Cellular/Molecular

Nerve Growth Factor Sensitizes Adult Sympathetic Neurons to the Proinflammatory Peptide Bradykinin

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Levels of nerve growth factor (NGF) are elevated in inflamed tissues. In sensory neurons, increases in NGF augment neuronal sensitivity (sensitization) to noxious stimuli. Here, we hypothesized that NGF also sensitizes sympathetic neurons to proinflammatory stimuli. We cultured superior cervical ganglion (SCG) neurons from adult male Sprague Dawley rats with or without added NGF and compared their responsiveness to bradykinin, a proinflammatory peptide. The NGF-cultured neurons exhibited significant depolarization, bursts of action potentials, and Ca^{2+} elevations after bradykinin application, whereas neurons cultured without NGF showed only slight changes in membrane potential and cytoplasmic Ca^{2+} levels. The NGF effect, which requires trkA receptors, takes hours to develop and days to reverse. We addressed the ionic mechanisms underlying this sensitization. NGF did not alter bradykinin-induced M-current inhibition or phosphatidylinositol 4,5-bisphosphate hydrolysis. Maxi-K channel-mediated current evoked by depolarizations was reduced by 50% by culturing neurons in NGF. Application of iberiotoxin or paxilline, blockers of Maxi-K channels, mimicked NGF treatment and sensitized neurons to bradykinin application. A calcium channel blocker also mimicked NGF treatment. We found that NGF reduces Maxi-K channel opening by decreasing the activity of nifedipine-sensitive calcium channels. In conclusion, culture in NGF reduces the activity of L-type calcium channels, and secondarily, the calcium-sensitive activity of Maxi-K channels, rendering sympathetic neurons electrically hyper-responsive to bradykinin.

Key words: **BK channel; M-current; Maxi-K channel; nifedipine-sensitive calcium channel; slo1 channel; superior cervical ganglion neurons**

Introduction

Here we describe the effect of chronically culturing adult sympathetic neurons with nerve growth factor (NGF). NGF was discovered as a secreted protein essential for the survival of developing sympathetic and peripheral sensory neurons (Levi-Montalcini and Booker, 1960a, b). A few weeks after birth, neurons lose this dependence on NGF for survival (Orike et al., 2001; Hefti et al., 2006), but they still express receptors for NGF (Reinhardt et al., 1994), suggesting that NGF plays other roles in adult neurons. Interestingly, increased NGF levels have been detected in adult animal models for disease conditions, such as pancreatitis, chronic headache, and peripheral neuropathies (Toma et al., 2000; Sarchielli et al., 2001; Peleshok and Ribeiro-da-Silva, 2012), which raises the question what role(s) NGF plays under such conditions.

One of the better understood effects of NGF in adulthood is sensitization of nociceptive neurons during inflammation, which leads to hyperalgesia. For example, thermal hyperalgesia can be mimicked by administration of a single dose of NGF (Lewin et al., 1994), and blocking the actions of NGF by injecting anti-NGF antibodies reduces hypersensitivity induced by inflammation (McMahon et al., 1995; Dmitrieva et al., 1997; Zahn et al., 2004; Ugolini et al., 2007). NGF-neutralizing antibodies are being assessed in clinical trials against chronic pain (Kumar and Mahal, 2012). On the cellular level, NGF regulates expression of several genes in nociceptive neurons and thereby increases the expression of proinflammatory molecules, such as substance P and calcitonin-gene related peptide (Lindsay and Harmar, 1989; Donnerer et al., 1992). In addition, NGF increases the excitability of nociceptive neurons by increasing the expression and trafficking of ion channels, such as TRPV1 (Zhang et al., 2005; Stein et al., 2006) and voltage-gated sodium channels (Friedel et al., 1997; Gould et al., 2000).

Adult sympathetic ganglion neurons also express NGF receptors and therefore can be affected by NGF during chronic inflammatory diseases (Peeker et al., 2000; Straub et al., 2006; Schnegelsberg et al., 2010; Longo et al., 2013). Here, we hypothesized that NGF sensitizes peripheral sympathetic neurons to proinflammatory molecules. One candidate is bradykinin (BK), a potent proinflammatory and hyperalgesic nonapeptide formed

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from plasma protein precursors after tissue injury. BK receptors are expressed in the sympathetic nervous system (Prado et al., 2002). BK can stimulate sympathetic neurons (Lewis and Reit, 1965) through activation of BK receptor Type 2 by closing K^+-permeable M-current channels (Jones et al., 1995). Here, we analyze how NGF alters the signaling pathway induced by BK in sympathetic neurons. We found a strong NGF-dependent sensitization of the electrical responses of sympathetic neurons to BK.

Materials and Methods

Culture of sympathetic neurons. Animals were handled according to guidelines approved by the University of Washington Institutional Animal Care and Use Committee. Neurons were isolated from superior cervical ganglia (SCG) of 7- to 12-week-old male Sprague Dawley rats by enzymatic digestion as described previously (Beech et al., 1991; Vivas et al., 2013). Isolated neurons were plated on poly-L-lysine (Sigma) coated glass chips and incubated in 5% CO_2 at 37°C in medium supplemented with 10% FBS. For many experiments, the cells were cultured in medium supplemented with either 1 ng/ml NGF (Invitrogen) or 2 μg/ml anti-NGF antibody (Abcam, catalog #ab6199, RRID:AB_2152414), but none of the recording solutions contained NGF or the anti-NGF antibody. For some experiments, the medium was supplemented with 200 nM K252a (LC Laboratories) in addition to NGF. Recordings were performed on neurons with a membrane capacitance between 50 and 100 pF.

Photometric recordings. Optical measurements of calcium and Förster resonance energy transfer (FRET) were performed on single cells with a grating-controlled monochromatic light source (Polychrome IV; TILL Photonics) by whole-cell photometry (not with images) as previously described (Falkenburger et al., 2010; Dickson et al., 2013). For measurements of cytoplasmic free Ca^{2+}, neurons were loaded for 40 min with 2 μM fura-2 AM (Invitrogen) dissolved in Ringer's solution containing 0.02% pluronic acid F-68, followed by a wash in Ringer's solution for 20 min. FRET was measured as the ratio of corrected fluorescence from YFP and CFP after excitation of CFP molecules and is reported as the fluorescence emission ratio FRETr, defined as YFP/CFP.

Electrophysiology. Electrophysiological recordings were made with an EPC9 patch-clamp amplifier (HEKA) using 4 MΩ patch pipettes. Membrane potentials were measured via current-clamp in the perforated-patch configuration with 200 μM amphotericin B (Sigma). The recording chamber was superfused at 2 ml/min with external solutions, permitting solution exchange with a time constant of 10 s. The bath solution (Ringer's solution) contained 150 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 8 mM glucose, adjusted with NaOH to pH 7.4. All agonists were applied for 20 s. Agonists and blockers were dissolved in Ringer's solution to obtain a final concentration of 250 nM BK, 10 μM oxotremorine methiodide (Oxo-M), 200 nM iberiotoxin (IbTX), 100 nM paxilline, 100 nM apamin, 200 nM TTX, 100 μM $CdCl_2$, or 5 μM nifedipine. For a nominally Ca^{2+}-free external solution, $CaCl_2$ was replaced by $MgCl_2$. The pipette solution contained 175 mM KCl, 5 mM $MgCl_2$, 5 mM HEPES, 0.1 mM K_4BAPTA, 3 mM Na_2ATP, and 0.1 mM Na_3GTP, adjusted with KOH to pH 7.2. Sometimes the Maxi-K channels were activated by clamping free intracellular Ca^{2+} to 10 μM, using a pipette solution containing 95 mM KCl, 20 mM K_4BAPTA, 19.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 3 mM Na_2ATP, and 0.1 mM Na_3GTP, adjusted with KOH to pH 7.2. Potassium currents (M-current and Ca^{2+}-activated K^+ currents) were recorded in whole-cell configuration with the described bath and pipette solutions. Series resistances of 6–10 MΩ were compensated by 50%–70%. Potassium currents were sampled at 2 kHz. M-current amplitude was measured from tail currents at −60 mV after a depolarizing voltage-step to −20 mV as the difference between the average current of the first 10–20 ms and the average current of the last 10 ms. Ca^{2+}-activated K^+ current was calculated by subtracting potassium currents recorded in the absence and presence of $CdCl_2$ or IbTX.

Single-channel currents were recorded in the excised inside-out patch-clamp configuration with symmetrical K^+ solutions, resulting in a potassium equilibrium potential of 0 mV. The bath solution mimicked the intracellular medium: 150 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM EGTA, 10 mM HEPES, adjusted with KOH to pH 7.3. The pipette solution contained 150 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 200 nM TTX, 10 mM HEPES, and was adjusted with KOH to pH 7.3. To calculate single-channel conductances, current amplitude histograms were constructed from recordings at test potentials of −80 mV and 80 mV and fitted with Gaussian curves to determine average current amplitudes for individual channel openings.

Transfection of sympathetic neurons. SCG neurons were transfected 18 h after isolation using an Eppendorf 5242 pressure microinjector and 5171 micromanipulator system (Eppendorf). cDNA was dissolved in 1 mg/ml dextran-fluorescein solution (10,000 kDa; Molecular Probes) to yield a final concentration of 50–150 ng/μl. cDNA was microinjected into the nucleus for 0.3 s at pressures of 80–120 hPa. Human eCFP-PH (phospholipase Cδ1 [PLCδ1]) and eYFP-PH (PLCδ1) plasmids were provided by K. Jalink (The Netherlands Cancer Institute, Amsterdam, Netherlands).

Data analysis. We used Igor Pro (Igor Software, RRID:nlx_156887) and Excel (Microsoft) to analyze data. Statistics are given as mean ± SEM. Student's *t* test was used to test for statistical significance. *p* values <0.05 were considered significant.

Results

NGF augments electrical responses of SCG neurons to BK stimulation

In this work, we cultured rat SCG neurons with or without NGF for 48 h. In both conditions, the neurons showed neurite outgrowth (Fig. 1*A*) and were able to fire action potentials upon stimulation. On average, there were more neurites with NGF. Culture with or without NGF did not affect the resting membrane potential (no NGF: −57.4 ± 0.9 mV, $n = 19$; +NGF: −55.8 ± 1.0 mV, $n = 20$; $p = 0.25$) or resting cytoplasmic Ca^{2+} levels (fura-2 ratio in cell cultured without NGF: 0.093 ± 0.002, $n = 18$; or with NGF: 0.096 ± 0.004, $n = 17$; $p = 0.55$).

BK directly excites sympathetic neurons of the rat and cat (Lewis and Reit, 1965; Jones et al., 1995). We asked whether incubation with NGF alters the electrical responses induced by BK. Rat SCG neurons cultured in the absence or presence of 1 ng/ml NGF for 48 h were studied with current-clamp recording in the perforated-patch configuration. In neurons cultured without NGF, application of 250 nM BK led to a small depolarization that evoked firing of action potentials in only 14% of cells ($n = 21$; Figs. 1*B* and 2*B*,*C*). In contrast, in neurons cultured for 48 h with NGF, BK elicited a significantly larger depolarization that evoked firing of action potentials in 80% of cells ($n = 25$; Figs. 1*C* and 2*B*,*C*). The following experiments suggest that sensitization can be seen even when most voltage-gated sodium channels were blocked by 200 nM TTX to preclude action potential firing. With TTX in the recording solution, BK application to cells cultured without NGF led again to only a small depolarization (3.5 ± 0.6 mV, $n = 5$; Fig. 1*D*), whereas BK application to cells cultured in NGF led to significantly larger depolarization (11.3 ± 1.1 mV, $n = 5$, $p = 0.002$; Fig. 1*E*).

NGF augments Ca^{2+} signals evoked by BK

SCG neurons show Ca^{2+} elevations in response to BK application. We asked whether incubation with NGF also alters these signals. The first experiments used no recording pipette or voltage control, and the cells were loaded with fura-2 AM as a Ca^{2+} indicator. In control cells cultured for 48 h in the absence of NGF, BK evoked small Ca^{2+} elevations (Fig. 1*F*,*G*, black line), as reported previously (Cruzblanca et al., 1998; Zaika et al., 2007). However, following culture with 1 ng/ml NGF for 48 h, BK evoked fourfold larger and longer cytoplasmic Ca^{2+} signals (Fig. 1*F*,*G*, red line). Presumably, unlike the control neurons, NGF-treated ones fired action potentials as in Figure 1*C*. We found that

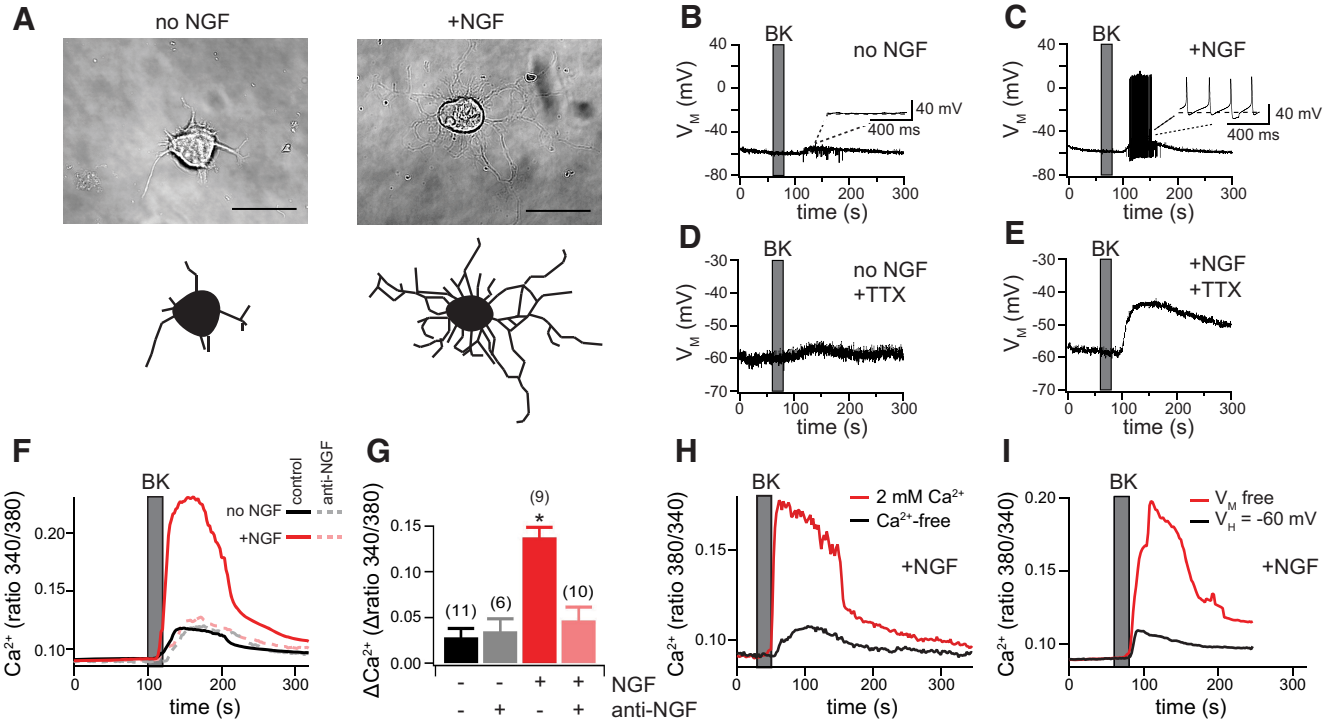

Figure 1. NGF increases BK-evoked electrical responses of SCG neurons and augments Ca^{2+} signals. ***A***, Top, Phase-contrast images of SCG neurons cultured for 48 h with 2 μg/ml of an anti-NGF antibody (left) or with 1 ng/ml NGF (right). Scale bar, 20 μm. Bottom, Schematic drawings of the neurons shown in the top. ***B–E***, Membrane potentials of SCG neurons were recorded in the perforated-patch configuration. Cells were cultured without or with 1 ng/ml NGF as indicated, but NGF was removed immediately before the recordings. BK (250 nM) was applied for 20 s. Recordings were done in the presence or absence of 200 nM TTX where indicated. ***F***, Cytoplasmic Ca^{2+} signals evoked by 250 nM BK application in neurons cultured with or without NGF and 2 μg/ml anti-NGF antibody. Traces represent time courses of the fluorescence ratio of the Ca^{2+} indicator fura-2. ***G***, fura-2 ratio changes measured in experiments illustrated in ***F***. Numbers in parentheses indicate number of cells. No NGF vs +NGF, $*p < 10^{-6}$; +NGF vs +NGF + anti-NGF, $*p = 10^{-4}$. ***H***, BK-evoked cytoplasmic Ca^{2+} signals in NGF-treated neurons in the presence and absence of external Ca^{2+}. ***I***, Cytoplasmic Ca^{2+} signals in NGF-cultured neurons clamped at a membrane potential of −60 mV or not manipulated (V_M free, no pipette).

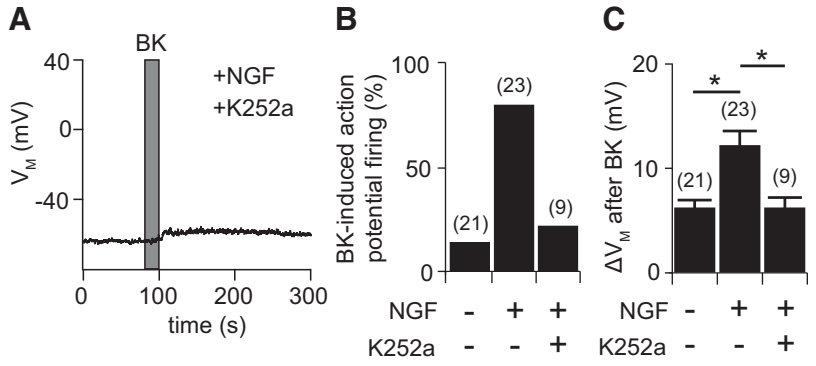

Figure 2. NGF-mediated sensitization to BK is dependent on trkA receptors. ***A***, SCG neurons were cultured with 1 ng/ml NGF and 200 nM K252a. Membrane potentials were measured in the perforated-patch configuration in the absence of TTX, and 250 nM BK was applied for 20 s. ***B***, Percentage of SCG neurons firing action potentials after BK application after different culture conditions as indicated. ***C***, Depolarization of membrane evoked by BK application in cells cultured as indicated: no NGF vs NGF, $*p = 0.0004$; NGF vs K252a, $*p = 0.001$. Numbers in parentheses indicate number of cells.

a much higher NGF concentration (50 ng/ml), often used in published experiments (Delmas et al., 1998; Zaika et al., 2007), was no more effective than 1 ng/ml in these cells from 7- to 12-week-old rats. To exclude the possibility that the control cultures contained significant NGF either because of secretion or from the culture medium, we added an anti-NGF antibody to the medium with no added NGF. After 48 h in the antibody, cells responded to BK with the same small Ca^{2+} responses as without the antibody (Fig. 1*F*,*G*, dashed gray line). To confirm the ability of the antibody to neutralize NGF, we added NGF at the usual concentration of 1 ng/ml to the antibody-containing culture medium and tested Ca^{2+} signals after 48 h. Under these conditions, the Ca^{2+} signals remained small and comparable with those from cells incubated without added NGF (Fig. 1*F*,*G*, dashed pink line). Thus, NGF incubation, even at modest concentrations, augments responses to BK, and our culture medium contains little NGF, except when it is added exogenously.

The augmented Ca^{2+} signal evoked by BK in NGF-treated neurons depends on external Ca^{2+} and depolarization

To determine the source of the additional Ca^{2+} observed in NGF-treated neurons, we used a nominally Ca^{2+}-free Ringer's solution before, during, and after BK application. NGF-treated SCG neurons superfused with Ca^{2+}-free Ringer's solution showed reduced Ca^{2+} signals, comparable with those in cells cultured in the absence of NGF (Fig. 1*H*). Evidently, the augmented Ca^{2+} signals in NGF-treated SCG neurons depend on external Ca^{2+}. We next asked whether a membrane depolarization underlies the extra Ca^{2+} influx. Therefore, in addition to loading the cells with fura-2 AM, we used perforated-patch electrical recording to control membrane potential changes. Under current clamp of NGF-treated cells, Ca^{2+} signals with BK were large (Fig. 1*I*, V_M free), whereas

under voltage clamp at −60 mV, Ca^{2+} signals were strongly reduced, becoming like those in neurons cultured in the absence of NGF (Fig. 1*I*). The need for external Ca^{2+} and for membrane depolarization suggests that culturing in NGF potentiates a BK-evoked influx of Ca^{2+} through voltage-gated calcium channels.

Sensitization of SCG neurons to NGF depends on trkA receptors

Two receptors for NGF have been found in SCG neurons: trkA and p75. trkA receptors bind NGF with high affinity (K_d = 10–100 pM), whereas p75 receptors bind NGF with low affinity (K_d = 100 pM to 2 nM) (Schechter and Bothwell, 1981; Hempstead et al., 1991; Bothwell, 1995; Ramer et al., 2001). Because 1 ng/ml NGF (~40 pM) was enough to sensitize neurons to BK, we tested for the involvement of trkA receptors in sensitization of the neurons. We cultured cells with NGF and an antagonist for trkA receptors, K252a, and measured the membrane potential responses to BK in perforated-patch current-clamp. Although 80% of neurons cultured with NGF alone fired action potentials upon stimulation with BK (Fig. 2*B*), only 22% of those cultured in NGF together with K252a fired (Fig. 2*A*,*B*). Similarly, the response rate was only 14% for neurons cultured in the presence of an anti-NGF antibody without added NGF (Fig. 2*B*). In addition, culturing neurons with K252a significantly reduced the membrane potential depolarization evoked by BK to the level observed in neurons cultured without NGF (Fig. 2*C*; $p = 0.001$). We conclude that high-affinity trkA receptors mediate the NGF-induced sensitization of SCG neurons to BK.

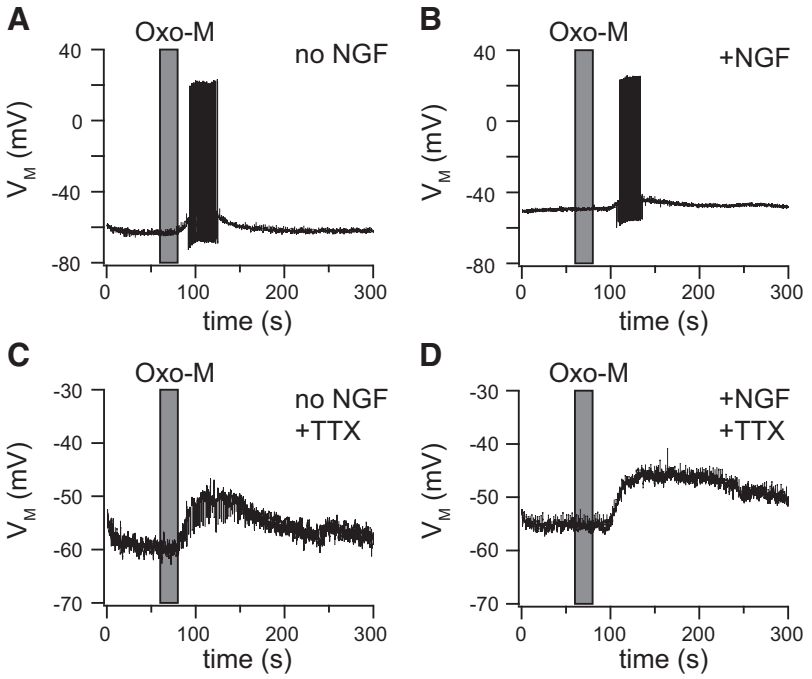

Figure 3. Response to muscarinic stimulation is not altered by NGF. ***A–D***, Membrane potentials of SCG neurons were measured in the perforated-patch configuration. Cells were cultured without or with 1 ng/ml NGF, as indicated, and stimulated for 20 s with 10 μM Oxo-M. ***C***, ***D***, TTX (200 nM) was present throughout the recordings.

Responses to muscarinic stimulation are not altered by NGF

Neurotransmission between preganglionic fibers and postganglionic SCG neurons is mediated by acetylcholine. We focused on the muscarinic acetylcholine action. As with BK receptors, activation of muscarinic acetylcholine receptors depolarizes sympathetic neurons (Brown and Adams, 1980). We tested whether NGF can also augment electrical responses of SCG neurons to muscarinic stimulation. In neurons cultured either with or without NGF, application of 10 μM of the general muscarinic agonist Oxo-M led to a strong depolarization and action potential firing (Fig. 3*A*,*B*). Even in TTX, muscarinic stimulation gave a large depolarization (with no action potentials) whether cells were cultured without NGF (7.3 ± 1.1 mV, $n = 5$; Fig. 3*C*) or with NGF (8.9 ± 1.0 mV, $n = 5$; Fig. 3*D*, not significantly different from Oxo-M without NGF, $p = 0.36$). The muscarinic cholinergic stimulation was not significantly different from BK stimulation in neurons cultured with NGF ($p = 0.2$) but was significantly larger than with BK in neurons cultured without NGF ($p = 0.03$).

Inhibition of M-current is not changed by NGF

Because BK induces larger depolarization in neurons cultured in the presence of NGF, even without sodium channel activity, we investigated other ion channels. We looked for an altered inhibition of the KCNQ potassium channels that are responsible for the M-current (I_M). The M-current is active at the resting membrane potential and thereby helps to regulate the electrical excitability of SCG neurons (Marrion, 1997). Activation of either BK or muscarinic receptors in SCG neurons inhibits these channels, depolarizes the membrane, and induces action potential firing (Brown and Adams, 1980; Jones et al., 1995; Brown et al., 2007; Zaika et al., 2007). Might BK be ineffective at suppressing KCNQ currents when sympathetic neurons are cultured without NGF? This hypothesis was not supported by our experiments, which showed similar amounts of I_M-inhibition upon BK or Oxo-M application independent of prior culture with or without NGF (Fig. 4*A–F*). In all variants of the experiment, the average inhibition of I_M was 70%–80% (Fig. 4*C*,*F*) without significant differences between the different experimental groups. In addition, we did not observe any significant differences in I_M density between SCG neurons cultured with or without NGF (no NGF: 7.2 ± 1.2 pA/pF, +NGF: 5.7 ± 1.0 pA/pF, $p = 0.36$).

I_M can be inhibited by two signaling pathways in SCG neurons: (1) net depletion of phosphatidylinositol (4,5)-bisphosphate ($PI(4,5)P_2$) and (2) binding of Ca^{2+}/calmodulin (Suh and Hille, 2002; Zhang et al., 2003; Zaika et al., 2007). Activation of BK receptors leads to activation of phospholipase C, which in turn hydrolyzes $PI(4,5)P_2$. This potentially depletes the $PI(4,5)P_2$ pool while also generating inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 releases Ca^{2+} from intracellular stores, which can lead to calmodulin-dependent inhibition of KCNQ channels (Zaika et al., 2007). The apparent lack of effect of NGF on the I_M inhibition by BK suggested that culturing with NGF does not alter signaling from BK receptors. Presumably comparable amounts of $PI(4,5)P_2$ depletion and IP_3 generation occur with and without NGF treatment.

To assess the activity of BK receptors more directly, we injected SCG neurons with plasmids for a pair of probes, CFP- and YFP-tagged pleckstrin-homology (PH) domains from PLCδ1, to measure $PI(4,5)P_2$ by FRET (van der Wal, 2001). The two probes

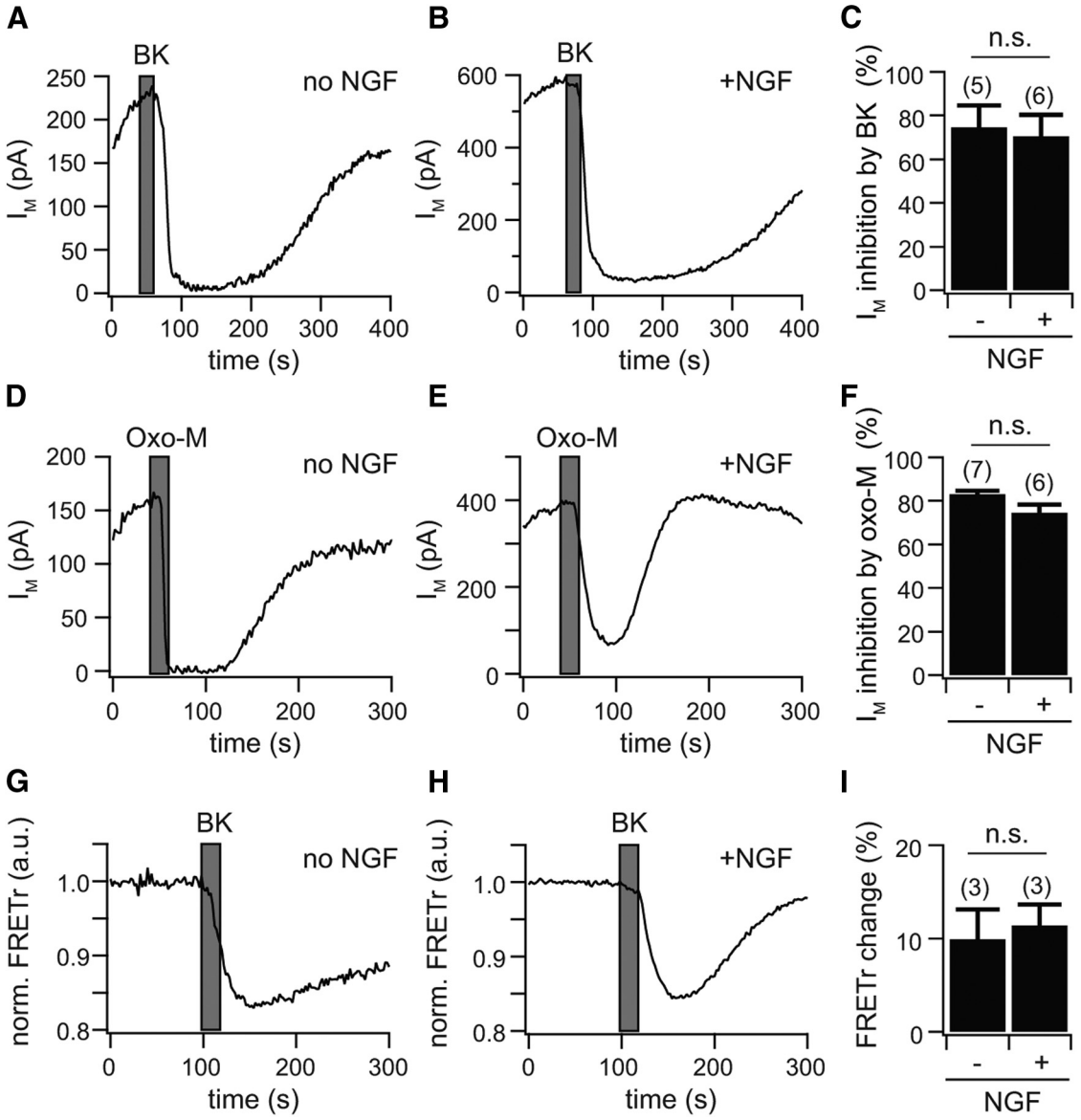

Figure 4. Inhibition of M-current, $PI(4,5)P_2$ hydrolysis, and IP_3 generation by BK receptor activation are independent of NGF-treatment. ***A***, ***B***, Amplitude of M-current in response to 250 nM BK application measured in whole-cell recordings in SCG neurons. Cells were cultured in the absence (***A***) or presence of 1 ng/ml NGF (***B***) for 48 h before experiments. ***C***, Percentage inhibition of M-current by BK. ***D–F***, Same experiment as in ***A–C***, but with stimulation by 10 μM Oxo-M instead of BK. ***G–I***, SCG neurons were injected with plasmids for CFP- and YFP-tagged PH probes from PLCδ1. Neurons were stimulated with BK, and decreases in FRET ratio were measured to monitor loss of $PI(4,5)P_2$ and generation of IP_3. Before experiments, neurons were cultured in the absence (***G***) or presence of 1 ng/ml NGF (***H***) for 48 h. ***I***, Percentage change of FRETr between CFP- and YFP-tagged PH probes after application of BK. Numbers in parentheses indicate number of experiments.

normally colocalize at the plasma membrane at rest by binding to $PI(4,5)P_2$ headgroups and show FRET between CFP and YFP. Net depletion of membrane $PI(4,5)P_2$ and generation of cytoplasmic IP_3 lead to translocation of these probes to the cytoplasm and a reduction in their FRET interaction (Jensen et al., 2009; Falkenburger et al., 2010). Application of BK to SCG neurons led to reductions in FRET of PH domains that were not significantly different between nontreated or NGF-treated cells (Fig. 4*G–I*). Such experiments suggested that NGF does not alter the activity of BK receptors.

Maxi-K channels control electrical responses of SCG neurons

Our measurements render unlikely models of the NGF effect that invoke changes of I_M modulation or changes of receptor activity. Therefore, we looked at other ion channels that might be affected by culturing in NGF. Because BK induces a larger calcium signal in the presence of NGF, we tested the calcium-dependent Maxi-K channel (also named "BK channel," an abbreviation we avoid here, or KCa1.1, or I_C), which is present in SCG neurons (Adams et al., 1982a, b; Brown et al., 1982). This channel, activated by Ca^{2+} and depolarization, shows a very large single-channel conductance, so opening of only a few Maxi-K channels would suffice to hyperpolarize the membrane (Rothberg, 2012). We probed with IbTX to block Maxi-K channels selectively. These experiments were done on SCG neurons cultured without NGF while recording the membrane potential in current clamp with the perforated-patch configuration. As before, control experiments without IbTX showed no action potential firing during application of BK (Fig. 1*B*). In contrast, with the channel blocker present before and during the application of BK, there was action potential firing in 80% of neurons, mimicking the effect of culturing neurons for 48 h with NGF (Fig. 5*A*; for comparison, see

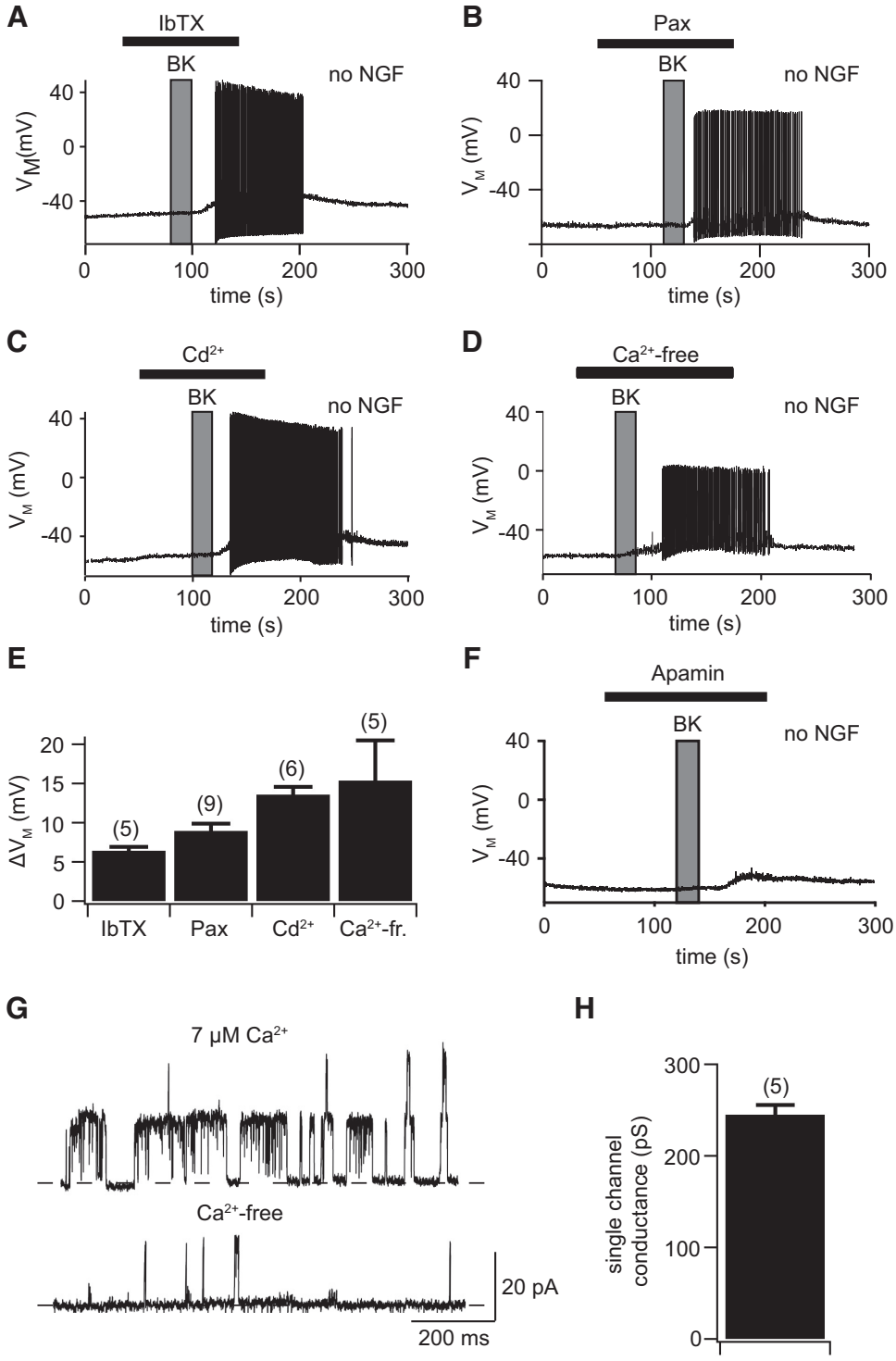

Figure 5. Maxi-K channel activity regulates electrical excitability of SCG neurons. ***A***, Neurons cultured for 48 h in the absence of 1 ng/ml NGF were exposed to 200 nM IbTX before, during, and after 250 nM BK application. Membrane potentials were recorded in current clamp. ***B–D***, Same experiment as in ***A***, but with superfusion of 100 nM paxilline (Pax) (***B***), Cd^{2+} (***C***), or calcium-free (Ca^{2+}-free) Ringer's solution (***D***) as indicated by black bars. ***E***, Quantification of BK-evoked change in membrane potential measured in experiments displayed in ***A–D***. ***F***, Same experiment as in ***A***, but with superfusion of 100 nM apamin. ***G***, Single-channel recordings acquired in the inside-out patch-clamp configuration from membrane patches excised from SCG neurons cultured without NGF. Recordings were performed at a membrane potential of 80 mV with either 7 μM free Ca^{2+} in the bath or a nominally Ca^{2+}-free solution. The channel closed level is indicated by a dashed line. ***H***, Calculated single-channel conductance from recordings as shown in ***G***. Numbers in parentheses indicate number of experiments.

Fig. 1*C*). Similar sensitization was observed with paxilline, a different inhibitor of Maxi-K channels (Fig. 5*B*). In the presence of paxilline, application of BK evoked action potentials in 67% of neurons. An alternative means to reduce the activation of Maxi-K channels is to reduce Ca^{2+} entry. We applied cadmium (Cd^{2+}) to block voltage-gated calcium channels before and during BK application or used Ca^{2+}-free bathing solutions while applying BK. Under both experimental conditions, BK evoked action po-

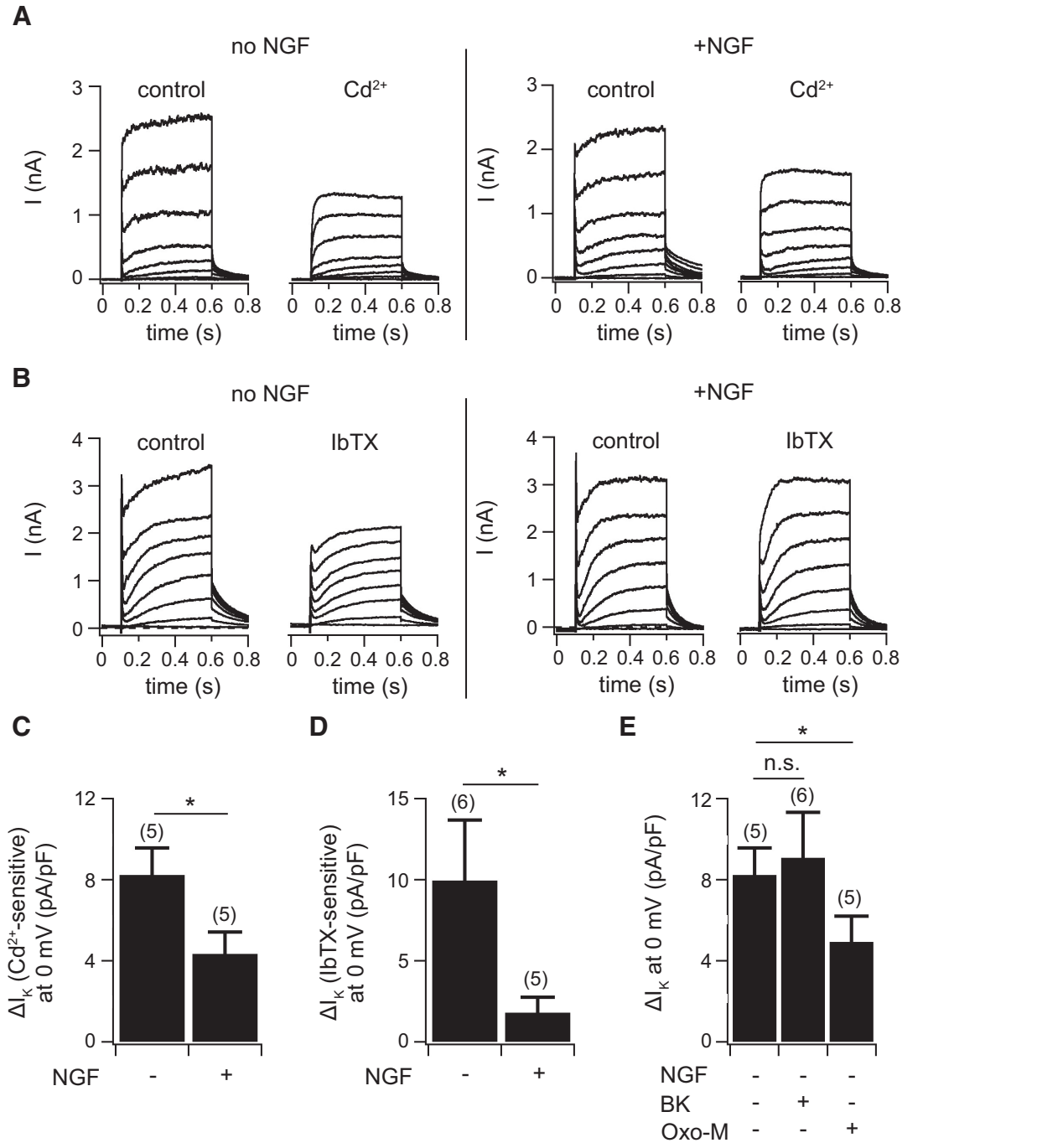

Figure 6. Maxi-K channel activity is regulated by NGF and muscarinic stimulation. ***A***, Left, Traces of potassium outward currents recorded in whole-cell configuration from a neuron cultured without NGF before and after addition of 100 μM Cd^{2+}. Pulse protocol: Cells were depolarized in 10 mV steps from a holding potential of −50 mV up to 20 mV for 500 ms per voltage step. Right, Same protocol as on the left, but with a cell cultured with 1 ng/ml NGF. ***B***, Same type of experiments as in ***A***, but with application 200 nM IbTX instead of Cd^{2+}. ***C***, ***D***, Difference current densities (ΔI_K, ± Cd^{2+} or ± IbTX) at a membrane potential of 0 mV calculated from recordings of neurons cultured without or with NGF as shown in ***A*** ($p = 0.04$) or (***B***) ($p = 0.05$). ***E***, SCG neurons cultured in the absence of NGF were treated for 20 s with 250 nM BK or 10 μM Oxo-M, respectively, whereas potassium outward currents were recorded as shown in ***A***. Bars represent difference current densities at a membrane potential of 0 mV under control conditions or after stimulation of cells with BK or Oxo-M (control vs Oxo-M $p = 0.03$, n.s., Not significant, $p = 0.78$). Numbers in parentheses indicate number of experiments.

tential firing in 80% of neurons despite culture without NGF (Fig. 5*C*,*D*). The observed changes in membrane potential are summarized in Figure 5*E*. In the presence of apamin, an inhibitor of small conductance calcium-activated potassium channels (SK channels), the application of BK induced only a small depolarization that was not enough to start a burst of action potentials (Fig. 5*F*), indicating that inhibition of Maxi-K channels, but not SK channels, mimics the NGF effect.

Using single-channel recording in excised inside-out membrane patches, we recorded activity attributable to Maxi-K channels in neurons cultured without NGF. Our recordings in symmetrical K^+ revealed large outward unitary currents at 80 mV that were dependent on intracellular Ca^{2+} concentration and corresponded to a single-channel conductance (238 ± 10 pS, $n = 5$ patches) in the range reported for Maxi-K channels (Fig. 5*G*,*H*) (Rothberg, 2012). Overall, these results confirm that

Maxi-K channels actively participate in regulating electrical excitability of SCG neurons. Together with I_M, Maxi-K channels help to dampen electrical responses. Unlike I_M, Maxi-K channels are not active at resting membrane potential and cytoplasmic Ca^{2+} concentrations, so block with IbTX, or paxilline does not induce depolarization in resting cells (Fig. 5*A*,*B*, before application of BK).

Maxi-K channel activity is sensitive to NGF treatment and needs Ca^{2+} influx

Our observations so far would be consistent with a reduction of Maxi-K channel activity in SCG neurons cultured with NGF. We tested this concept by voltage clamp. We first estimated Maxi-K channel-mediated current by subtracting total potassium outward currents during depolarizing steps before and after the application of 100 μM Cd^{2+} (Fig. 6*A*). These measurements revealed a reduction by at least 50% of the calcium-sensitive outward current after 48 h culture with NGF (Fig. 6*C*). Repeating the experiment with 200 nM IbTX instead of Cd^{2+} gave the same result: culture in NGF had reduced the evoked Maxi-K current (Fig. 6*B*,*D*). Such findings might explain why culturing SCG neurons with NGF makes them more responsive to BK but not why the neurons are easily excited by Oxo-M even when not cultured with NGF. We hypothesized that Oxo-M alone depresses evoked Maxi-K channel activity. We recorded potassium outward currents before and after the application of 100 μM Cd^{2+} in the presence of Oxo-M or BK in neurons cultured in the absence of NGF and found indeed that Oxo-M, but not BK, reduces depolarization-evoked Cd^{2+}-sensitive outward currents (probably Maxi-K current) on its own (Fig. 6*E*).

In many cells, the Ca^{2+} entry that activates Maxi-K channels is mediated by L-type voltage-gated calcium channels (Rothberg, 2012; Rehak et al., 2013). We tested this possibility using the L-type Ca^{2+} channel blocker nifedipine. We performed current-clamp measurements in the perforated-patch configuration on SCG neurons cultured without NGF and applied nifedipine before and during application of BK. In the presence of nifedipine, BK initiated a strong depolarization, leading to action potential firing in 60% of neurons (Fig. 7*A*,*B*), whereas without nifedipine there was as before only a moderate depolarization and no action potential firing (Fig. 7*B*). Under voltage clamp, application of nifedipine led to a reduction of Maxi-K-mediated potassium current (Fig. 7*C*) similar to that observed with Cd^{2+} (Fig. 6*A*,*C*) or IbTX (Fig. 6*B*,*D*). Although >80% of the peak inward Ca^{2+} current in rat SCG neurons is carried by N-type Ca^{2+} channels (Plummer et al., 1989; Mathie et al., 1992), Maxi-K channel opening seems to depend on activation of the minority L-type Ca^{2+} channels. These two channel types must be near each other in the membrane. Apparently, even the small depolarization induced by BK in neurons cultured without NGF activates enough L-type Ca^{2+} channels to open Maxi-K channels.

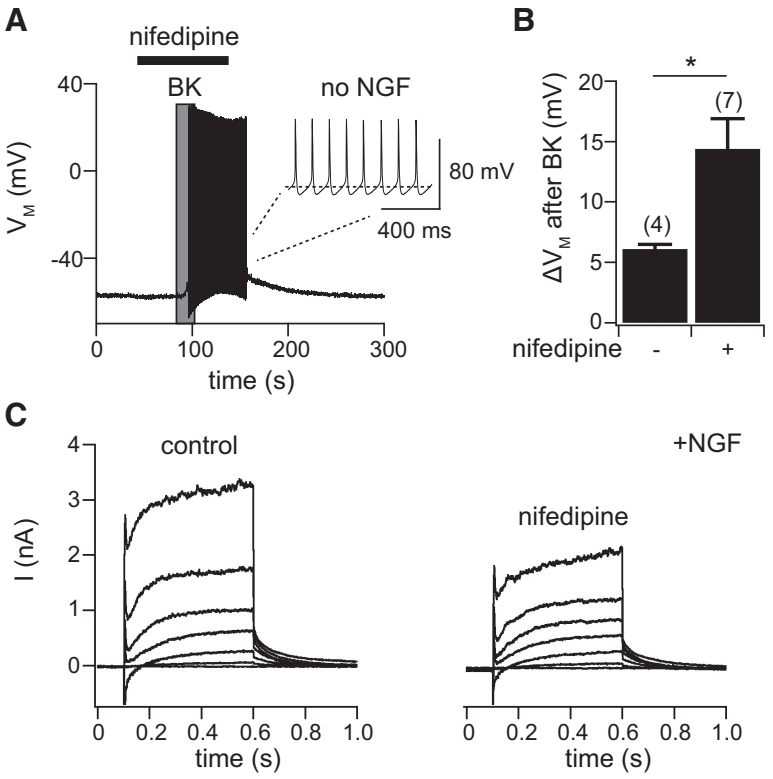

Figure 7. Inhibition of voltage-gated calcium channels by nifedipine sensitizes SCG neurons to BK. ***A***, Current-clamp recording in the perforated-patch configuration of a neuron cultured without NGF. Nifedipine (5 μM) and BK (250 nM) were applied as indicated. ***B***, Quantification of BK-induced depolarization without or with nifedipine in neurons cultured without NGF. *$p < 0.02$. Numbers in parentheses indicate number of experiments. ***C***, Left, Traces of potassium outward currents recorded from a neuron cultured with NGF. Right, Same cell recorded after addition of 5 μM nifedipine. Voltage protocol: Cells were depolarized in 10 mV steps from a holding potential of −50 mV up to 10 mV for 500 ms per voltage step.

NGF reduces activation of Maxi-K channels by reducing L-type calcium channel activity

Our findings led us to ask whether culture in NGF decreases the density of Maxi-K channels in the plasma membrane or decreases their activation. We assessed Maxi-K channel density by recording potassium currents in voltage clamp with elevated Ca^{2+} in the pipette and nifedipine in the bath. The pipette solution contained 20 mM BAPTA and sufficient $CaCl_2$ to raise cytoplasmic free Ca^{2+} to ~10 μM Ca^{2+}. Under these recording conditions, Maxi-K channels are activated by the free Ca^{2+} in the pipette solution, rather than by L-type Ca^{2+} channels. We elicited potassium outward currents by depolarizing voltage steps from a holding potential of −50 mV (Fig. 8*A*). The IbTX-sensitive potassium currents showed indistinguishable current densities in cells cultured with or without NGF (Fig. 8*B*). Hence, incubation with NGF may not reduce the density of Maxi-K channels at the cell surface but may instead reduce their activation.

We next hypothesized that NGF reduces Maxi-K channel currents by reducing Ca^{2+} entry via L-type calcium channels. We compared the nifedipine-sensitive calcium currents of neurons cultured without or with NGF (Fig. 8*C*). As predicted, nifedipine inhibited more current in neurons cultured without NGF (16 ± 3%, $n = 7$) than in those cultured with NGF (6 ± 3%, $n = 5$, $p = 0.035$; Fig. 8*D*), whereas total calcium channel-mediated current density was unaltered (no NGF: −31.0 ± 2.3 pA/pF, +NGF: −26.8 ± 2.2 pA/pF, $n = 25$, $p = 0.20$). Thus, NGF treatment reduces L-type calcium current that governs the activity of Maxi-K channels.

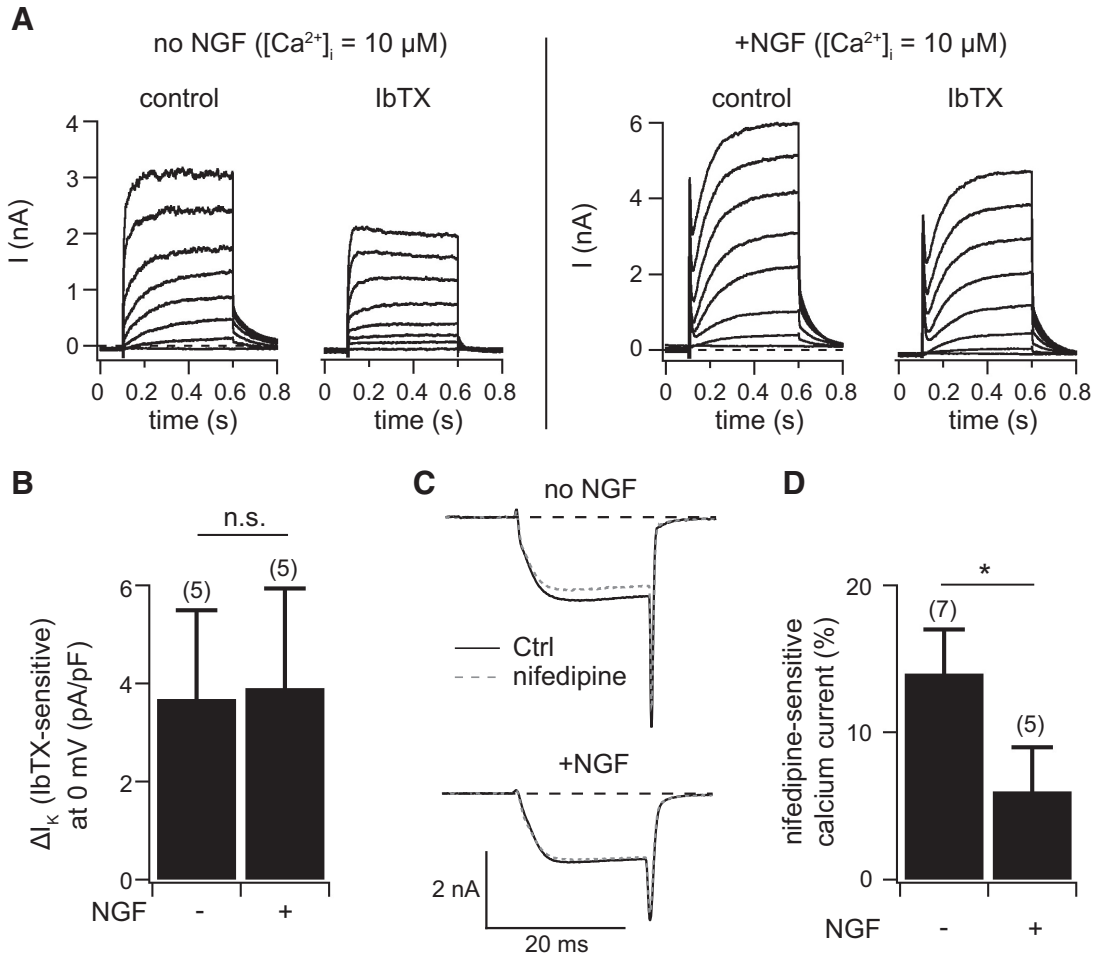

Figure 8. NGF reduces the activating Ca^{2+} entry rather than Maxi-K channel density. ***A***, Left, Potassium currents recorded from a neuron cultured without NGF before and after addition of 200 nM IbTX. The pipette solution contained a calcium-BAPTA mixture that buffered cytoplasmic free Ca^{2+} to 10 μM Ca^{2+}. The bath solution contained 10 μM nifedipine to inhibit L-type Ca^{2+} channel activity. Right, Same type of experiment, but with a cell cultured with 1 ng/ml NGF. ***B***, Current densities of IbTX-sensitive I_K at a membrane potential of 0 mV in neurons cultured without or with NGF as shown in ***A***. n.s., Not significant; $p = 0.94$. ***C***, Traces of total calcium inward currents during a step to 0 mV from a holding potential of −80 mV from neurons cultured without (top) or with NGF (bottom), before and after application of 5 μM nifedipine. Pulse protocol: P/4. ***D***, Percentage of nifedipine-sensitive calcium current in recordings as shown in ***C***. *$p = 0.035$. Numbers in parentheses indicate number of experiments.

Some properties of acutely isolated SCG neurons are like those of cells cultured in the absence of NGF

The obvious effect of NGF on neuronal responses made us wonder whether the native state of these cells was more like the cultures that had NGF or the cultures that lacked it. As a first approach, we compared electrical responses of the 48 h cultured neurons to those of acutely dissociated neurons. We isolated neurons (~2 h) and allowed them to adhere to poly-L-lysine-covered glass chips for only 30 min. Soon afterward, the cells were analyzed by current-clamp recordings in the perforated-patch configuration, and BK was applied. Like cells cultured without NGF, only 11% of these uncultured, acutely dissociated neurons fire action potentials upon BK application and showed only a small depolarization of the membrane potential (Fig. 9*A*,*C*).

Might these acutely dissociated neurons be temporarily damaged by the enzymes and mechanical trauma of the isolation procedure? As one control, we cultured cells for 48 h in NGF, so they reached a sensitized state and then treated them with enzymes again to mimic the acute isolation procedure. The cultured cells were incubated for 20 min with collagenase and dispase at 37°C. Perforated-patch current-clamp recordings then revealed action potential firing and significant depolarization of the membrane potential after BK application (Fig. 9*B*,*C*). Therefore, we conclude that incubation with these enzymes does not reverse NGF sensitization of BK responses.

To understand better how SCG neurons are sensitized by NGF, we determined the induction time of the NGF action. The experiment began with neurons in an NGF-free initial condition. After culture for 48 h with an anti-NGF antibody, we changed the culture medium to one containing 1 ng/ml NGF and no antibody. Using the perforated-patch configuration, we recorded membrane potential responses periodically starting 1 h after the switch to NGF. Depolarizations evoked by BK began to increase after 6 h and grew gradually over 48 h (Fig. 9*D*). The percentage of neurons firing action potentials after BK application increased in parallel, starting at only 17% after 1 h in NGF and reaching 72% after 24 h (Figs. 9*E* and 2*B*). To study the time course of reversal of these effects, neurons were cultured for 48 h with 1 ng/ml NGF and then rinsed with medium lacking NGF and cultured with an anti-NGF antibody. Interestingly, the membrane potential depolarizations after BK remained high for 24 h without NGF and were only partially decreased at 48 h (Fig. 9*D*). Similarly, the reduction in the percentage of cells firing action potentials after BK application was slow (Fig. 9*E*). Even 48 h after NGF removal,

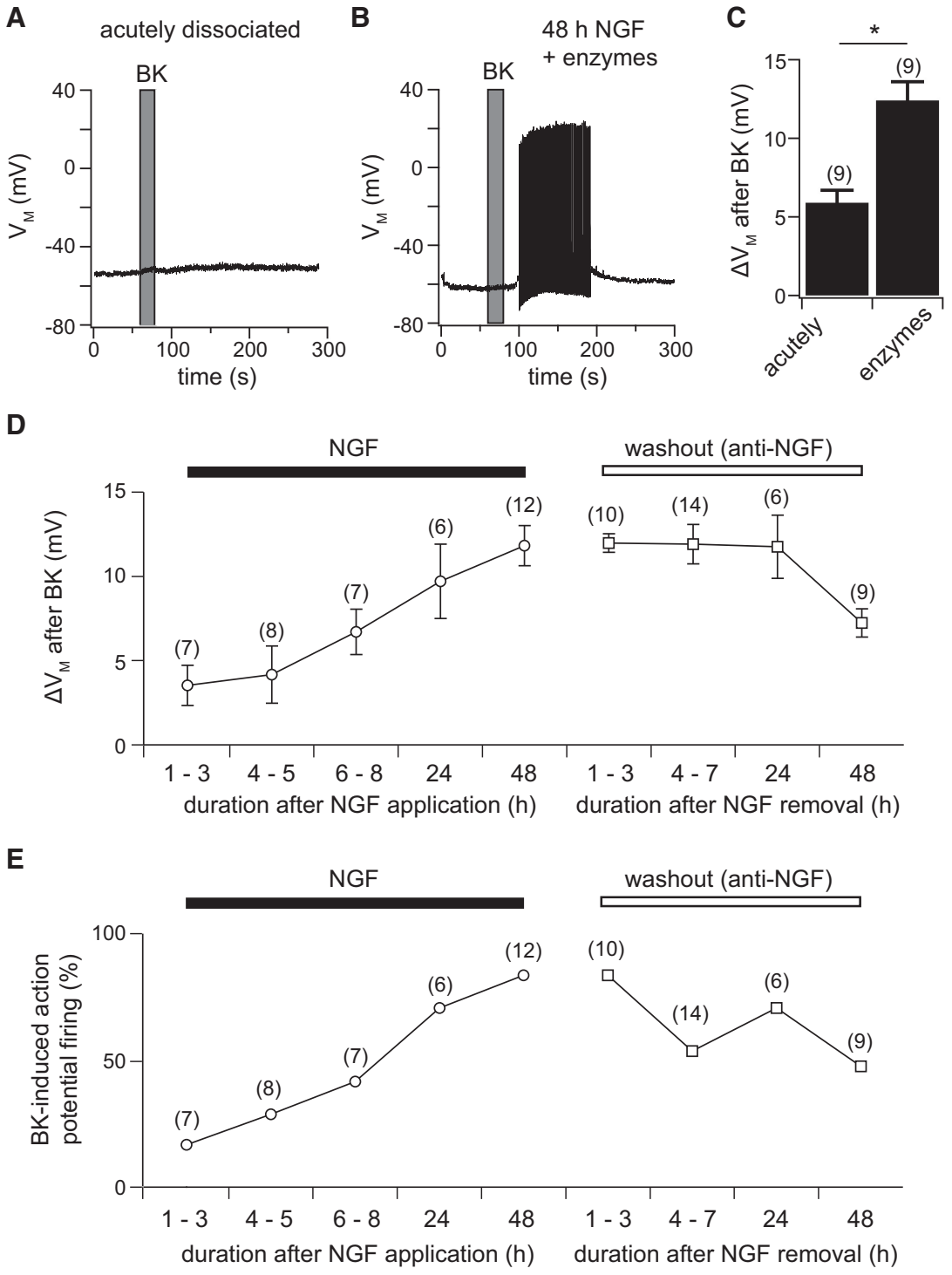

Figure 9. Acutely dissociated SCG neurons show electrical excitability similar to cells cultured without NGF. ***A***, Current-clamp recording in the perforated-patch configuration of a neuron acutely isolated from SCG. BK (250 nM) was applied as indicated. ***B***, Same type of recording as in ***A***, but from a neuron cultured for 48 h with 1 ng/ml NGF and then treated with collagenase and dispase for 20 min before the recording. ***C***, Quantification of changes in membrane potential after BK application from cells either acutely dissociated or cultured for 48 h with NGF and followed by collagenase and dispase for 20 min ("enzymes"). *$p < 0.001$. ***D***, Left, Mean membrane potential depolarizations evoked by a 20 s BK application measured in perforated-patch recordings from SCG neurons cultured for the indicated amount of time with 1 ng/ml NGF (closed bar) after prior 48 h culture without NGF. Right, Same type of experiment, but neurons were cultured for 48 h with 1 ng/ml NGF and subsequently cultured in medium with an anti-NGF antibody (open bar) for the indicated amount of time. ***E***, Percentage of cells firing action potentials after BK application in the same experiment as in ***D***. Numbers in parentheses indicate number of cells.

we still observed action potential firing in 44% of neurons compared with 14% in control neurons (Fig. 2*B*). Thus, it takes hours after first exposure to NGF before SCG neurons show measurable increases in excitability and several days for recovery. Considering that to record from acutely isolated neurons it took <2 h after killing the animal, and that the NGF effect reverses only partially after 48 h, we concluded that our results with acutely dissociated neurons are not due to NGF loss during the dissection protocol.

In summary, acutely isolated neurons behave like cells cultured without NGF and because the reversibility of NGF effect takes days, we suggest that these resemble the native state of SCG neurons.

Discussion

We tested the effects of NGF on adult sympathetic neurons and found that culturing in NGF sensitizes adult sympathetic neurons to BK. Only after treatment with NGF does BK cause sympathetic neurons to depolarize enough to fire action potentials. NGF treatment does not alter the ability of BK to activate PLC and inhibit M current. Instead, it reduces L-type calcium channel activity, thereby decreasing the depolarization-evoked activation of Maxi-K channels and increasing electrical excitability of these neurons.

First, we discuss NGF-mediated sensitization observed in other types of adult neurons. For example, NGF sensitizes responses of adult, but not neonatal, nociceptive neurons to noxious stimuli in minutes (Shu and Mendell, 1999; Bonnington and McNaughton, 2003; Zhu et al., 2004; Zhang et al., 2005). NGF also renders capsaicin-sensitive small sensory neurons more responsive to BK (Kasai et al., 1998; Kasai and Mizumura, 1999). On a longer time scale, exposure of cells to NGF for 4–48 h also sensitizes bladder afferent neurons (Yoshimura et al., 2006) and brainstem neurons (Bie et al., 2010), and exposure to NGF for several days sensitizes skin nociceptors yielding heightened responses to touch and induction of behaviors that protect the affected area (Hefti et al., 2006; Pezet and McMahon, 2006; Rukwied et al., 2013). Sensitization of bladder afferents correlates with overreactivity of the bladder (Yoshimura et al., 2006), and sensitization of brainstem neurons to opioids promotes mechanisms predisposing for drug addiction (Bie et al., 2012). We find that NGF sensitizes sympathetic neurons to the proinflammatory molecule BK.

NGF levels change during pathological conditions. Basal NGF levels in serum are normally low (2–40 pg/ml) (Toma et al., 2000; Sarchielli et al., 2001; Peleshok and Ribeiro-da-Silva, 2012; Diniz et al., 2013; Palumbo et al., 2013; Peng et al., 2013) and increase threefold to fivefold during urinary or abdominal inflammation (Lewin et al., 1994; Dmitrieva et al., 1997; Sarchielli et al., 2001; Jiang et al., 2013; Liu et al., 2013), diseases with involvement of the sympathetic nervous system (Hohenfellner et al., 1992; Peeker et al., 2000; Buffington, 2004; Lutgendorf et al., 2004; Straub et al., 2006; Schnegelsberg et al., 2010). In agreement with low resting levels of NGF *in vivo*, we found that acutely dissociated sympathetic neurons were not depolarized much by BK, whereas culture for >6 h in NGF was enough to sensitize them to BK. Further experiments comparing *in vivo* NGF effects in control and in inflamed models would help to establish a physiological significance for our findings.

Much literature about electrical excitability and Ca^{2+} signals of isolated SCG neurons concerns cells from neonatal to 2-week-old rats. The survival of these young cells still depends on NGF. Commonly, 25–100 ng/ml NGF is added to supplement the culture medium of neonatal neurons (Lindsay and Harmar, 1989; Donnerer et al., 1992; Cruzblanca et al., 1998; Brown et al., 2007; Zaika et al., 2007; Brown and Passmore, 2009). In our study, we cultured neurons from older rats that apparently no longer require NGF to survive (Orike et al., 2001; Ramer et al., 2001; Hefti et al., 2006). They survived for several days in the absence of NGF with no apparent morphological or functional signs of stress. Indeed, even culturing the cells in the presence of an NGF-neutralizing anti-NGF antibody did not alter cell survival rates, cell morphology, action potential firing patterns, or potassium channel activity. Thus, we do not need to invoke extraneous sources of NGF in our cultures to explain cell survival and activity, and we confirm that sympathetic neurons from adult rats lose their dependence on NGF for survival and cellular activity. Nonetheless, culture in a low concentration of NGF still sensitizes adult SCG neurons to proinflammatory molecules, such as BK, and it promotes greater outgrowth of neurites.

We found that NGF reduces L-type calcium current and hence the evoked activity of Maxi-K channels. Other sensitization mechanisms have been proposed for sensory and brainstem neurons. Acute application of NGF to sensory neurons increases surface expression of channels like TRPV1, sensors of noxious levels of heat, lowering the threshold for pain (Zhang et al., 2005; Stein et al., 2006), whereas long-term application of NGF to brainstem neurons increases the surface expression of δ-opioid receptors (Bie et al., 2010). We did not find evidence for an alteration in BK receptor Type 2 activity by NGF, as indicated by similar amounts of M current inhibition and $PI(4,5)P_2$ hydrolysis. The ability of NGF to regulate ion channels has been documented in numerous reports. For instance, NGF increases both TTX-resistant and TTX-sensitive sodium channel activities in PC12 cells, sensory neurons, and bullfrog sympathetic ganglion neurons on a rapid time scale of a few minutes up to 24 h (Garber et al., 1989; Friedel et al., 1997; Lei et al., 1997; Gould et al., 2000; Zhang et al., 2002). NGF also increases or decreases voltage-gated potassium currents on a similar time scale (Sharma et al., 1993; Luther and Birren, 2006, 2009; Bai et al., 2010). After 2 d in NGF, we found a significant reduction in L-type calcium channel currents in adult SCG neurons, secondarily reducing evoked currents in Maxi-K channels. In preliminary experiments not shown, we also tested for an NGF effect on Maxi-K current on small sensory neurons, but we did not find evidence for one. Other studies find increases in L-type calcium channel currents in adult bullfrog sympathetic ganglion B neurons (Ford et al., 2008), somatotrope-like GH3 cells (López-Domínguez et al., 2006), and pancreatic β-cells (Rosenbaum et al., 2002). Future experiments need to be done to explain why the L-type channels of SCG neurons respond to NGF differently and to identify the underlying molecular mechanisms.

In conclusion, our working model postulates that BK application first inhibits M-current. This leads to an initial small depolarization of the membrane potential that activates L-type calcium channels and subsequently Maxi-K channels. Activation of Maxi-K channels counterbalances the closure of M channels, restricts any membrane depolarization, and prevents action potential firing. After culture in NGF, the calcium influx through L-type calcium channels is reduced, with fewer Maxi-K channels open; therefore, BK depolarizes neurons enough to evoke action potential firing. Finally, as a consequence of action potential firing, calcium enters into the cell through N-type calcium channels and causes a large transient increase in cytoplasmic calcium. This NGF-mediated sensitization is observed for stimulation by BK but not by Oxo-M. Insensitivity of muscarinic acetylcholine signaling to NGF is probably explained by the ability of muscarinic receptors to inhibit L-type calcium channels in these neurons preventing activation of Maxi-K channels (Mathie et al., 1992; Zaika et al., 2007).

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