neurons (Fig. 3A). All of the test conditions had curves that well matched the activation curve fitted with Boltzmann kinetics (Fig. 3A).

Changing the V_h from -90 to -40 mV produced a similar reduction in the peak I_{Ba} density for acute, 6 d cultured, and 6 d cultured in the presence of LHRH cells (Fig. 2D–F), suggesting that there was no alteration in the steady-state inactivation of I_{Ba} . This was also reflected in the isochronic-inactivation curves (Fig. 3B). For 4 sec before the test pulse to -10 mV, cells were held at a range of prepulse potentials from -105 to -30 mV. The test pulse current (normalized as g/g_{max}) was plotted against the holding potential (V_h) of the prepulse. This does not yield a true h_∞ plot, because the removal of inactivation of g_{Ca} is very slow (Jones

A. Voltage-Dependence of Activation

B. Voltage-Dependence of Inactivation

Figure 3. Effects of LHRH on voltage dependence of I_{Ba} activation and inactivation. *A*, Activation curves for normalized g_{Ba} for acutely dissociated cells and cells cultured in the presence or absence of LHRH. I_{Ba} was evoked by a series of 20 msec command potentials. Tail currents at the end of each pulse were recorded at -40 mV. Tail current amplitudes were estimated by fitting the data to a monoexponential function and extrapolating the amplitude at zero time. The solid line represents a plot fitted to a Boltzmann equation: $g/g_{max} = 1/[1 + \exp((V_{1/2} - V)/V_s)]^2$, where $V_{1/2}$ is the midpoint voltage (-4.7 mV) and V_s is the steepness factor (10.5 mV) (Sala 1991). *B*, Normalized isochronic-inactivation curves for the three experimental conditions. I_{Ba} was evoked at 0 mV after holding cells at various potentials for 4 sec. In all of the data for both figures, $n > 20$. Error bars indicating SEM are not visible when error is less than the symbol used to designate the data point.

and Marks, 1989), so that a steady state may not have been attained with a 4 sec prepulse. There was no difference between the normalized inactivation curves for 6 d cultured cells in the presence or absence of LHRH (Fig. 3*B*). Compared with acutely dissociated cells, the isochronic-inactivation curves for 6 d cultured cells both with and without LHRH were shifted to the left (Fig. 3*B*). The effect of LHRH on voltage dependence of g_{Ba} inactivation thus differs from that of NGF, because NGF induced a rightward shift in the curve so as to restore the voltage dependence of Ca^{2+} channel inactivation to that of acutely isolated neurons (Lei et al., 1997).

Long-term exposure to LHRH therefore caused an increase in the total amount of I_{Ba}/g_{Ba} without altering either activation or inactivation and without affecting cell size. Increases in conductance, in the absence of changes in inactivation, would be consistent with an increase in the total number of functional Ca^{2+} channels available.

Functional LHRH receptors present at cell surface after 6 d culture with LHRH

Prolonged exposure of BFGS B-neurons to LHRH throughout the 6 d time course of our culture experiments may promote desensitization, internalization, and/or alterations in expression

of functional LHRH receptors or the cell membrane. We therefore used the acute, inhibitory effects of LHRH on I_{Ba} (Elmslie et al., 1990) to assess the level of functional LHRH receptors during the course of our experiments. Cells were examined 18 – 24 hr after the last daily replenishment on medium containing 450 nM LHRH.

Cells maintained in the presence of 450 nM LHRH for 6 d showed significantly reduced I_{Ba} suppression by acutely applied 1 μ M LHRH. A suppression of $36.2 \pm 3.8\%$ ($n = 19$) was seen compared with control cultured cells in which a $48.2 \pm 5.0\%$ suppression was seen ($p < 0.05$) (data not shown). This implies that functional LHRH receptors persist on BFGS neurons throughout the 6 – 7 d period of chronic peptide exposure. The reduced responsiveness may imply some desensitization, downregulation, or internalization of LHRH receptors per se or some general downregulation of G-protein-coupled receptor signaling. To distinguish between these possibilities, we compared the effect of NA on I_{Ba} in 6 – 7 d control cultured neurons with its effect on I_{Ba} in neurons that had been chronically exposed to 450 nM LHRH. There was no significant difference in the amount of I_{Ba} suppression induced by 100 μ M NA in control cells ($33.4 \pm 1.6\%$; $n = 6$) compared with LHRH-treated cells ($32 \pm 2.3\%$; $n = 6$; $p > 0.6$). The reduced responsiveness to LHRH during chronic exposure may therefore reflect alterations in LHRH receptor expression or function rather than changes in G-protein signaling.

Regulation of N- and L-type Ca^{2+} channels by LHRH

In BFGS, N-type channels carry $\sim 90\%$ of the total I_{Ca} , whereas L-type channels carry most of the remaining amount (Jones and Elmslie, 1992). Studies in pheochromocytoma (PC12) cells and in BFGS neurons have shown that the nifedipine-sensitive L-type Ca^{2+} current ($I_{Ca,L}$) and the ω -conotoxin GVIA-sensitive N-type Ca^{2+} current ($I_{Ca,N}$) are differentially regulated by NGF (Lewis et al., 1993; Lei et al., 1997). We therefore compared the pharmacology of I_{Ba} recorded from 6 d cultured and 6 d LHRH-treated cells to see whether LHRH was also capable of differentially regulating these two types of Ca^{2+} channels in BFGS. Currents were generated once every 60 sec by voltage steps to 0 mV (from V_h of -90 mV). Cells cultured for 6 – 7 d with or without LHRH were first exposed to ω -CgTX (300 nM) for 45 min to allow for full inhibition of N-type Ca^{2+} channels to develop. This was followed by 15 min exposure to 1 μ M nifedipine. The contribution of L- and N-type Ca^{2+} channels to total I_{Ba} was determined as the amount of total current density suppressed by ω -CgTX or nifedipine in individual cells. The current density that remained after ω -CgTX and nifedipine treatment was defined as $I_{Ba,other}$. In cultured cells ($n = 9$), the ω -CgTX-sensitive current density was 45.0 ± 8.8 pA/pF ($67.4 \pm 6.9\%$); 11.25 ± 3.8 pA/pF ($20 \pm 5.5\%$) was nifedipine sensitive, and 7.5 ± 1.3 pA/pF ($12.6 \pm 3.6\%$) was ω -CgTX and nifedipine resistant (Fig. 4). In LHRH-treated cells ($n = 10$), 67.5 ± 6.5 pA/pF ($85 \pm 3.5\%$) of the current density was ω -CgTX sensitive, 5.0 ± 1.3 pA/pF ($6.7 \pm 2.6\%$) was nifedipine sensitive, and 6.3 ± 1.3 pA/pF ($8.2 \pm 1.6\%$) was ω -CgTX and nifedipine resistant (Fig. 4). LHRH thus induced a significant increase in the density of $I_{Ba,N}$ ($p < 0.05$), whereas $I_{Ba,L}$ ($p > 0.1$) and $I_{Ba,other}$ ($p > 0.4$) were unchanged. Although NGF also increases $I_{Ba,N}$ in BFGS neurons, its effect differs from that of LHRH, because NGF also produces a large increase in $I_{Ba,L}$ (Lei et al., 1997).

Cut C-fibers in vivo

We next examined whether the effects of LHRH that we observed *in vitro* could also be seen *in vivo*. As mentioned above, pregan-

Figure 4. Densities of ω -Conotoxin GVIA-sensitive $I_{Ba,N}$ (N-type), nifedipine-sensitive $I_{Ba,L}$ (L-type), and ω -Conotoxin GVIA/nifedipine-resistant $I_{Ba,other}$ (Other) in cells cultured for 6 d in the presence ($n = 10$) or absence ($n = 9$) of 450 nM LHRH. ω -Conotoxin GVIA (300 nM) was applied initially for 45 min to allow for full $I_{Ba,N}$ suppression to occur, before 15 min application of 1 μ M nifedipine. Error bars indicate SEM. Asterisk designates $p < 0.05$, compared with 6 d culture without LHRH.

glionic B-fibers emerge from the CNS in spinal nerves IV, V, and VI, whereas preganglionic C-fibers emerge in spinal nerves VII and VIII (Horn and Stofer, 1988). By selectively cutting the rami communicantes connecting spinal nerves VII and VIII with their associated ganglia, it was possible to interrupt C-fiber transmission selectively while leaving B-cell–B-fiber cholinergic transmission intact (Fig. 5*A,B*). This manipulation prevents the tonic release of LHRH from C-fiber terminals, because it is known that 5 d after denervating preganglionic C-fibers, 95% of ganglionic LHRH is removed (Jan et al., 1979). Acutely dissociated B-neurons from BFSG that had preganglionic C-fibers cut for 10 d exhibited peak I_{Ba} density of 127.4 ± 10.3 pA/pF ($n = 20$). Recordings from a representative cell are illustrated in Figure 5*C*. The current density was significantly less than the current density seen in B-cells derived from the contralateral control side of the same animals (176.2 ± 22.3 pA/pF; $n = 18$; $p < 0.05$) (Fig. 5*D,E*). Thus, I_{Ba} density is 38% larger in cells in which the preganglionic C-fibers are intact. In spite of changes seen in current density, cutting C-fibers had no effect on either the voltage dependence of g_{Ba} activation or inactivation (data not shown).

LHRH-induced changes are transcription dependent

The DNA transcription inhibitor actinomycin D (0.02 μ g/ml) was used to test whether LHRH-induced changes in I_{Ba} involved an alteration in gene expression. The effect of this concentration on the initial increases in I_{Ba} amplitude and cell size (C_{in}) seen after 6 d in culture was not significant. Thus, I_{Ba} in acutely dissociated cells was 3.9 ± 0.3 nA ($n = 35$), and this increased to 9.1 ± 0.4 nA after 6 d in culture (Figs. 1*B* and 2*A*) and to only 7.6 ± 0.4 nA in the presence of actinomycin D ($n = 14$; $p < 0.095$, compared with 6 d cultures). C_{in} of acutely dissociated cells was 41.2 ± 3.0 pF ($n = 35$). This increased to 85.1 ± 3.6 pF ($n = 49$) in 6 d cultures (Fig. 2*B*) but to only 72.8 ± 4.8 pF in cells cultured for 6 d with actinomycin D ($n = 14$; $p < 0.06$, compared with control 6 d cultures). I_{Ba} density was unchanged. Thus, I_{Ba} density after 6 d in actinomycin was 108.8 ± 7.1 nA/pF ($n = 14$) compared with 117.7 ± 6.8 ($n = 49$) in 6 d cultured cells ($p > 0.5$). Actinomycin D did, however, prevent the LHRH-induced increase in current density. Accordingly, the current density of BFSG B-neurons recorded in the presence of actinomycin D for 6 d (108.8 ± 7.1 nA/pF; $n = 14$) was indistinguishable from that of neurons recorded in the presence of actinomycin D plus LHRH for 6 d (94.9 ± 7.3 nA/pF; $n = 23$; $p > 0.15$). This suggests that LHRH induces alterations in the level of functional Ca^{2+}

Figure 5. Cutting preganglionic C-fiber axons to prevent the release of LHRH at C-fiber terminals reduces B-cell I_{Ba} . *A,B*, Diagrams of experimental situations. *C*, Typical Ba^{2+} currents recorded at V_h of -90 mV, from an acutely dissociated B-cell derived from a ganglion in which the preganglionic C-fibers had been cut 10 d before. *D*, Typical control Ba^{2+} currents recorded from an acutely dissociated B-cell derived from a ganglion that had the preganglionic C-fibers left intact. *E*, Peak I_{Ba} density–voltage relationships for B-neurons derived from ganglia in which the preganglionic C-fibers had been either cut 10 d prior or left intact; $n \geq 17$ for both groups. Error bars indicating SEM are not visible when error is less than the symbol used to designate the data point.

channels via signaling through the nucleus to alter gene expression.

Discussion

Our primary finding is that long-term application of the neuropeptide transmitter LHRH upregulates Ca^{2+} channel density in fully differentiated, adult sympathetic neurons both *in vitro* and *in vivo*. We therefore propose that, in addition to its previously known role as a ganglionic neurotransmitter, LHRH also functions in a neurotrophic manner to regulate the functional expression of Ca^{2+} channels in adult BFSG.

Regulation of I_{Ba} by LHRH

LHRH selectively increases $I_{Ba,N}$ density without any significant effect on $I_{Ba,L}$ or $I_{Ba,other}$ (Fig. 4). Because $I_{Ba,N}$ is the dominant contributor to total I_{Ba} in BFSG (Jones and Elmslie, 1992), total current density increased (Fig. 2*C*). The result with actinomycin D shows that gene transcription is required for this effect. Because it had no effect on the voltage dependence of activation or inactivation, LHRH is unlikely to have affected the expression of Ca^{2+} channel β subunits or other proteins that may affect channel properties (Varadi et al., 1991). It is tempting to speculate, therefore, that LHRH increased the functional expression of (N-type Ca^{2+} channel) $\alpha 1B$ subunits. It is, of course, possible that

the peptide promoted expression of N-type Ca^{2+} channels with greater single-channel conductance. This possibility remains to be investigated.

Comparison of actions of NGF and LHRH

The long-term effect of NGF on cultured adult BFGS B-neurons involves an increase in both N- and L-type I_{Ba} ($I_{\text{Ba,N}}$ and $I_{\text{Ba,L}}$) and a decrease in inactivation of the total Ba^{2+} conductance (Lei et al., 1997). These effects likely involve the Ras–MAPK pathway (Lei et al., 1998). Although we have preliminary data that the LHRH-induced increase in g_{Ca} is prevented by inhibitors of the Ras–MAPK pathway (Ford and Smith, 2000), its effects differ from those of NGF. Although there is also an increase in the total g_{Ba} and an increase in $I_{\text{Ba,N}}$ with LHRH, there is no decrease in inactivation of the total conductance, and LHRH does not increase $I_{\text{Ba,L}}$. The causes of these differences and the details of the LHRH transduction mechanism thus remain to be elucidated.

Although LHRH and NGF both upregulate Ca^{2+} channels, the action of LHRH does not seem to be a consequence of improved health of cultured B-cells. Cells cultured in either the presence or absence of LHRH showed no obvious morphological differences, and there were no differences in the extent or dimensions of axonal sprouting (data not shown). This is similar to the effect of other neurotrophins that modulate ion channels without the initiation of a growth response (Levine et al., 1995; Lei et al., 1997). Furthermore, the effects of LHRH in upregulating I_{Ba} density appear to be specific, because Na^+ conductance is not altered by culturing BFGS B-cells in the presence of LHRH (Lu et al., 2002).

Physiological significance

Previous reports have shown that various growth factors and the activation of MAPK is important for the regulation of neuronal ion channels (Huang and Rane, 1994; Levine et al., 1995; Pollock and Rane, 1996; Fitzgerald and Dolphin, 1997; Lei et al., 1997, 2001; Fitzgerald, 2000). However, we believe that we are the first to report that a neurotransmitter that likely acts via a G-protein can regulate the expression of Ca^{2+} channels both *in vivo* and *in vitro*. Selectively cutting preganglionic C-fibers reduced the I_{Ba} density by ~28%. This procedure selectively removes nearly all of the ganglionic LHRH (Jan et al., 1979), without altering cholinergic B-fiber–B-cell transmission. In BFGS, LHRH is the only known transmitter able to diffuse from C-fibers to affect B-cells. Thus, we believe that the only effect of cutting preganglionic C-fibers on B-cells was the interruption of LHRH transmission. This implies that the reduction in I_{Ba} was a consequence of the loss of LHRH.

Whereas NGF upregulation of Ca^{2+} channel expression in BFGS persists as long as target-derived neurotrophin is available, the neurotrophic effect of LHRH is more labile, because it depends on peptide release and hence neuronal activity in preganglionic nerve fibers. Because neuropeptide release is favored by intense neuronal activity (Peng and Horn, 1991), regulation of Ca^{2+} channels by LHRH may couple preganglionic activity to alterations in the electrical properties of postganglionic cells. The release of neuropeptides from preganglionic fibers also causes long-term increases in tyrosine hydroxylase activity and noradrenaline synthesis (McKeon and Zigmond, 1993). Because of the strong correlation between Ca^{2+} influx and neurotransmitter release, increased Ca^{2+} conductance at sympathetic postganglionic terminals may augment sympathetic outflow to target tissues. This effect may be relevant to understanding disease processes, such as hypertension and congestive heart failure, both of

which involve increases in sympathetic outflow to blood vessels, the heart, and other visceral organs.

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