The Journal of Neuroscience, November 15, 1998, *18*(22):9294–9302

Depolarization Stimulates Initial Calcitonin Gene-Related Peptide Expression by Embryonic Sensory Neurons *In Vitro*

Xingbin Ai, Sally E. MacPhedran, and Alison K. Hall

Department of Neurosciences, Case Western Reserve University, Cleveland, Ohio 44106-4975

The neuropeptide calcitonin gene-related peptide (CGRP) is expressed by one-third of adult rat lumbar dorsal root ganglion (DRG) neurons, many of which mediate pain sensation or cause vasodilation. The factors that regulate the developmental expression of CGRP are poorly understood. Embryonic DRG neurons initially lack CGRP. When these neurons were stimulated in culture by serum or persistent 50 mM KCl application, the same percentage of CGRP-immunoreactive (CGRP-IR) neurons developed *in vitro* as was seen in the adult DRG *in vivo*. The addition of the L-type calcium channel blockers, 5 μM nifedipine or 10 μM verapamil, dramatically decreased the proportion of CGRP-IR neurons that developed, although the N-type calcium channel blocker, 2.5 μM ω-conotoxin, was less effective. By contrast, the sodium channel blocker 1 μM tetrodotoxin had no effect on CGRP expression after depolarization. Fura-2 ratiometric imaging demonstrated that mean intracellular free calcium levels increased from 70 to 135 nM with chronic depolarization, and the addition of nifedipine inhibited that increase. Only a subpopulation of neurons had elevated calcium concentrations during chronic depolarization, and they were correlated with CGRP expression. Key signal transduction pathways were tested pharmacologically for their role in CGRP expression after depolarization; the addition of the CaM kinase inhibitor KN-62 reduced the proportion of CGRP-IR neurons to basal levels. By contrast, protein kinase A and protein kinase C were not implicated in the depolarization-induced CGRP increases. These data suggest that depolarization and the subsequent Ca^{2+}-based signal transduction mechanisms play important roles in the *de novo* expression of CGRP by specific embryonic DRG neurons.

Key words: sensory ganglion; calcitonin gene-related peptide; depolarization; signal transduction; ion channel; calcium imaging

Adult sensory ganglia are composed of distinct neuronal populations, but the factors that generate this diversity remain unclear. For example, neuropeptides, including calcitonin gene-related peptide (CGRP), often are found in small-sized neurons that constitute approximately one-third of the dorsal root ganglion (DRG) and that can innervate skin and viscera (Lee et al., 1985; Gibbins et al., 1987; Molander et al., 1987; O'Brien et al., 1989; McCarthy and Lawson, 1990; Noguchi et al., 1990; Kashiba et al., 1991). CGRP is localized in polymodal nociceptors and is a potent vasodilator (Brain et al., 1985; Wallengren and Hakanson, 1987; Holzer, 1988; Scott, 1992). Although CGRP first appears in lumbar DRG *in vivo* when peripheral target connections are functional on embryonic day 18 (E18) (Narayanan et al., 1971; Saito, 1979; Kudo and Yamada, 1985; Marti et al., 1987; Kucera et al., 1988; Fitzgerald, 1991), cell culture studies with E14 rat DRG indicate that *de novo* CGRP expression occurs in the absence of those target contacts (Hall et al., 1997). Transcription and steady-state CGRP mRNA levels in cell lines can be upregulated by cAMP, forskolin, phorbol ester, and nerve growth factor or can be inhibited by glucocorticoids, retinoic acid, and vitamin D (deBustros et al., 1985, 1986; Haller-Brem et al., 1988; Naveh-Many and Silver, 1988; Lindsay and Harmar, 1989; Russo et al., 1992; Tverberg and Russo, 1992). Although these studies have identified candidate CGRP regulators, little is known about the initiation of CGRP expression in primary sensory neurons. At least one report suggests caution in assuming that primary sensory neurons and cell lines use equivalent CGRP regulatory mechanisms (Watson et al., 1995). The required combination of factors needed for the generation of CGRP containing neurons in the DRG remains poorly defined.

One important cue that can regulate neuropeptide expression is neuronal activity. In peripheral neurons and neural cell lines, membrane depolarization increases vasoactive intestinal peptide, substance P (Sun et al., 1992; Adler and Fink, 1993), neuropeptide Y (Higuchi et al., 1996), preprotachykinin mRNA (Noguchi et al., 1988), or pituitary adenylate cyclase-activating polypeptide (Brandenburg et al., 1997). In many neurons the elevation of extracellular potassium leads to an increase in intracellular calcium, which can interact with calmodulin and modulate calcium-binding enzymes such as CaM kinases, protein kinases, and adenylyl cyclases. These effectors for second messenger molecules then mediate cellular responses by activating transcription factors that alter immediate early and delayed response gene expression (Misra et al., 1994; Ghosh and Greenberg, 1995; Bito et al., 1997). In most cases, depolarization increases peptide expression in neurons that already synthesize the neuromodulator. By contrast, the early development of the catecholaminergic phenotype in some sensory neurons also can be regulated by neuronal activity (Hertzberg et al., 1995; Brosenitsch et al., 1998). It is not known whether depolarization can affect *de novo* peptide expression. Embryonic DRG neurons are spontaneously active during the period just preceding and during the formation of functional peripheral target contacts (Fitzgerald, 1987). Thus, early neuro-

Received June 1, 1998; revised Aug. 4, 1998; accepted Aug. 17, 1998.

This study was supported by National Institutes of Health (NS-30842 and NS-23678) and the March of Dimes (FY98-0506). We thank Drs. Richard Zigmond and Ben Strowbridge for helpful critiques of this manuscript.

Correspondence should be addressed to Dr. Alison K. Hall, Department of Neurosciences, Case Western Reserve University, School of Medicine, Cleveland, OH 44106-4975.

nal activity may play a critical role in the neuropeptide differentiation of sensory neurons.

To test the role of neuronal activity in the onset of CGRP expression and to elucidate factors that regulate CGRP expression, embryonic rat DRG neurons were cultured in chemically defined medium and depolarized them with 50 mM KCl. Because little is known about the link between calcium entry and the regulation of delayed response genes important for neuronal function, the role of calcium influx and calcium-mediated signaling pathways was examined by ratiometric imaging and by pharmacological analyses. These studies demonstrate that depolarization is sufficient to induce CGRP expression in DRG neurons. Further, molecular components of the signal transduction systems that regulate the onset of CGRP expression were identified.

MATERIALS AND METHODS

Cell culture. E14.5 DRG were dissected from Sprague Dawley rats (Zivic Miller, Zelienople, PA). The dissociation, plating, and survival assays were as described (Hall et al., 1997). Approximately 4000 DRG cells were plated in each 0.32 cm^2 well. Growth medium consisted of Neurobasal chemically defined medium (Life Technologies, Grand Island, NY) with B-27 serum-free supplement (Life Technologies), 3 mM glutamine, and nerve growth factor (NGF; 25 ng/ml, Austral Biological, San Ramon, CA). The potassium concentration of Neurobasal growth medium (NB) was 5 mM (NB5). To depolarize cells, we added a stock solution of 4 M KCl to vary the $[K^+]$ between 10 and 75 mM. In some cases, 5% heat-inactivated rat serum (RS) was added to L15/CO_2 medium with NGF. Iso-osmolar control medium consisted of 1% mannose in NB5 medium, which empirically produced an osmolarity equivalent to Neurobasal medium with 50 mM KCl (NB50).

In some experiments, pharmacological agents were added to cultures in NB50 at the time of plating, and one-half of the medium was exchanged with fresh medium every 1 or 2 d. The agents used were the following: 1 μM tetrodotoxin (TTX; Na^+ channel blocker; Calbiochem, La Jolla, CA) (Elliott and Elliott, 1993), 10 μM nifedipine or 10 μM verapamil (Calbiochem) (Gault and Siegel, 1997), 2.5 μM ω-conotoxin GVIA (ω-Con; Sigma, St. Louis, MO) (McCleskey et al., 1987), 1 μM {1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine} (KN-62; Calbiochem) (Tokumitsu et al., 1990), 2 μM {*N*-[2-((*p*-bromocinnamyl;)amino)ethyl]-5-isoquinolinesulfonamide, HCl} (H-89; Calbiochem) (Findik et al., 1995), and 1 μM bisindolylmaleimide III (BIM III; Calbiochem) (Toullec et al., 1991). For compounds dissolved in dimethyl sulfoxide (DMSO), the control cultures included 0.1% DMSO in the growth medium.

Immunocytochemistry. Cells were fixed in 4% paraformaldehyde and 0.1 M PO_4, pH 7.4, for 30 min at room temperature (RT). After being washed, the cells were permeabilized with dilution buffer containing 0.2% Triton X-100/PBS, 20% goat serum, and 0.01% sodium azide for 2 hr and then were incubated in primary antibody (rabbit anti-CGRP, 1:300; Amersham, Arlington Heights, IL) overnight at 4°C. After being rinsed, the secondary antibody (biotin-conjugated goat anti-rabbit IgG, 1:250; Chemicon, Temecula, CA) was applied to the cultures. The staining was visualized with avidin–horseradish peroxidase and a diaminobenzidine (DAB) chromagen. In each case at least 200 neurons were counted per well, and triplicate wells in each of at least two independent experiments were quantified. Data were compared between groups by using an unpaired Student's *t* test.

Calcium measurement. Intracellular free Ca^{2+} was monitored with the fluorescent dye fura-2 AM (Molecular Probes, Eugene, OR). Cells were grown on poly-L-lysine/laminin-coated No. 1 glass coverslips affixed to 60 mm Petri plates (Fisher Scientific, Pittsburgh, PA), and cells were loaded with 4 mM fura-2 AM in NB growth medium at 37°C for 20 min. After being washed, the cultures were replaced with fresh growth medium and maintained at 37°C for an additional 20 min to allow for complete hydrolysis of the dye. Coverslips then were placed on the stage of a Zeiss Axiovert 405M microscope (Oberkochen, Germany) prewarmed to 37°C, and intracellular fura-2 was excited at 350 and 380 nm with a xenon lamp. A Hamamatsu SIT camera or Princeton CCD camera was used to collect information. The fluorescence signal at 510 nm was collected, and the ratio of the fluorescence at the two excitation wavelengths was calculated by MetaFluor analysis software (Universal Imaging, West Chester, PA). Areas on the same coverslips without cells were recorded as background images. Intracellular free $[Ca^{2+}]$ was calculated according to Grynkiewicz et al. (1985): $[Ca^{2+}] = K_D \times F_{min}/F_{max} \times (R - R_{min}/R_{max} - R)$, where $K_D = 225$ nM, R is the ratio value, R_{min} is the ratio value in a calcium-free solution, R_{max} is the ratio value of a saturated calcium solution (1 mM $CaCl_2$), F_{min} is the fluorescence intensity of the calcium-free solution at 380 nm, and F_{max} is the intensity of a 1 mM calcium solution at 380 nm. Actual values were obtained by using point-to-point correlations obtained from specific free calcium standards between 0 and 40 μM (Molecular Probes). In some cases, cells cultured in NB50 for 4 d on a glass-etched grid (Bellco Glass, Vineland, NJ) were subjected to calcium imaging, followed by immunocytochemistry for CGRP.

RNA isolation and PCR. Total cellular RNA was isolated with RNAzol (Sigma) from each well of a six well plate containing 100,000 DRG cells. Five micrograms of total RNA was DNase I-treated and reverse-transcribed. Primers for CGRP (sense primer 5′-ATGCAGATGAAAGTCAGGGA-3′ and antisense primer 5′-GGGGCTATTATCTGTTCAAG-3′, recommended by Andy Russo, University of Iowa) or elongation factor 1α (sense 5′-TTCACTGCTCAGGTGATTATCC-3′ and antisense 5′-GGCAGCATCACCAGACTTCAAGA-3′) were added in 20 mM Tris-HCl, pH 8.4, 50 mM KCl, and 2 mM $MgCl_2$, layered with mineral oil, and subjected to 35 cycles of PCR amplification (denatured at 94°C for 45 sec, annealed at 55°C for 45 sec, and extended at 72°C for 1 min) in a PTC-200 Peltier thermal cycler. PCR products were separated in a 2% agarose gel and detected after ethidium bromide staining and UV illumination.

RESULTS

Embryonic sensory ganglia harvested before CGRP was detectable and before functional connection with targets had occurred *in vivo* subsequently became CGRP-immunoreactive (CGRP-IR) over time in serum-containing cultures, demonstrating that CGRP expression is an intrinsic property of a subpopulation of DRG neurons (Hall et al., 1997). However, this culture system was not appropriate for identifying factors that regulate CGRP expression because of the uncertainties as to the nature and activity of trophic substances within RS. To understand further the cellular and molecular mechanisms that regulate *de novo* CGRP expression, we developed a chemically defined culture system and investigated the factors required for CGRP expression.

Depolarization elicited CGRP expression in embryonic sensory neurons

To test the role of depolarization in CGRP expression, we placed E14.5 DRG cells in basal NB5 or depolarizing NB50 medium for 8 d, a period in which CGRP expression develops and stabilizes. The stimulus used, 40–50 mM KCl, is a common tissue culture manipulation used to mimic aspects of neuronal depolarization, and it can alter neuropeptide expression in a variety of neurons (for recent examples, see Rao et al., 1992; Higuchi et al., 1996; MacArthur and Eiden, 1996; Brandenburg et al., 1997; Brosenitsch et al., 1998). CGRP expression was examined with immunocytochemistry (Fig. 1*A*). Few neurons maintained in NB5 medium for 8 d were CGRP-IR (8% ± 0.8). In contrast, CGRP immunoreactivity was detected in approximately one-third of the neurons in depolarizing NB50 (29.3% ± 1.7). The same proportion of CGRP-IR neurons was present in NB50 and in serum-containing media ($p = 0.73$), and depolarization in the presence of RS did not increase the percentage of CGRP-IR neurons further ($p = 0.11$). From these data we infer that depolarization and serum affected a single responsive population of DRG neurons. Neuronal survival in all conditions was ~80% (Fig. 1*B*), suggesting that the changes in CGRP-IR neurons were attributable to CGRP regulation rather than to selective survival. To rule out the possibility that the increase in the percentage of CGRP-IR neurons in NB50 medium was attributable to an osmolarity change, we added 1% mannose to NB5 medium to

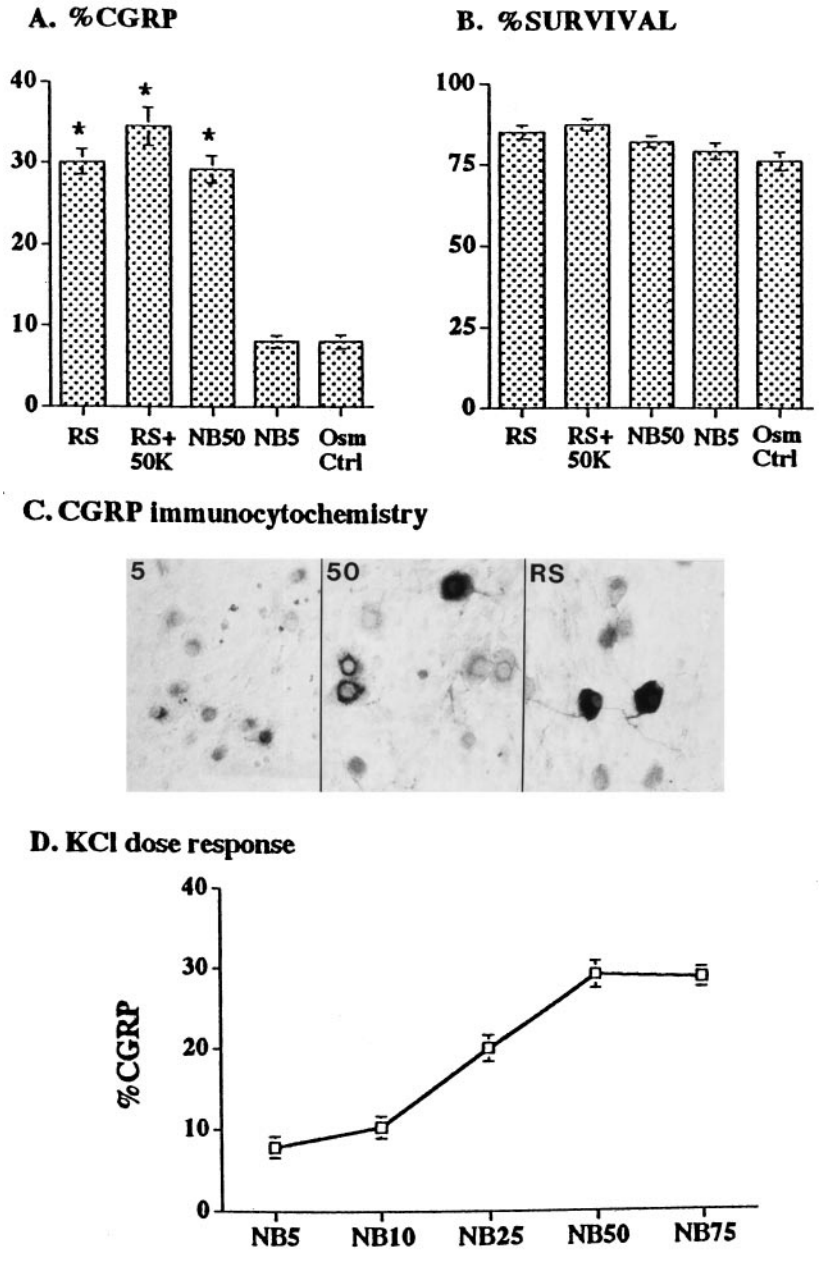

Figure 1. CGRP expression was induced by serum or depolarization. Dissociated lumbar E14.5 DRGs were plated in basal defined NB5 medium containing 5 mM KCl or in depolarizing NB50 medium containing 50 mM KCl and compared with cultures in rat serum (*RS*). After 8 d the CGRP-IR neurons were quantified. *A*, The percentage of CGRP-IR neurons. In either depolarizing medium or in serum a similar proportion of neurons expressed CGRP, and the effects of depolarization and serum were not additive. By contrast, few neurons expressed CGRP in NB5 medium or in the iso-osmolarity control. *Asterisks* indicate the conditions under which CGRP-IR neurons were equivalent ($p > 0.1$) and that also differed from basal NB5 values ($p < 0.0001$). *B*, Cell survival was good in all conditions, with ~80% of neurons alive after 8 d. Four independent experiments with each variable in triplicate were performed for *A* and *B*. *C*, CGRP immunocytochemistry in cultures with NB5 (*5*), NB50 (*50*), and *RS*. Few neurons in NB5 were lightly CGRP-immunoreactive (CGRP-IR). In NB50, some neurons had intense staining in the cell body and particularly in the perinuclear region. Intense CGRP staining was observed in the whole cell body of some neurons cultured in *RS*. *D*, The proportion of CGRP-IR neurons increased with increased KCl concentration. The concentration of KCl was varied from basal levels (*NB5*) to medium containing 75 mM KCl (*NB75*); CGRP-IR neurons were counted after 8 d in culture. Five or six independent experiments with each concentration were performed in triplicate. Approximately 85% of the neurons survived in each condition.

produce a control medium with osmolarity empirically similar to NB50; in such mannose-containing medium, CGRP expression remained as low as that in basal NB5 medium ($p = 0.98$). Neurons cultured in different media maintained their pseudounipolar morphology (Fig. 1*C*), although their overall size and CGRP distribution varied. In depolarizing medium, neuronal perikarya were approximately the same diameter as in RS but were bigger than those in basal medium. In serum-containing cultures, CGRP immunoreactivity often was detected throughout the whole cell body and processes. However, the majority of neurons cultured in depolarizing NB50 had CGRP immunoreactivity in a perinuclear, Golgi-like distribution.

To determine how much KCl was required to induce CGRP immunoreactivity, we varied the concentration of KCl in Neurobasal growth medium. Increases in CGRP-IR neurons were observed only when KCl was applied at a concentration of 25 mM or higher (Fig. 1*D*). The maximal induction of CGRP expression was reached when cells were cultured in NB growth media containing 50 mM KCl. No further increase of the percentage of CGRP-IR neurons was detected at a KCl concentration of 75 mM. Because the maximal induction of CGRP expression in embryonic sensory neurons was reached with 50 mM KCl (Fig. 1*D*), NB50 was used in subsequent studies.

Maintained depolarization was required for increased CGRP expression

To learn whether depolarization-induced increases in CGRP expression required a transient or prolonged signal or if neurons had a critical period in which stimulation must occur, we exposed cells to depolarizing NB50 medium for portions of the culture period and then switched to the basal NB5 medium, or vice versa. After a total of 8 or 10 d in culture, cells were fixed and stained for CGRP (Fig. 2). No difference in cell survival was observed in any condition, such that ~80% neurons present on day 1 were

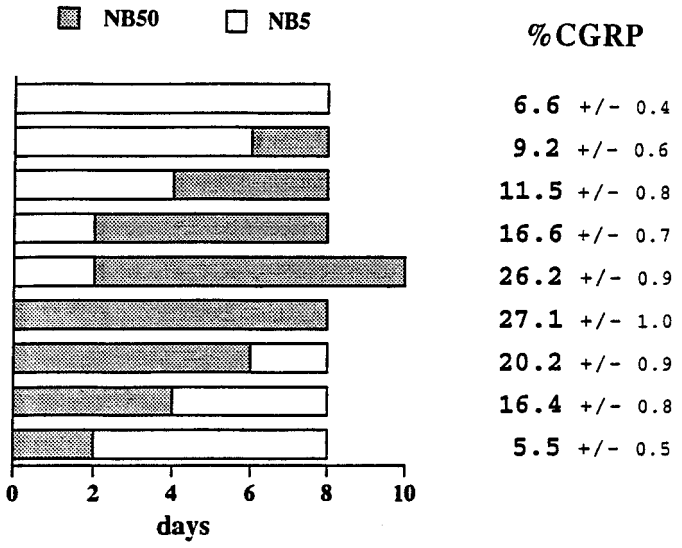

Figure 2. Maintained depolarization was required to elicit CGRP expression. Dissociated E14.5 DRG cells were maintained in NB5 or NB50. Growth media were switched at days 2, 4, or 6. After a total of 8 or 10 d the cultures were processed for CGRP immunocytochemistry. The proportion of CGRP-IR neurons was quantified, and the data represent the mean and SEM of eight independent experiments. The highest proportion of CGRP-IR neurons was seen with 8 d of depolarization (chronic or applied after 2 d in NB5 had the same results; $p = 0.73$). CGRP expression decreased if NB50 was removed during the culture period.

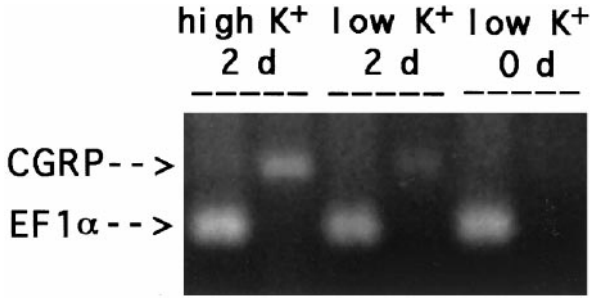

Figure 3. CGRP mRNA increased in DRG cultures with depolarization. Dissociated E14.5 DRG cells were placed in tissue culture in basal or depolarizing conditions. At the time indicated, total RNA was extracted and prepared for RT-PCR with primers specific for α-CGRP or the transcription factor elongation factor 1α (EF1α). In control cultures total RNA was extracted immediately (low K^+, 0 d), and no CGRP mRNA was detected. After 2 d, CGRP mRNA was present in depolarizing (high K^+, 2 d) and basal (low K^+, 2 d) conditions. This result was obtained in three independent assays.

alive after 8 or 10 d in culture. The highest level of CGRP expression was observed in cultures maintained in NB50 for 8 d. At least 4 d in depolarizing medium were required to increase the proportion of CGRP-IR neurons above basal levels. The proportion of CGRP-IR neurons depended on the length of time in depolarizing medium and was not influenced by whether depolarization occurred early or late in the culture period. These data suggest that neurons maintained in basal medium for at least 4 d remained competent to respond to depolarizing signals delivered later in the culture period and that there did not appear to be a critical period during which depolarization had an effect.

Depolarization *in vitro* increased the amount of CGRP mRNA in DRG cultures (Fig. 3). A 4 d period was required for sufficient CGRP peptide to be present in the cells so that it could be detected by immunohistochemistry. CGRP mRNA is likely to be present before peptide can be detected. For that reason, to learn if CGRP mRNA was affected by treatments even before peptide changes were apparent, we assayed cultures at earlier times. CGRP mRNA was assayed by RT-PCR from DRG cultures grown in NB5 or NB50 and compared with the expression of the transcription factor elongation factor 1α mRNA. As expected, no CGRP mRNA was present in dissociated DRG from which total RNA was immediately extracted. However, CGRP mRNA was present in both depolarized and nondepolarized neurons after 2 d.

L-type calcium channel activity was necessary for depolarization-induced CGRP expression

Because depolarization leads to ionic fluxes across the plasma membrane, we examined the possibility that voltage-activated calcium or sodium channels were required for the CGRP response by adding specific pharmacological agents to NB50 (Table 1). DRG neurons express a variety of calcium and sodium ion channels (Fedulova et al., 1985, 1994; Scroggs and Fox, 1991, 1992; Ogata and Taebayashi, 1992; Elliott and Elliott, 1993). The addition of the L-type calcium channel blockers, 10 μM nifedipine or verapamil, for the entire culture period reduced the proportion of CGRP-IR neurons in depolarizing medium by 60 and 75%, respectively, suggesting that calcium influx is important for CGRP expression. The effect of nifedipine was concentration-dependent, with similar reductions in CGRP expression observed at 20, 10, and 5 μM nifedipine (unpaired t test, $p > 0.1$), but in control CGRP expression was observed at 1 μM nifedipine ($p > 0.4987$; from triplicate wells in two experiments). The N-type Ca^{2+} channel blocker ω-conotoxin reduced CGRP expression, but less effectively than L-type blockers. By contrast, the inhibition of sodium channels with tetrodotoxin had little or no effect on CGRP expression. It is important to note that neuronal survival remained high even in these drugs, indicating no overt effects on survival.

Neurons had elevated intracellular calcium in depolarized cultures

To test that calcium was involved in increased CGRP expression under chronic depolarizing conditions, we visualized intracellular free calcium concentrations by ratiometric imaging with fura-2 AM (Fig. 4). In general, intracellular free calcium levels were low in unstimulated cultures (NB5). Chronically depolarized neurons (NB50 at 4 d) had higher mean intracellular free calcium levels. The average intracellular calcium concentration in neurons cultured in NB5 for 4 d was 70 nM, whereas depolarized neurons had an average intracellular calcium concentration of 135 nM. The calcium elevation in depolarized cultures was inhibited completely by the L-type calcium channel blocker nifedipine (Fig. 4*D*).

Depolarized neurons not only had an increased mean calcium level, but a subpopulation of depolarized DRG neurons at 4 d had fura-2 fluorescence ratios higher than any neurons observed in nondepolarizing media (Fig. 4*C,E*). The responsive "higher calcium" population with mean values above 0.2 μM was not the only group that could respond to depolarizing signals. When cultures in basal NB5 medium were depolarized with 50 mM KCl for 2 hr before being loaded with fura-2 AM, all neurons responded uniformly, and the mean intracellular free calcium increased to 597 nM. Thus, all neurons in these cultures were capable of responding to short-term depolarization, but only a subpopula-

Table 1. CGRP expression after depolarization required Ca^{2+} channel activity

Treatment	Blocks	Survival (%)	CGRP (%)
NB5	—	109.7 ± 8.2	7.0 ± 0.6
NB50	—	85.3 ± 2.2	26.4 ± 1.6
NB50 + tetrodotoxin	Na^+ channel	81.4 ± 3.6	21.4 ± 1.6
NB50 + ω-conotoxin	N-type Ca^{2+} channel	86.4 ± 3.6	19.1 ± 2.3*
NB50 + verapamil	L-type Ca^{2+} channel	89.6 ± 1.6	14.4 ± 0.7**
NB50 + nifedipine	L-type Ca^{2+} channel	85.9 ± 0.6	12.0 ± 0.7***

E14.5 DRG cells were maintained in NB50 medium with 1 μM tetrodotoxin, 2.5 μM ω-conotoxin GVIA, 10 μM verapamil, or 10 μM nifedipine for 8 d before immunocytochemistry for CGRP. The percentage of CGRP-IR and surviving neurons was determined. Cell survival in all conditions was good, with 80% of neurons alive after 8 d (unpaired t test, $p > 0.20$). CGRP expression was inhibited by nifedipine, verapamil, and ω-conotoxin GVIA. In contrast, tetrodotoxin had no effect (unpaired t test, $p = 0.14$). Similar values were obtained in at least three independent experiments for each condition. Data are mean and SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$.

tion exhibited elevated intracellular calcium levels after chronic stimulation.

To test the hypothesis that these "higher calcium" neurons expressed CGRP, we grew neurons on gridded coverslips in NB50 medium for 4 d. The neurons were subjected to calcium imaging, followed immediately by immunocytochemistry for CGRP. E14 neurons grown for 4 d have only just begun to express CGRP, and the peptide is detectable by immunochemistry in approximately one-half the neurons that will eventually be CGRP-IR at 8 d *in vitro* (Hall et al., 1997). Nonetheless, CGRP-IR neurons were correlated with neurons that had higher free calcium levels (Fig. 5). In a total of 737 neurons from four independent experiments, 63 were CGRP-IR and 181 were "higher calcium" neurons. Twenty-eight CGRP-IR neurons were in the "higher calcium" subpopulation. Thus, higher calcium neurons were twice as likely to be CGRP-IR as average calcium neurons (15.1% ± 2.7 SEM of high Ca^{2+} neurons were CGRP-IR, whereas 6.8% ± 1.4 SEM of non-high Ca neurons visualized at 4 d were CGRP-IR; $p = 0.028$).

By contrast, the addition of the calcium ionophore ionomycin did not increase CGRP immunoreactivity. Ionomycin (1 μM) application rapidly increased free calcium levels (mean free calcium concentration ~360 nM) as compared with control cultures (~50 nM), but chronic application of ionomycin for 2 or 8 d did not increase CGRP expression or affect survival (data not shown). These data support the notion that the route of calcium entry may be important for neuronal phenotypic differentiation (Ghosh and Greenberg, 1995).

Depolarization-induced CGRP expression was dependent on CaM kinase pathways

To begin to understand which signaling cascades were involved in the depolarization-induced increase of CGRP, we added agents that inhibit CaM kinases, PKA or PKC, to depolarizing NB50 medium. In general, neuronal survival was good in all drugs, although the PKC inhibitor BIM III (Table 2) reduced neuronal survival ($p < 0.05$). KN-62 (1 μM), which inhibits CaM kinase (Tokumitsu et al., 1990; Enslen et al., 1994), completely blocked the effects of 50 mM KCl on CGRP expression such that the percentage of CGRP-IR neurons dropped to basal levels. In fact, KN-62 concentrations between 0.1 and 2 μM were equally effective in blocking CGRP increases ($p < 0.45$). By contrast, H-89, a selective PKA inhibitor, did not have inhibitory effects at 2 μM ($p > 0.05$). This concentration of H-89 is sufficient to inhibit PKA activity in other studies (Findik et al., 1995). Although the PKC inhibitor BIM III reduced CGRP-IR neurons by ~40% ($p < 0.05$), this effect may have been the result of differential neuronal survival. The concentrations of KN-62 and H-89 used in these experiments were close to or in excess of their pKi values of 0.9 and 0.048 μM (Chijiwa et al., 1990; Tokumitsu et al., 1990). These studies suggest that depolarization-mediated effects on CGRP induction are mediated via calcium and a calcium/calmodulin kinase signal transduction pathway.

Whereas in some cases KN-62 can block L-type calcium channels (Li et al., 1992), the addition of this drug in short-term studies did not alter free intracellular calcium increases after depolarization. To determine whether KN-62 altered free calcium, we examined DRG neurons cultured for 2 d in nondepolarizing medium for free calcium changes after depolarization with NB50 and after depolarization in the presence of nifedipine or KN-62. The addition of KCl to raise the potassium concentration to 50 mM resulted in rapid increases in free calcium (average fluorescence ratio, 0.39 ± 0.17 SEM) in most neurons at 20 min after stimulation. The addition of nifedipine, which blocks L-type channels, reduced intracellular calcium levels (to average fluorescence ratios of 0.22 ± 0.01 SEM), whereas intracellular calcium fluorescent ratios with KN-62 were unaffected (0.35 ± 0.19 SEM; $p < 0.0711$). Thus, the reduction in CGRP expression with the addition of KN-62 resulted from an inhibition of a CAM kinase pathway rather than from an alteration in free calcium.

DISCUSSION

The same proportion of CGRP-IR neurons that develop *in vivo* (Hall et al., 1997) was elicited *in vitro* by stimulating embryonic sensory neurons with high potassium, suggesting that membrane depolarization and perhaps neuronal activity play critical roles in the initial development of this sensory neuron phenotype. This observation not only confirmed our previous finding that initial CGRP expression is independent of target tissues but also provides an important model system for investigating the cellular and molecular mechanisms that regulate *de novo* CGRP expression. Our analysis with pharmacological agents and calcium ratiometric imaging suggested that intracellular calcium changes and subsequent calcium-based signal transduction pathways were required for activity-induced CGRP expression in embryonic sensory neurons. An important aspect of this study is that it links depolarization with neuropeptide regulation via a CaM kinase pathway.

These experiments make no assumption about the order of action of particular signaling components and are intended to begin to identify "players" in the signaling cascade. Given the lag time between depolarization and the observed increases in CGRP immunoreactivity, a complex cascade of intervening

A

	Mean Ratio	Mean Conc. (nM)	Count
NB5	.214 +/-.039	70	331
NB50+Nif	.188 +/-.040	50	316
NB50	.290 +/-.126	135	433

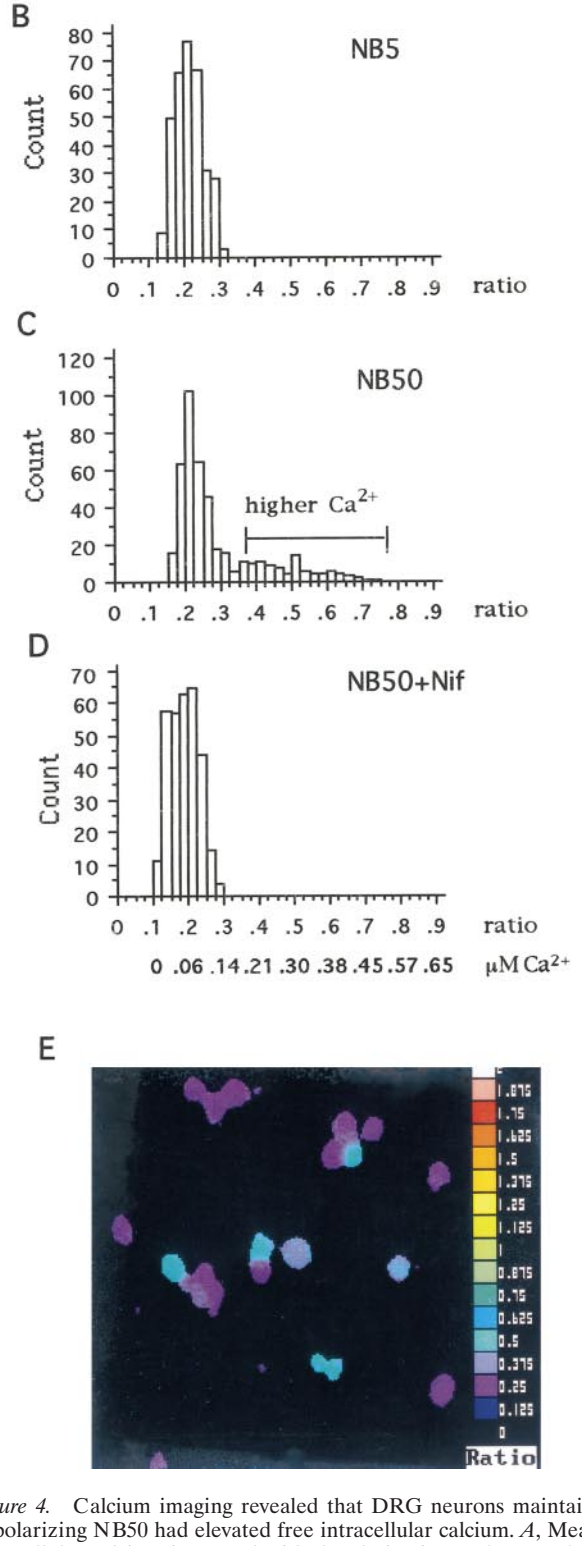

Figure 4. Calcium imaging revealed that DRG neurons maintained in depolarizing NB50 had elevated free intracellular calcium. *A*, Mean free intracellular calcium increased with depolarization and was reduced to basal levels after nifedipine treatment. Free intracellular Ca^{2+} levels were measured in neurons at day 4. DRG cells were grown for 4 d in NB5, NB50, or NB50 with 10 μM nifedipine before they were loaded with fura-2 AM. Free [Ca^{2+}] inside the cell was quantified. At least 300 neurons from three independent experiments were analyzed for each condition. *B–D*, Ratio histograms. The distributions of free calcium values from neurons cultured in NB5, NB50, or NB50 with 10 μM nifedipine for 4 d are shown in histograms. Most neurons in NB50 had ratios similar to those in NB5, whereas a distinct subpopulation had "higher Ca^{2+}" levels. *E*, Free calcium levels were higher in a subpopulation of neurons cultured in NB50 for 4 d, shown in the ratio measurement with *light blue* pseudocolor. Similar observations were obtained in six independent experiments.

events involving changes in second and third messengers may be very time-dependent.

Depolarization of developing sensory neurons in high potassium *in vitro* may mimic physiological processes. Sensory afferents of DRG neurons have a high level of activity during embryogenesis, but not at birth or in the adult (Fitzgerald, 1987). *In vivo*, lumbar DRG neurons begin to fire on E16, and the firing peaks at E18/19, just the time period in which the functional contacts have been established and CGRP first can be detected. The firing may be triggered by chemical stimuli from peripheral targets or membrane properties of the DRG neurons themselves (Scott, 1992).

In our study, Ca^{2+} entry via L-type calcium channels was important for depolarization-induced CGRP expression in embryonic sensory neurons. Voltage-dependent calcium channels, especially L-type channels, have a long open time and are implicated in increasing the expression of *c-fos* (Greenberg et al., 1986; Bading et al., 1993; Misra et al., 1994), tyrosine hydroxylase (Brosenitsch et al., 1998), nicotinic ACh receptor subunit genes (DeKoninck and Cooper, 1995), and GABA receptor genes (Gault and Siegel, 1997). Because L-type calcium channels require strong depolarization for activation, they usually are triggered experimentally in cultured cells by increasing extracellular potassium to 50 mM. In the present study, nifedipine and verapamil dramatically decreased the proportion of CGRP-IR neurons. The blockade of Ca^{2+} entry by nifedipine was confirmed by direct visualization of free intracellular Ca^{2+} with calcium imaging on cultures at day 4. Although their effects were dramatic, none of these calcium channel blockers showed complete inhibition of the depolarization-induced CGRP expression. One explanation for the partial effect by channel blockers is that the activation of CGRP expression by depolarization was not linked to a single type of voltage-activated Ca^{2+} channel. Another possibility is that calcium-induced calcium release from intracellular stores contributes to the intracellular free Ca^{2+} level (Clapham, 1995). This notion may be unlikely, because ionomycin treatment did not increase CGRP. A third possibility is that a Ca^{2+}-independent mechanism also contributes to CGRP induction after depolarization. Future studies using combinations of drugs or agents that deplete intracellular Ca^{2+} stores will differentiate among these possibilities. Although our data implicate calcium entry via L-type calcium channels in CGRP regulation, the activity of other calcium channels cannot be ruled out. Chronic depolarization by high potassium is likely to inactivate other channels, including the N-type channels. By contrast, L-type calcium channels have been implicated in the regulation of a number of neuronal genes (Higuchi et al., 1996; Gault and Siegel, 1997; Brosenitsch et al., 1998).

The elevation of intracellular calcium in DRG neurons maintained in depolarizing conditions may reflect calcium entry through voltage-sensitive or ligand-gated channels as well as cal-

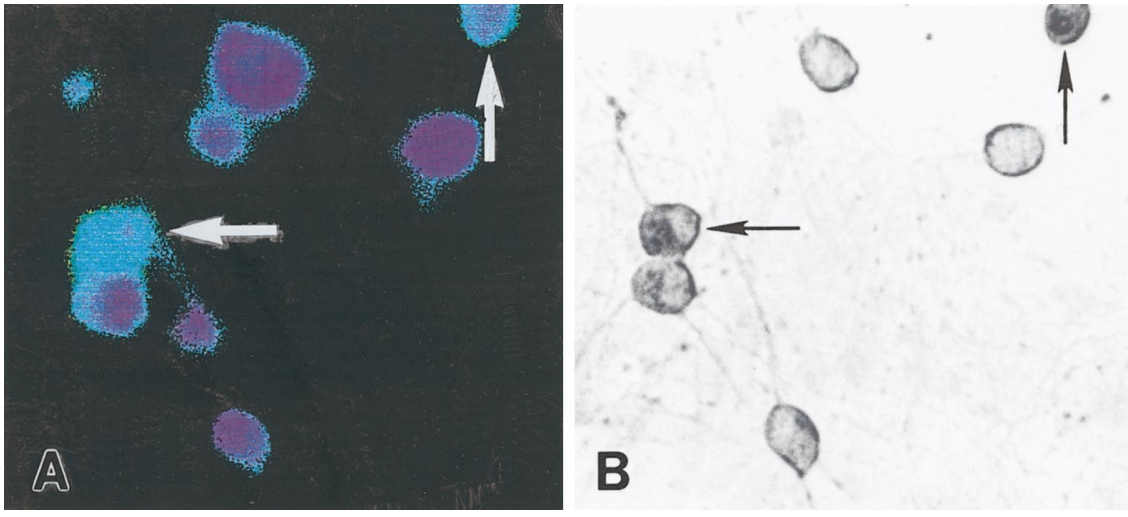

Figure 5. Higher intracellular calcium levels were correlated with CGRP immunoreactivity. DRG cells were grown for 4 d in depolarizing NB50 on gridded coverslips, and free intracellular calcium was visualized by fura-2 fluorescence ratiometric imaging (*A*). Although many neurons had basal free calcium levels, some contained higher free calcium and appeared *blue* in pseudocolor (*arrows*). The cells were fixed and processed for CGRP immunocytochemistry (*B*), and the same neurons were located on gridded coverslips. Peptide expression was only just detectable at this early stage and appeared in a perinuclear, Golgi-like location in neurons. CGRP immunoreactivity was more likely to be found in neurons with elevated calcium (*arrows*).

Table 2. CaM kinases were required for increased CGRP expression after depolarization

Treatment	Blocks	Survival (%)	CGRP (%)
NB5	—	91.2 ± 6.5	8.6 ± 1.0
NB50	—	83.7 ± 6.9	31.4 ± 1.6
NB50 + KN-62	CaM kinase	85.3 ± 4.5	6.1 ± 2.4***
NB50 + Bim III	PKC	67.5 ± 2.7	20.1 ± 1.8**
NB50 + H-89	PKA	93.2 ± 8.2	24.7 ± 4.3

Dissociated E14.5 DRG cells were grown in NB5 or NB50 for 8 d before immunocytochemistry for CGRP. Kinase inhibitors, 1 μM KN-62, 1 μM Bim III, or 1 μM H-89, were applied at the time of plating, and the medium was replenished every 1–2 d. Complete inhibition of CGRP expression was observed with KN-62 (unpaired *t* test, $p < 0.0001$). Cell survival was good except in cultures in Bim III (unpaired *t* test, $p < 0.05$); thus, partial CGRP inhibition by Bim III may be the result of differential neuronal survival. No inhibition was detected with H-89 (unpaired *t* test, $p > 0.05$). Values obtained represented the mean and SEM of three independent experiments. $^{**}p < 0.001$; $^{***}p < 0.0001$.

cium release from intracellular stores. DRG neurons also express a variety of receptor-mediated Ca^{2+} channels, such as NMDA receptors, that can be associated with depolarization-induced gene expression (Bading et al., 1993). However, the addition of 100 μM AP-V, a potent and selective NMDA receptor blocker, failed to inhibit CGRP expression (data not shown), suggesting that NMDA receptor was not involved in depolarization-induced CGRP expression in developing sensory neurons.

CGRP expression in depolarized sensory neurons was detected when the mean free intracellular Ca^{2+} level was elevated in the population. Indeed, a distinct subset of "higher Ca^{2+}" neurons in depolarized cultures at day 4 was correlated with the CGRP-IR neurons just detectable in the population. Because the calcium measurements we have done revealed the intracellular Ca^{2+} level at the specific time these neurons were imaged, it is not clear whether this subset of "higher Ca^{2+}" neurons differed uniquely from others by their electrical properties so that they were capable of maintaining high calcium levels or if they represented a changing subpopulation of DRG neurons that happened to have higher free calcium levels at that instant. It will be important to observe calcium changes over time in these cultures to learn whether neurons sustain increased calcium for long periods. Interestingly, no such "higher Ca^{2+}" subsets were observed in depolarized cultures at day 2 (data not shown), further suggesting that calcium homeostasis changes over time in culture.

A common mechanism by which elevated intracellular Ca^{2+} regulates gene expression is by the activation of calmodulin. The calcium/calmodulin complex then binds and modulates multiple regulatory proteins, such as CaM kinases and calcineurin (Enslen and Soderling, 1994; Bito et al., 1996, 1997). KN-62, which abolished the increase in CGRP caused by depolarization, blocks CaM kinase IV and V as well as CaM kinase II (Tokumitsu et al., 1990; Enslen et al., 1994), a finding that suggests that membrane depolarization acts via CaM kinase pathways to initiate CGRP expression. One CaM kinase, CaM kinase IV, is localized in many DRG neurons (Sakagami et al., 1994) and is implicated in regulating gene expression (Sun et al., 1996). Previous studies on CaM kinase IV in embryonic and adult DRG indicated that it is detectable in rat DRG as early as E15 and is expressed preferentially in small neurons in sensory ganglion (Sakagami et al., 1994; Ji et al., 1996). However, CaM kinase IV expression is not well correlated with CGRP in adult DRG neurons and was not confined to CGRP-IR neurons in our RS-containing cultures (data not shown). It is important to recognize that pharmacological probes, but not direct biochemical enzyme assays, implicate these messengers in effecting CGRP expression in the developing DRG. Alternatively, changes in neuronal gene expression can be regulated by neuronal activity via the phosphorylation of cAMP response element-binding protein (CREB) by various Ca^{2+}-dependent protein kinases, including CaM kinases (Misra et al., 1994; Bito et al., 1996). However, our preliminary immunocytochemical study with antibodies against pCREB did not reveal a correlation of pCREB with CGRP at 4 d (data not shown), suggesting either that the depolarization-induced CGRP expression is not mainly attributable to the activation of CREB or that pCREB-mediated events occurred at earlier times.

Stimulation by depolarization or serum resulted in the same proportion of CGRP-IR neurons with a similar onset of expression, suggesting that these stimuli are eliciting the phenotype in a

competent population rather than inducing plastic populations of neuronal precursors to express CGRP. For this reason, to understand how that subpopulation becomes defined during development, we must address which factors restrict CGRP to one-third of DRG neurons. It is not clear which active agent in RS results in CGRP expression.

Although this study demonstrates changes in CGRP immunoreactivity that follow specific stimuli, it reflects steady-state CGRP amounts and does not identify which cellular processes limit the CGRP changes. In particular, it is not clear if depolarization directly increases transcription or if increases in CGRP immunoreactivity reflect changes in peptide processing. The amount of CGRP mRNA present after 2 d of stimulation is increased with high KCl, suggesting but not proving that transcriptional events are regulated by depolarization. It is unlikely that depolarization increases peptide storage, however, because depolarization generally causes peptide release. CGRP gene expression is regulated in part at the transcriptional level, as indicated by promoter analysis in thyroid C cell lines (deBustros et al., 1985, 1986; Haller-Brem et al., 1988; Naveh-Many and Silver, 1988; Lindsay and Harmar, 1989; Russo et al., 1992; Tverberg and Russo, 1992). The 5′ flanking sequence of the calcitonin/CGRP gene contains a cAMP-responsive element (CRE; Watson and Latchman, 1995) and an E-box/helix–loop–helix (HLH) enhancer region (Peleg et al., 1990; Tverberg and Russo, 1993). It is interesting to speculate that the E-box/HLH imparts depolarization sensitivity of CGRP expression, because this motif acts as a depolarization response element in other neuronal genes (Su et al., 1995; Higuchi et al., 1996; Walke et al., 1996).

In combination, these data demonstrate that CGRP expression during embryonic development of the DRG is sensitive to depolarization and raise the possibility that neuronal activity plays an important role in the differentiation of DRG neuronal phenotypes.

REFERENCES

Adler EM, Fink JS (1993) Calcium regulation of vasoactive intestinal polypeptide mRNA abundance in SH-SY5Y human neuroblastoma cells. J Neurochem 61:727–737.

Bading H, Ginty DD, Greenberg ME (1993) Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. Science 260:181–186.

Bito H, Deisseroth K, Tsien RW (1996) CREB phosphorylation and dephosphorylation: a Ca stimulus duration-dependent switch for hippocampal gene expression. Cell 87:1203–1214.

Bito H, Deisseroth K, Tsien RW (1997) Ca^{2+}-dependent regulation in neuronal gene expression. Curr Opin Neurobiol 7:419–429.

Brain SD, Williams TJ, Tippins JR, Morris HR, MacIntyre I (1985) Calcitonin gene-related peptide is a potent vasodilator. Nature 313:54–56.

Brandenburg CA, May V, Braas KM (1997) Identification of endogenous sympathetic neuron pituitary adenylate cyclase-activating polypeptide (PACAP): depolarization regulates production and secretion through induction of multiple propeptide transcripts. J Neurosci 17:4045–4055.

Brosenitsch TA, Salgado-Commissariat D, Kunze DL, Katz DM (1998) A role for L-type calcium channels in short-term regulation and long-term potentiation of tyrosine hydroxylase expression in developing primary sensory neurons. J Neurosci 18:1047–1055.

Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T, Hidaka H (1990) Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase *N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. J Biol Chem 265:5267–5272.

Clapham D (1995) Calcium signaling. Cell 80:259–268.

deBustros A, Baylin SB, Berger CL, Rosos BA, Leong SS, Nelkin BD (1985) Phorbol esters increase calcitonin gene transcription and decrease *c-myc* mRNA levels in cultured human medullary thyroid carcinoma. J Biol Chem 260:98–104.

deBustros A, Baylin SB, Levine MA, Nelkin BD (1986) Cyclic AMP and phorbol esters separately induce growth inhibition, calcitonin secretion, and calcitonin gene transcription in cultured human medullary thyroid carcinoma. J Biol Chem 261:8036–8041.

DeKoninck P, Cooper E (1995) Differential regulation of neuronal nicotinic ACh receptor subunit genes in cultured neonatal rat sympathetic neurons: specific induction of $\alpha 7$ by membrane depolarization through a Ca/calmodulin-dependent kinase pathway. J Neurosci 15:7966–7978.

Elliott AA, Elliott JR (1993) Characterization of TTX-sensitive and TTX-resistant sodium currents in small cells from adult rat dorsal root ganglion. J Physiol (Lond) 463:39–56.

Enslen H, Soderling TR (1994) Roles of calmodulin-dependent protein kinases and phosphatase in calcium-dependent transcription of immediate early genes. J Biol Chem 269:20872–20877.

Enslen H, Sun P, Brickey D, Soderling SH, Klamo E, Soderling TR (1994) Characterization of Ca^{2+}/calmodulin-dependent protein kinase IV. J Biol Chem 269:15520–15527.

Fedulova SA, Kostyuk PG, Veselovsky NS (1985) Two types of calcium channels in the somatic membrane of newborn rat dorsal root ganglion neurones. J Physiol (Lond) 359:431–446.

Fedulova SA, Kostyuk PG, Veselovsky NS (1994) Comparative analysis of ionic current in the somatic membrane of embryonic and newborn rat sensory neurons. Neuroscience 58:341–346.

Findik D, Song Q, Hidaka H, Lavin M (1995) Protein kinase A inhibitors enhance radiation-induced apoptosis. J Cell Biochem 57:12–21.

Fitzgerald M (1987) Spontaneous and evoked activity of fetal primary afferents *in vivo*. Nature 326:603–605.

Fitzgerald M (1991) A physiological study of the prenatal development of cutaneous sensory inputs to dorsal horn cells in the rat. J Physiol (Lond) 432:473–482.

Gault L, Siegel R (1997) Expression of the $GABA_A$ receptor δ-subunit mRNA is selectively modulated by depolarization in cultured rat cerebellar granule neurons. J Neurosci 17:2391–2399.

Ghosh A, Greenberg ME (1995) Calcium signaling in neurons: molecular mechanisms and cellular consequences. Science 268:239–247.

Gibbins IL, Furness JB, Costa M (1987) Pathway-specific patterns of the co-existence of substance P, calcitonin gene-related peptide, cholecystokinin, and dynorphin in neurons of the dorsal root ganglia of the guinea-pig. Cell Tissue Res 248:417–437.

Greenberg ME, Ziff EB, Greene LA (1986) Stimulation of neuronal nicotinic acetylcholine receptors induces rapid gene transcription. Science 234:80–83.

Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450.

Hall AK, Ai X, MacPhedran SE, Nduaguba CO, Robertson CP (1997) The generation of neuronal heterogeneity in a rat sensory ganglion. J Neurosci 17:2775–2784.

Haller-Brem S, Muff R, Fischer JA (1988) Calcitonin gene-related peptide and calcitonin secretion from a human medullary thyroid carcinoma cell line: effects of ionomycin, phorbol ester, and forskolin. J Endocrinol 119:147–152.

Hertzberg T, Brosenitsch T, Katz DM (1995) Depolarizing stimuli induce high levels of dopamine synthesis in fetal rat sensory neurons. NeuroReport 7:233–237.

Higuchi H, Nakano K, Kim CH, Li BS, Kuo CH, Taira E, Miki N (1996) Ca^{2+}/calmodulin-dependent transcriptional activation of neuropeptide Y gene induced by membrane depolarization: determination of Ca^{2+} and cyclic AMP/phorbol 12-myristate 13-acetate responsive elements. J Neurochem 66:1802–1809.

Holzer P (1988) Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide, and other neuropeptides. Neuroscience 24:739–768.

Ji RR, Shi TJ, Zhang ZQ, Sakagami H, Kondo H, Hokfelt T (1996) Ca^{2+}/calmodulin-dependent protein kinase type IV in dorsal root ganglion: colocalization with peptides, axonal transport, and effect of axotomy. Brain Res 721:167–173.

Kashiba H, Senba E, Ueda Y, Tohyama M (1991) Cell size and cell-type analysis of calcitonin gene-related peptide containing cutaneous and splanchnic sensory neurons in the rat. Peptides 12:101–106.

Kucera J, Walro JM, Reichler J (1988) Innervation of developing intrafusal muscle fibers in the rat. Am J Anat 183:344–358.

Kudo N, Yamada T (1985) Development of the monosynaptic stretch reflex in the rat: an *in vitro* study. J Physiol (Lond) 369:127–144.

Lee Y, Takami K, Kawai Y, Girgis S, Hillyard CJ, MacIntyre I, Emson PC, Tohyama M (1985) Distribution of calcitonin gene-related peptide in the rat peripheral nervous system with reference to its coexistence with substance P. Neuroscience 15:1227–1237.

Li G, Hidaka H, Wollheim CB (1992) Inhibition of voltage-gated Ca^{2+} channels and insulin secretion in HIT cells by the Ca^{2+}/calmodulin-dependent protein kinase II inhibitor KN-62: comparison with antagonists of calmodulin and L-type Ca^{2+} channels. Mol Pharmacol 42:489–498.

Lindsay RM, Harmar AJ (1989) Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. Nature 337:362–364.

MacArthur L, Eiden L (1996) Neuropeptide genes: targets of activity-dependent signal transduction. Peptides 17:721–728.

Marti E, Gibson SJ, Polak JM, Facer P, Springall DR, Aswegen GV, Aitchson M, Koltzenburg M (1987) Ontogeny of peptide- and amine-containing neurones in motor, sensory, and autonomic regions of rat and human spinal cord, dorsal root ganglia, and rat skin. J Comp Neurol 266:332–359.

McCarthy PW, Lawson SN (1990) Cell-type and conduction velocity of rat primary sensory neurons with calcitonin gene-related peptide like immunoreactivity. Neuroscience 34:623–632.

McCleskey EW, Fox AP, Feldman DH, Cruz LJ, Olivera BM, Tsien TW, Yoshikami D (1987) ω-Conotoxin: direct and persistent blockade of specific types of calcium channels in neurons but not muscle. Proc Natl Acad Sci USA 84:4327–4331.

Misra RP, Bonni A, Miranti CK, Rivera VM, Sheng M, Greenberg ME (1994) L-type voltage-sensitive calcium channel activation stimulates gene expression by a serum response factor-dependent pathway. J Biol Chem 269:25483–25493.

Molander C, Ygge J, Dalsgaard CJ (1987) Substance P-, somatostatin-, and calcitonin gene-related peptide-like immunoreactivity and fluoride-resistant acid phosphatase activity in relation to retrogradely labeled cutaneous, muscular, and visceral primary sensory neurons in the rat. Neurosci Lett 74:37–42.

Narayanan CH, Fox MW, Hamburger V (1971) Prenatal development of spontaneous and evoked activity in the rat. Behaviour 40:100–134.

Naveh-Many T, Silver J (1988) Regulation of calcitonin gene transcription by vitamin D metabolites *in vivo* in the rat. J Clin Invest 81:270–273.

Noguchi K, Morita Y, Kiyama H, Ono K, Tohyama M (1988) A noxious stimulus induces the preprotachykinin A gene expression in the rat dorsal root ganglion: a quantitative study using *in situ* hybridization histochemistry. Mol Brain Res 4:31–35.

Noguchi K, Senba E, Morita Y, Sato M, Tohyama M (1990) Coexpression of α-CGRP and β-CGRP mRNAs in the rat dorsal root ganglion cells. Neurosci Lett 108:1–5.

O'Brien C, Woolf CJ, Fitzgerald M, Lindsay RM, Molander C (1989) Differences in the chemical expression of rat primary afferent neurons which innervate skin, muscle, or joint. Neuroscience 32:493–502.

Ogata N, Taebayashi H (1992) Ontogenic development of the TTX-sensitive and TTX-insensitive Na channels in neurons of the rat dorsal root ganglion. Dev Brain Res 65:93–100.

Peleg S, Abruzzese RV, Coper CW, Gagel RF (1990) Downregulation of calcitonin gene transcription by vitamin D requires two widely separated enhancer sequences. Mol Endocrinol 7:999–1008.

Rao MS, Tyrrel S, Landis SC, Patterson PH (1992) Effects of ciliary neurotrophic factor (CNTF) and depolarization on neuropeptide expression in cultured sympathetic neurons. Dev Biol 150:281–293.

Russo AF, Lanigan TM, Sullivan BE (1992) Neuronal properties of a thyroid C cell line: partial repression by dexamethasone and retinoic acid. Mol Endocrinol 6:207–218.

Saito K (1979) Development of spinal reflexes in the rat fetus studies *in vitro*. J Physiol (Lond) 294:581–594.

Sakagami H, Tsubochi H, Kondo H (1994) Immunohistochemical localization of Ca^{2+}/calmodulin-dependent protein kinase type IV in the peripheral ganglion and paraganglia of developing and mature rats. Brain Res 666:173–181.

Scott SA (1992) Sensory neurons: diversity, development, and plasticity. New York: Oxford UP.

Scroggs RS, Fox AP (1991) Distribution of dihydropyridine and ω-conotoxin-sensitive calcium currents in acutely isolated rat and frog sensory neuron somata: diameter-dependent L channel expression in frog. J Neurosci 11:1334–1346.

Scroggs RS, Fox AP (1992) Calcium current variation between acutely isolated rat dorsal root ganglion neurons of different size. J Physiol (Lond) 445:639–658.

Su C, Huang C, Schmidt J (1995) The depolarization response element in acetylcholine receptor genes is a dual function E-box. FEBS Lett 366:131–136.

Sun P, Lou L, Maurer RA (1996) Regulation of activating transcription factor-1 and the cAMP response element-binding protein by Ca^{2+}/calmodulin-dependent protein kinases type I, II, and IV. J Biol Chem 27:3066–3073.

Sun Y, Rao MS, Landis SC, Zigmond RE (1992) Depolarization increases vasoactive intestinal polypeptide and substance P-like immunoreactivities in cultured neonatal and adult sympathetic neurons. J Neurosci 12:3717–3728.

Tokumitsu H, Chijiwa T, Hagiwara M, Mizuntani A, Teraswas M, Hidaka H (1990) KN-62, 1-[*N*,*O*-Bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine, a specific inhibitor of Ca^{2+}-calmodulin-dependent kinase II. J Biol Chem 265:4315–4320.

Toullec D, Pianetti P, Cost H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, Duhamel L, Charon D, Kirilovsky J (1991) The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J Biol Chem 266:15771–15781.

Tverberg LA, Russo AF (1992) Cell-specific glucocorticoid repression of calcitonin/CGRP transcription: localization to an 18 bp basal enhancer element. J Biol Chem 267:17567–17573.

Tverberg LA, Russo AF (1993) Regulation of the calcitonin/calcitonin gene-related peptide gene by cell-specific synergy between helix-loop-helix and octamer-binding transcription factors. J Biol Chem 268:15965–15973.

Walke W, Xiao G, Goldman D (1996) Identification and characterization of a 47 base pair activity-dependent enhancer of the rat nicotinic acetylcholine receptor δ-subunit promoter. J Neurosci 16:3641–3651.

Wallengren J, Hakanson R (1987) Effects of substance P, neurokinin A, and calcitonin gene-related peptide in human skin and their involvement in sensory nerve-mediated responses. Eur J Pharmacol 143:267–273.

Watson A, Latchman D (1995) The cyclic AMP response element in the calcitonin/calcitonin gene-related peptide gene promoter is necessary but not sufficient for its activation by nerve growth factor. J Biol Chem 270:9655–9660.

Watson A, Ensor E, Symes A, Winter J, Kendall G, Latchman D (1995) A minimal CGRP gene promoter is inducible by nerve growth factor in adult rat dorsal root ganglion neurons but not in PC12 pheochromocytoma cells. Eur J Neurosci 7:394–400.