A Role for L-Type Calcium Channels in Developmental Regulation of Transmitter Phenotype in Primary Sensory Neurons

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To examine the influence of activity-dependent cues on differentiation of primary afferent neurons, we investigated the short- and long-term effects of depolarization and calcium influx on expression of transmitter traits in sensory ganglion cell cultures. We focused on expression of tyrosine hydroxylase (TH), a marker for dopaminergic neurons, in developing petrosal ganglion (PG), nodose ganglion, and dorsal root ganglion neurons grown in the presence or absence of depolarizing concentrations of KCl. Exposure to 40 mM KCl increased the proportion of TH-immunoreactive neurons in all three ganglia in a developmentally regulated manner that corresponded to the temporal pattern of dopaminergic expression in vivo. PG neurons, for example, were most responsive to elevated KCl on embryonic day 16.5 (E16.5), the age at which the dopaminergic phenotype is first detectable in vivo. However, KCl was relatively ineffective at increasing TH expression in neonatal PG, indicating a critical period for induction of this phenotype by depolarization. Detailed analysis of TH induction in PG neurons demonstrated that, although N-type calcium channels carried the majority of the high voltage-activated calcium current, only L-type calcium channel blockade inhibited the effect of elevated KCl. Further studies revealed that after removal of high KCl, neurons remained sensitized to subsequent stimulation for >1 week. Specifically, cultures exposed to KCl beginning on E16.5 (the conditioning stimulus), then returned to control medium, and subsequently re-exposed to elevated KCl after 9 d (the test stimulus) contained fourfold more TH-positive neurons than did cultures exposed to the test stimulus alone. Moreover, blockade of L-type calcium channels during the conditioning stimulus completely abolished long-term potentiation of the TH response to elevated KCl. These findings demonstrate a novel role for L-type calcium channels in activity-dependent plasticity of transmitter expression in sensory neurons and indicate that exposure to depolarizing stimuli during early development may alter neuronal response properties at later ages.

Key words: Bay K-8644; ω-conotoxin GVIA; depolarization; dopaminergic; long-term potentiation; nimodipine; petrosal ganglion; primary sensory neurons; transmitter plasticity; tyrosine hydroxylase; voltage-dependent calcium channels

Activity of developing primary sensory neurons plays a critical role in regulating maturation of postsynaptic target cells in the CNS. For example, blocking the activity of vestibulocochlear afferents alters gene transcription, protein synthesis, and the size of neurons in the brainstem cochlear nucleus (Sie and Rubel, 1992; Garden et al., 1995), and the activity of primary olfactory afferents is thought to regulate developmental expression of dopaminergic transmitter properties in the olfactory bulb (Baker and Farbman, 1993). Although the importance of afferent activity in the development of second-order sensory neurons seems clear, it is unknown whether maturation of primary afferents themselves is also regulated by activity-dependent cues.

In mature primary sensory neurons, activity regulates quantitative expression of transmitter properties, such as preprotachykinin mRNA (Noguchi et al., 1988). Therefore, to explore the possibility that activity may also play a role in sensory neuron differentiation, we have been examining the influence of depolarizing stimuli on development of sensory transmitter phenotypes (Fan, 1995; Hertzberg et al., 1995). We have focused in particular on dopaminergic traits, which are expressed by subpopulations of sensory neurons in cranial and spinal ganglia (Katz et al., 1983; Price and Mudge, 1983). In vivo, the magnitude and time course of dopaminergic phenotypic expression vary widely among different sensory ganglia. In adult dorsal root ganglia (DRG), for example, only 1–4% of the total neuronal population is dopaminergic, depending on axial level (Price and Mudge, 1983; Vega et al., 1991). Tyrosine hydroxylase (TH) immunoreactivity and dopamine histofluorescence are first detectable in lumbar DRG neurons 1–2 weeks after birth (Price and Mudge, 1983). In contrast, dopaminergic afferents in cranial sensory ganglia are more abundant and differentiate earlier. In the adult glossopharyngeal petrosal ganglion (PG), for example, at least 10–20% of the neuron population is dopaminergic (Katz and Black, 1986), and TH immunoreactivity is first detectable in these cells on embryonic day 16.5 (E16.5) (Katz and Erb, 1990).

Recently, we found that expression of dopaminergic properties by developing cranial and spinal sensory neurons is highly regulated by depolarizing stimuli in vitro, indicating that activity-dependent mechanisms may influence the number of sensory neurons that express a dopaminergic phenotype (Fan, 1995; Hertzberg et al., 1995). Specifically, treatment of fetal cranial sensory neurons with depolarizing concentrations of veratridine (10 μM) or KCl (40 mM) increased the proportion of ganglion cells that expressed TH and elevated dopamine synthesis by up to 10-fold (Hertzberg et al., 1995). Thus, although dopaminergic
properties are normally expressed by a relatively small subset of primary sensory neurons in vivo (Katz et al., 1983), exposure to depolarizing stimuli can unmask a widespread potential for dopaminergic phenotypic expression in these cells, raising the possibility that activity plays an important role in sensory neuron differentiation.

The present study was designed to examine the relationship between sensory neuron responsiveness to depolarizing stimuli in vitro and dopaminergic development in vivo and to begin defining mechanisms that underlie depolarization-mediated TH expression. We found that KCl-induced depolarization increases the proportion of dopaminergic neurons in sensory ganglia in a developmentally regulated manner that corresponds to the temporal pattern of dopaminergic expression in vivo. In addition, we made the unexpected discovery that transient exposure of sensory neurons to depolarizing stimuli during fetal development leads to long-term changes in the regulation of TH expression. Moreover, calcium influx through L-type channels is required for both short-term regulation and long-term potentiation to occur.

MATERIALS AND METHODS

Cell culture

Pregnant dams (Sprague Dawley rats; Zivic-Miller, Zelienople, PA) were deeply anesthetized by exposure to carbon dioxide. The uterine horn was removed and placed in PBS containing 10% glucose, and the embryos were excised. To assign gestational ages, we designated the day after mating E0.5. Newborn (P0) and 1-week-old (P7) pups were killed with an overdose of sodium pentobarbital (6.0 mg/kg, i.p.). Dissociate cultures of E13.5, E14.5, E16.5, E19.5, P0, and P7 PG, nodose ganglia (NG), and cervical DRG were grown in Leibovitz’s L-15/CO2 medium containing 10% NuSerum (Collaborative Biomedical Products, Bedford, MA), 5% heat-inactivated rat serum, fresh vitamin mixture (Mains and Patterson, 1973), penicillin (50 IU/ml; Gibco BRL, Gaithersburg, MD), and streptomycin (50 μg/ml; Gibco BRL). Embryonic bronchial cells were digested in Dispase (Collaborative Biomedical Products; diluted 1:1 in PBS) for 1 hr at 37°C followed by trituration through fire-polished Pasteur pipettes; P0 and P7 ganglia were digested in 0.5% trypsin (Worthington, Freehold, NJ) for 20 min at 37°C followed by trituration. Embryonic and newborn cells were plated onto glass coverslips coated with poly-d-lysine (0.1 mg/ml) and laminin (0.3 mg/ml), whereas P7 cells were grown on growth factor-reduced Matrigel matrix (Collaborative Biomedical Products; diluted 1:5). All cultures were supplemented with recombinant human brain-derived neurotrophic factor (BDNF; Regeneron Pharmaceuticals, Inc., Tarrytown, NY) at a concentration of 10 ng/ml. DRG cultures were supplemented with, in addition to BDNF, 10 ng/ml nerve growth factor (NGF; Dr. Kenneth Neet, Chicago Medical College) and 10 ng/ml neurotrophin-3 (NT-3; Regeneron Pharmaceuticals, Inc.). Depolarizing conditions were produced by supplementing cultures with 34 mM KCl to achieve a final concentration of 40 mM. Control cultures were not adjusted to isomolarity because we found previously that TH expression in these cells was unaffected when the concentration of NaCl, rather than KCl, was raised to 40 mM (Hertzberg et al., 1995). In most experiments, neurons were cultured for a total of 3 d in the presence or absence of depolarizing concentrations of KCl. In some experiments, neurons were cultured for 2 d in control medium containing 6 mM KCl and then transferred for 24 hr to medium containing 40 mM KCl either with or without a selective calcium channel antagonist. Specifically, the N-type calcium channel antagonist w-conotoxin GVIA (1 μM; Sigma, St. Louis, MO) and the L-type calcium channel antagonists nifedipine (1 μM; Calbiochem, San Diego, CA), nimodipine (2 μM; a gift from Miles Pharmaceuticals, West Haven, CT), and verapamil (10 μM; Sigma) were used. In other experiments, an L-type calcium channel agonist, (±)-Bay K 8644 (1 μM; Calbiochem), was added at the beginning of the 3 day culture period. In long-term experiments (15 d), neurons were cultured on growth factor-reduced Matrigel. During the initial 3 d of culture, cytosine β-D-arabinofuranoside (10 μM; Sigma) was added to the culture medium to eliminate non-neuronal cells.

Immunocytochemistry

All cultures were fixed with 4% paraformaldehyde in 0.1 m sodium phosphate buffer, pH 7.4, overnight at 4°C. Double immunostaining was performed using polyclonal anti-DN (Pel-Freeze Biologics, Rogers, AR), polyclonal anti-sense P (SP) (Innate Corporation, Stilwater, MN), monoclonal anti-neurofilament protein (NF160; Sigma), goat anti-rabbit IgG-FITC (for TH staining, Boehringer Mannheim, Indianapolis, IN; for SP staining, Cappel, West Chester, PA), and goat anti-mouse IgG-rhodamine (Cappel) antibodies.

TH immunostaining. Cells were (1) incubated overnight at room temperature in anti-TH (1:200) and anti-NF (1:100) diluted in PBS containing 0.5% Triton X-100, (2) washed three times in PBS, (3) incubated for 1 hr at room temperature in goat anti-rabbit IgG-FITC (1:200) plus goat anti-mouse IgG-rhodamine (1:200) diluted in PBS–Triton containing 10% goat serum and 10% rat serum, (4) washed in PBS, (5) incubated in pH-phenylenediamine (1 mg/ml) for 1 min, (6) washed in PBS, and (7) coverslipped with glycerol gel.

Substance P (SP) immunostaining. The same protocol described for TH immunostaining was used except that the primary incubation was performed in anti-SP (1:2000) and anti-NF (1:50) diluted in PBS containing 0.5% Triton X-100, 2% rat serum, and 2% goat serum.

Cell counts and statistical analysis. The two fluorophores were distinguished using an Olympus fluorescence microscope (model BH-2) with rhodamine and FITC filter cubes. The number of TH– and NF–immunoreactive neurons in each culture was estimated by counting all cells in a measured area of each coverslip. All experiments were performed at least three times with three cultures per experimental group. Percentages were normalized (arcsine transformation), and values were compared using ANOVA followed by Scheffe’s multiple comparison procedure (Kleinbaum and Kupper, 1978). p < 0.05 was considered significant.

Electrophysiology

Calcium currents in dissociated E16.5 PG neurons were examined using conventional whole-cell recording techniques (Hamill et al., 1981). Experiments were performed at room temperature (25°C). −20 hr after dissociation. Currents were amplified (80 dB; decade low-pass Bessel filter set at a ~3 dB frequency of 10 kHz; Axopatch-1C; Axon Instruments, Inc., Foster City, CA), digitized (100 points/point), and analyzed using Clampfit (pClamp v. 6.0; Axon Instruments, Inc.), whereas whole-cell current measurements were made in response to voltage protocols controlled by pClamp v. 5.6 (Axon Instruments, Inc.).

Solutions. The compositions of solutions used to isolate calcium currents were as follows (in mM): for the pipette solution, 124.0 CsCl, 11.0 EGTA, 1.0 CaCl2, and 10.0 HEPES, pH 7.4, and for the bath solution, 140.0 TEA (tetraethylammonium chloride), 5.0 4-AP (4-aminopyridine), 15.0 HEPES, 2.0 CaCl2, and 20.0 glucose, pH 7.4. In one series of experiments, membrane potentials were examined in a bath solution containing L-15 serum-free media and varying concentrations of KC1 (6, 15, and 40 mM). In these experiments, the pipette solution was 124.0 CsCl, 14.0 MgCl2, 5.0 HEPES, 1.0 EGTA, 2.0 MgCl2, 5.0 NaCl, and 10.0 glucose, pH 7.2. The membrane potential was defined as the voltage (in millivolts) inside, relative to outside, the cell and was measured at zero current in current-clamp mode.

Calcium channel modulators. For electrophysiological measurements, N-(±)-Bay K 8644 (Research Biochemicals, Natick, MA) was dissolved in ethanol and stored in a 1 mM stock solution, nimodipine was dissolved in methanol and stored at a concentration of 0.5 mM, and w-conotoxin GVIA was dissolved in distilled water and stored at a concentration of 100 μM.

RESULTS

Sensory neuron responsiveness to depolarizing stimuli is developmentally regulated

We found previously that chronic exposure of E16.5 PG, NG, or DRG neurons to depolarizing stimuli in vitro can markedly increase both the percentage of ganglion cells that express TH and dopamine synthesis (Hertzberg et al., 1995). However, the proportion of neurons in which TH expression could be evoked varied from ~8% in the DRG to almost 100% in the PG (Hertzberg et al., 1995). These differences could reflect an underlying heterogeneity in phenotypic potential among different sensory neuron populations. Alternatively, responsiveness to de-
polarizing stimuli may be temporally regulated and exhibit a different time course in each ganglion. To explore this issue further, we compared the effect of KCl-mediated depolarization on TH expression in PG, NG, and DRG neurons cultured at various ages between E13.5 and P7. Neurons were grown for 3 d in the presence of control medium (6 mM KCl) or medium containing a depolarizing concentration of KCl (40 mM) and subsequently were processed for TH and NF immunostaining. An exposure time of 3 d was chosen because preliminary experiments demonstrated that, although 6 hr of KCl treatment was sufficient to induce TH expression, a 3 day exposure produced the greatest induction. The membrane potential of neurons grown for 3 d in control medium was between $-62$ and $-66$ mV ($n = 6$), compared with $-18$ to $-20$ mV ($n = 6$) in neurons grown for the same period of time in 40 mM KCl.

The percentage of TH-positive (TH$^+$) neurons in all three ganglia was significantly increased by KCl treatment; however, the magnitude and time course of the response were different in each population (Fig. 1). PG neurons exhibited the largest and earliest peak response (92% TH$^+$ neurons on E16.5), followed by the NG (55% TH$^+$ neurons on E19.5) and the DRG (22% TH$^+$ neurons on P0). In addition, KCl treatment seemed to define a window of responsiveness in TH regulation; in the PG, for example, KCl treatment was 2.5- and 5-fold less effective at inducing TH expression in E13.5 and P7 cultures, respectively, than in E16.5 cultures.

To determine whether depolarization increased the percentage of TH$^+$ cells by raising TH expression per cell or by selectively increasing either proliferation or survival of TH-immunoreactive neurons, we compared total neuron numbers in the absence or presence of depolarizing concentrations of KCl. In NG and DRG cultures, neuronal survival was unaffected by KCl treatment at all ages examined (Fig. 2). In the PG, KCl treatment had no effect on neuronal survival at E13.5, E14.5, E16.5, and P7 but significantly increased survival in E19.5 and P0 cultures (Fig. 2). However, when P0 cultures were grown on Matrigel, a basement membrane-rich substrate that augments neuronal attachment and survival, KCl evoked a comparable increase in the percentage of TH$^+$ neurons without altering neuronal survival [%TH$^+$ neurons, $16.1 \pm 1.7$ (control) vs $51.2 \pm 4.1$ (KCl-treated); number of NF-positive (NF$^+$) neurons, $235 \pm 36$ (control) vs $247 \pm 50$ (KCl-treated)]. Thus, altered survival or proliferation of TH-immunoreactive neurons cannot explain the increase in the percentage of TH$^+$ cells in KCl-treated cultures compared with age-matched controls. Moreover, PG neurons are postmitotic by E13–E14 (Altman and Bayer, 1982), i.e., before the age at which we observed the greatest percentage of TH$^+$ neurons in the presence of high KCl.

**Substance P expression in NG and PG neurons is unaffected by depolarization**

To determine whether depolarizing stimuli act selectively to upregulate dopaminergic traits, we examined expression of another neurotransmitter, the neuropeptide SP in PG, NG, and DRG cultures. SP is normally expressed by a subset of neurons in all three ganglia; however, TH and SP are not localized in the same cells (Price, 1985; Kummer et al., 1990; Finley et al., 1992). In marked contrast to TH, the percentage of PG and NG neurons exhibiting SP immunoreactivity (SP$^+$) was unchanged by KCl treatment at all ages examined (Fig. 3), indicating that depolarization does not increase transmitter traits in these ganglia in a nonselective manner. KCl treatment did, however, increase the percentage of SP neurons in E16.5 DRG cultures.

**Transient exposure to high KCl leads to long-term potentiation of TH expression**

The KCl-mediated increase in TH expression in E16.5 PG cultures is completely reversed after a return to control medium for...
12 d [compare Figs. 1 and 4, 91.6 ± 0.9% (3d KCl) vs 2.1 ± 0.3% (3d KCl + 12d Con) TH+ neurons]. To determine whether transient depolarization altered neuronal responsiveness to subsequent stimulation, E16.5 PG cultures were initially depolarized for 3 d (conditioning stimulus), returned to control medium for 9 d, and then depolarized a second time for 3 d (test stimulus). TH expression in these cultures was compared with that in cultures that received only the test stimulus during the last 3 d of the 15 d culture period. Controls included neurons cultured for the entire 15 d in medium without additional KCl, as well as neurons that received only the initial conditioning stimulus. Cultures grown in either of these control conditions contained few TH-immunoreactive neurons at the end of the culture period (<2.5% of the total population; Fig. 4, 15d Con and 3d KCl + 12d Con). Conversely, cultures that received the conditioning stimulus contained 29.7 ± 2.1% TH-positive neurons, a fourfold increase compared with cultures exposed to the test stimulus alone (Fig. 4, 7.8 ± 1.3%, 12d Con + 3d KCl). Total neuronal survival was equivalent in all experimental groups [NF+ cells/ganglion, 114 ± 6.5 (15d Con), 132 ± 10.3 (3d KCl + 12d Con), 124 ± 17.2 (12d Con + 3d KCl), and 130 ± 16.1 (3d KCl + 9d Con + 3d Con)].
E16.5 cultures examined immediately after 3 days of KCl treatment were refractory to the effect of KCl depolarization with time. (Fig. 1). These data indicate that at least some neurons became exhibiting TH immunoreactivity at the end of the culture period. Con indicated. Each neuron. Dissociated E16.5 PG neurons were cultured for a total of 15 d as holding potential of +40 mV. Depolarizing voltage steps (400 msec in duration) were applied from a depolarization-mediated gene expression, including regulation of TH (Kilbourne and Sabban, 1990; Menezes et al., 1996; Bito et al., 1997). To determine whether calcium entry is also important for short-term regulation and long-term potentiation of TH expression in developing sensory neurons, we first characterized the calcium currents present in E16.5-dissociated PG neurons using whole-cell recording techniques. We focused on PG neurons because these cells exhibited the greatest potential to express dopaminergic traits in response to depolarization (Fig. 1). These data indicate that at least some neurons became refractory to the effect of KCl depolarization with time.

**N-type channels carry a majority of the high voltage-activated calcium current in embryonic PG neurons**

Previous studies using the pheochromocytoma cell line PC12 demonstrated that calcium entry into the cell is required for depolarization-mediated gene expression, including regulation of TH (Kilbourne and Sabban, 1990; Menezes et al., 1996; Bito et al., 1997). To determine whether calcium entry is also important for short-term regulation and long-term potentiation of TH expression in developing sensory neurons, we first characterized the calcium currents present in E16.5-dissociated PG neurons using whole-cell recording techniques. We focused on PG neurons because these cells exhibited the greatest potential to express dopaminergic traits in response to depolarization (Fig. 1). Depolarizing voltage steps (400 msec in duration) were applied from a holding potential of −100 mV to potentials ranging from −80 to +40 mV. The current–voltage relationship consisted of two major components: a small, low threshold (−50 mV) rapidly activating and inactivating T-type current and a larger, high threshold (−30 mV) partially inactivating current. The average peak current evoked at 0 mV from the holding potential of −100 mV was 648 ± 169 pA (Fig. 5A). To further characterize the calcium currents, we repeated these experiments in the presence of selective calcium channel antagonists. Conotoxin (1 μM), an N-type calcium channel antagonist, reduced the average peak current by 68% to 196 ± 36 pA (Fig. 5C). In contrast, nimodipine (2 μM), which blocks L-type calcium currents, reduced the average peak current by only 16% to 539 ± 140 pA (Fig. 5B). To examine the voltage-dependent inactivation of calcium currents in PG neurons, we applied conditioning voltage steps ranging from −120 to 0 mV for 2 sec, before pulsing the neurons with a test voltage of 0 mV. The holding potential in these experiments was −100 mV. Inactivation of the calcium current began gradually but became steeply voltage dependent at conditioning voltages between −70 and −10 mV (Fig. 6). At the 0 mV step, most of the current was inactivated with only 6.7% of the peak current remaining. In the presence of Conotoxin (1 μM), a profound inhibition was observed at all conditioning voltages, whereas nimodipine (2 μM) had only a small inhibitory effect on total current at each step (Fig. 6).

**Calcium entry via L-type channels mimics depolarization-dependent induction of TH**

To determine whether calcium entry through a specific channel type is important for short-term regulation of TH expression, we exposed E16.5 PG neurons to KCl for 24 hr in the presence or absence of specific calcium channel antagonists. Application of Conotoxin (1 μM) had no effect on TH induction by KCl (Fig. 7). However, application of nimodipine (2 μM), nifedipine (1 μM), or verapamil (10 μM), L-type calcium channel antagonists, significantly inhibited depolarization-dependent induction of TH. Nimodipine completely abolished the TH response to elevated KCl (Fig. 7), whereas nifedipine and verapamil resulted in 37 and 51% inhibition, respectively (data not shown).

To examine whether activation of L-type channels was sufficient to increase TH expression, we exposed E16.5 PG neu-
rons to Bay K-8644, a selective L-type channel agonist (Nilius et al., 1985; Nowycky et al., 1985). Because Bay K-8644 is most effective when neurons are slightly depolarized, we first determined a KCl concentration that depolarized the neurons without altering TH expression. In the presence of 15 mM KCl, neurons were depolarized to approximately -42 mV (range between -41 and -43 mV; n = 4), but TH expression remained unchanged (Fig. 8). However, Bay K-8644, in the presence of 15 mM KCl, induced an increase in the percentage of TH⁺ neurons that was similar in magnitude to that produced by 40 mM KCl alone (Fig. 8). Moreover, at -40 mV, Bay K-8644 produced a substantial increase in calcium current (Fig. 9), indicating that the increase in TH expression observed in the presence of Bay K-8644 was indeed associated with an increase in calcium entry.
Long-term potentiation of TH regulation requires calcium entry through L-type channels

Because activation of L-type channels can mimic depolarization-dependent induction of TH expression in PG neurons, we wondered whether calcium entry was required for KCl-induced long-term potentiation of TH expression. Therefore, we examined whether blockade of L-type channels by nifedipine during the conditioning stimulus would alter the TH response to a subsequent KCl test stimulus. These experiments followed the same protocol as the conditioning experiments described above. At the end of the 15-day culture period, the percentage of TH neurons in cultures exposed to nifedipine (1 μM) during the initial 3 d of culture only. Comparisons were made using ANOVA followed by Scheffe’s test; *p < 0.05; ***p < 0.001.

DISCUSSION

Our data demonstrate that depolarizing stimuli and subsequent activation of L-type calcium channels can produce both short- and long-term changes in dopaminergic phenotypic expression in developing primary sensory neurons in vitro. These observations raise the possibility that activity-dependent mechanisms and transmitter differentiation may be linked. In particular, our findings indicate a critical period during which exposure to depolarizing stimuli can unmask a relatively widespread potential for dopaminergic expression, most notably in developing nodose and petrosal cranial sensory neurons. Moreover, the time course of neuronal responsiveness to depolarizing stimuli in vitro correlates well with developmental expression of dopaminergic traits in vivo.

Inhibition of long-term potentiation of TH expression by L-type calcium channel blockade. Dissociated E16.5 PG neurons were grown for a total of 15 d as indicated. Each bar shows the mean percentage (± SEM) of TH neurons exhibiting TH immunoreactivity at the end of the culture period. Black bars represent the percentage of TH-positive neurons in controls exposed to 1 μM nifedipine during the initial 3 d of culture only. Comparisons were made using ANOVA followed by Scheffe’s test; *p < 0.05; ***p < 0.001.
to develop a dopaminergic phenotype in vivo [a subset of carotid body afferents is also nondopaminergic (Finley et al., 1992); however, at least some, such as those that express SP, do not form synapses with chemoreceptor cells (Chen et al., 1986; Kummer et al., 1989)]. We hypothesize, therefore, that selective expression of dopaminergic traits by carotid body afferents, and not gustatory afferents, is related to the asynchronous functional maturation of these two populations of cells (Fig. 11). In particular, we propose that the early, tonic activity of carotid body afferents in vivo potentiates TH expression in a manner analogous to the effect of chronic depolarization in vitro. However, depolarization alone is unlikely to account completely for neuronal commitment to the dopaminergic phenotype, because the increase in TH expression induced by high KCl in vitro is reversible after return to control medium. Recent experiments in our laboratory support a role for activity-dependent mechanisms in regulating development of the sensory dopaminergic phenotype in vivo. Specifically, exposure of newborn rats to mild hypoxia, which increases carotid body chemosensory afferent impulse activity (Bisgard and Neubauer, 1995), significantly increases the number of PG neurons that express TH (J. T. Erickson, T. Hertzberg, and D. M. Katz, unpublished observations).

Our data indicate that activation of L-type calcium channels is sufficient to reproduce the effects of KCl depolarization on TH expression in developing sensory neurons. However, we cannot exclude a potential role for other channel subtypes as well. For example, the inability of ω-conotoxin to block depolarization-dependent induction of TH in PG neurons may reflect an inactivation of N-type channels during long-term exposure to high KCl. Specifically, neurons treated with 40 mM KCl exhibited membrane potentials of approximately −20 mV, and after a 2 sec conditioning pulse of −20 mV, only 28% of the total calcium current remained (Fig. 6). Alternatively, the induction of TH expression by depolarization might require calcium entry through specific channel subtypes, such as the L-type, and not through others, because of activation of channel-specific intracellular signaling cascades. Greenberg and colleagues have proposed such a mechanism based on studies of hippocampal neurons (Gallin and Greenberg, 1995; Ghosh and Greenberg, 1995). In these cells, calcium entry through either NMDA or L-type channels increases expression of the immediate-early response gene c-fos (Bading et al., 1993). However, in each case distinct signaling cascades are recruited to induce this expression; calcium/calmodulin-dependent (CaM) kinase is required for c-fos induction after activation of L-type channels but not after stimulation of NMDA receptors (Bading et al., 1993). In addition, unique promoter elements in the c-fos gene are required for calcium-induced c-fos expression, depending on whether calcium enters through L-type channels or NMDA receptors (Bading et al., 1993).

Our finding that calcium entry through L-type channels is required for the long-term effect of transient depolarization on TH induction suggests possible mechanisms by which this potentiation may occur. Early depolarization could change the sensitivity of second messenger pathways to subsequent calcium influx, a mechanism proposed by Schwartz and Greenberg (1987) as a molecular substrate for memory. CaM kinase II, which is required for some forms of long-term potentiation in the hippocampus (Malinow et al., 1989; Silva et al., 1992), has been proposed as a memory store because it can remain active in the absence of external stimuli (Lisman, 1994). Alternatively, depolarization of PG neurons may produce a lasting change in the transcription rate of the TH gene and thereby potentiate TH induction by subsequent stimulation.

Experiments in progress are designed to differentiate among these potential mechanisms.

Our finding that exposure to depolarizing stimuli can produce long-term phenotypic changes in developing sensory neurons is reminiscent of the sensitization observed in mature nociceptive afferents. During nociceptor sensitization, neuronal excitability is enhanced for many days after an intense stimulus (Levine et al., 1993). Coincident with this change in excitability, transmitter expression and release are increased (Levine et al., 1993). The mechanisms underlying these long-term changes are not well understood, although there is evidence that alterations in ion channels may be involved. For example, agents that sensitize sensory neurons increase Na+ current in DRG cells (Gold et al., 1996) and inhibit calcium-dependent K+ current in nodose neurons (Weinreich and Wonderlin, 1987). Our finding that L-type calcium channels are important for long-term potentiation of TH expression in developing sensory neurons raises the possibility that these channels may contribute to sensitization of transmitter expression in adult sensory neurons as well.
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