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Spontaneous Calcium Transients Are Required for Neuronal Differentiation of Murine Neural Crest

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We have shown that cultured mouse neural crest (NC) cells exhibit transient increases in intracellular calcium. Up to 50% of the cultured NC-derived cells exhibited calcium transients during the period of neuronal differentiation. As neurogenic activity declined, so did the percentage of active NC-derived cells and their calcium spiking frequency. The decrease in calcium transient activity correlated with a decreased sensitivity to thimerosal, which sensitizes inositol 1,4,5-triphosphate receptors. Thimerosal increased the frequency of oscillations in active NC-derived cells and induced them in a subpopulation of quiescent cells. As neurogenesis ended, NC-derived cells became nonresponsive to thimerosal. Using the expression of time-dependent neuronal traits, we determined that neurons exhibited spontaneous calcium transients as early as a neuronal phenotype could be detected and continued through the acquisition of caffeine sensitivity, soon after which calcium transient activity stopped. A subpopulation of nonneuronal NC-derived cells exhibited calcium transient activity within the same time frame as neurogenesis in culture. Exposing NC-derived cells to 20 mM Mg^{2+} blocked calcium transient activity and reduced neuronal number without affecting the survival of differentiated neurons. Using lineage-tracing analysis, we found that 50% of active NC-derived cells gave rise to clones containing neurons, while inactive cells did not. We hypothesize that calcium transient activity establishes a neuronal competence for undifferentiated NC cells.

Key Words: **differentiation; lineage analysis; cell autonomous; IP_3; neuronal progenitor.**

INTRODUCTION

The neural crest (NC) consists of a heterogeneous population of multipotent progenitors from which all of the neurons of the peripheral autonomic nervous system and dorsal root ganglia are derived (Le Douarin, 1982). During embryogenesis, these multipotent NC cells become restricted to sublineages that generate different parts of the peripheral nervous system (Henion and Weston, 1997; LaBonne and Bronner-Fraser, 1998; Stemple and Anderson, 1993; Weston, 1991). Studies have shown that lineage restriction of NC cells occurs prior to or soon after onset of migration (Baroffio *et al.*, 1990; Bronner-Fraser and Fraser, 1988, 1989; Frank and Sanes, 1991; Fraser and Bronner-Fraser, 1991; Raible and Eisen, 1994; Sieber-Blum and Cohen, 1980; Vogel and Weston, 1988). This restriction in developmental potential creates intrinsic differences between early NC cells that play an important role in the process of diversification. As yet, we do not understand the mechanisms that regulate lineage restriction of NC cells.

One possible mechanism involves intracellular calcium signaling. Dynamic changes in intracellular calcium have been linked to many developmental processes including proliferation (Berridge, 1995; Petr *et al.*, 1998), differentiation (Ferrari *et al.*, 1996; Jones *et al.*, 1995; Sauer *et al.*, 1998; Spitzer, 1995), and maturation (Sato-Bigbee *et al.*, 1999; Spitzer and Ou, 1997). Calcium regulates many of these processes by modulating signal transduction pathways that activate transcription factors, such as CREB and SRF (Hardingham *et al.*, 1997; Miranti *et al.*, 1995; Sheng *et al.*, 1990). In PC12 cells, calcium regulates CREB activity by modulating the efficacy of the Ras–MAPK pathway at various levels: tyrosine kinase receptor (Rosen and Greenberg, 1996), Ras (Rosen *et al.*, 1994), and Raf (Farnsworth *et al.*, 1995). Experimental manipulations that inhibit the interaction between calcium and the Ras–MAPK pathway perturb various aspects of neuronal differentiation of PC12

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cells, including survival, differentiation, and plasticity (for review, Finkbeiner and Greenberg, 1996). Variations in the calcium signaling parameters, i.e., frequency, duration, amplitude, and source of calcium, determine the signal transduction pathway activated and the extent to which it is activated (Bading *et al.*, 1997; Dolmetsch *et al.*, 1997, 1998; Fields *et al.*, 1997; Sheng *et al.*, 1993). Calcium signaling is distinct between cell types, since the factors that regulate the calcium signaling parameters are differentially expressed (Bennett *et al.*, 1996; De Smedt *et al.*, 1997; DeLisle *et al.*, 1996; Fujino *et al.*, 1995; Giannini *et al.*, 1995; Lievremont *et al.*, 1996; Miyakawa *et al.*, 1999; Sharp *et al.*, 1999; Sugiyama *et al.*, 1994).

Calcium transients are observed during the differentiation of many types of cells; inhibiting these calcium transients affects many aspects of their development. In *Xenopus*, calcium transients exhibited by myocytes regulate cytoskeletal organization during myofibrillogenesis (Ferrari *et al.*, 1996). Calcium signaling in *Xenopus* neuroblasts is required for neurogenesis (Jones *et al.*, 1995; Jones and Ribera, 1994) and neuronal maturation: GABA expression (Spitzer *et al.*, 1993), maturation of a K^+ conductance (Desarmenien and Spitzer, 1991), and axon extension (Spitzer, 1995). In the mouse, primitive endodermal cells exhibit calcium transients to regulate exo/endocytotic vesicle shuttling during their differentiation into parietal and visceral endoderm (Sauer *et al.*, 1998). In the subventricular zone, mouse CNS progenitors exhibit calcium transients and the frequency of calcium transient activity increases as they migrate to the neuronal layers (Owens and Kriegstein, 1998).

There have been no published reports describing the role of calcium transients in the development of the mammalian PNS. In this study, we show that cultured mouse NC-derived cells exhibit spontaneous calcium transients. Furthermore, these cells exhibit calcium transients only during the period of neuronal differentiation. We have determined, using immunocytochemistry and calcium pharmacology, that developing neurons exhibit calcium transient activity as well as a subpopulation of nonneuronal NC-derived cells. Using elevated extracellular Mg^{2+}, we provide evidence that blocking calcium transient activity inhibits neurogenesis without affecting neuronal survival or cell proliferation. Finally, using lineage-tracing dyes, we show that NC-derived cells that exhibit calcium transients have the potential to differentiate into neurons, while inactive cells do not.

MATERIAL AND METHODS

Neural Crest Cultures

Details of the culture system have been described elsewhere (Matsumoto, 1994a). Briefly, neural tubes from somite level 10, posterior to unsegmented somitic mesoderm, were dissected from embryonic day 9.5–10 Swiss–Webster mouse embryos. Cleaned neural tubes were placed onto an air-dried collagen substrate. NC cells were allowed to migrate from the neural tubes for 18–24 h. The tubes were then removed using a sharpened surgical knife. The cultures were washed twice and fed 1.5 ml of our standard culture medium (see below). The cultures were fed every 3–4 days.

The culture medium consisted of L-15 medium (Flow) modified for a 5% CO_2 atmosphere (Mains and Patterson, 1973). For the first 24 h of plating, the culture medium was supplemented with 2.5% (v/v) rat serum (Harlan), penicillin/streptomycin (100 U/ml/100 μg/ml; Gibco), 2 mM glutamine (Gibco), and 1% rat embryo extract. Twenty-four hours after plating, the embryo extract was replaced with four growth factors: glial-derived neurotrophic factor (Amgen), neurotrophin-3 (R & D), bone morphogenic protein-2 (Genetics Institute), and rat recombinant ciliary neurotrophic factor (a gift from Rae Nishi, OHSU), all at 10 ng/ml. This was our standard culture medium.

Imaging Intracellular Calcium

Neural crest cultures were loaded in the dark with the Ca^{2+} indicator dye, Oregon green 1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-1 (OGB; Molecular Probes). To do this, NC cultures were incubated (room temperature) for at least 30 min in our basic calcium recording solution (CaRec) plus the acetoxymethyl ester form (membrane permeable) of OGB (OGB-AM) at 2 μM. The cultures were then washed with CaRec for 10 min prior to imaging. The composition of CaRec is as follows (in mM): 160 NaCl, 3 KCl, 10 dextrose, 10 Hepes, 0.8 $MgCl_2$, and 5 $CaCl_2$. OGB-AM was dissolved in DMSO plus 0.2% pluronic to make 2 mM aliquots. This OGB-AM stock solution was diluted 1:1000 in CaRec for loading NC cultures.

To calcium-image NC cultures, the OGB-loaded culture was placed on the stage of an inverted light microscope (Nikon Diaphot 200) and continuously superfused with CaRec at room temperature (21–25°C). Illumination was provided with a Hg lamp. A dichroic cube in the path of the light set up an excitation wavelength of 488 nm and collected emissions of wavelengths longer than 515 nm. The illumination intensity was reduced using a series of neutral density filters (ND4, ND2, and either ND 0.3 or 0.6) to reduce photobleaching and photodynamic damage to the cells. Images were acquired using an intensified (Hamamatsu) CCD camera (Cohu) controlled by a PowerPC 7100 (Macintosh) computer running Cytos image acquisition software (ASI, Eugene, OR). Images were acquired every 3 s with each image consisting of an average of 8 frames. The exposure of the image field was computer regulated by rotating a filter wheel (Lambda 10; Sutter Instruments) from a closed position to a second position containing either an ND 0.3 or ND 0.6 neural density filter and back to the closed position. The fluorescence micrographs were digitized, and relative changes in $[Ca^{2+}]_i$ were determined for selected cells using data analysis software (IGOR, Wavemetrics) on a Macintosh computer. The data are expressed as the percentage change in fluorescence (fluorescence(t) − baseline fluorescence) over baseline fluorescence (% $\Delta F/F_0$). The baseline fluorescence was defined as the average of five minimum-level images for each trial. Events were counted if transient elevations in $[Ca^{2+}]_i$ exceeded 50% of baseline fluorescence. Calcium transient duration was estimated by measuring the time from the initial deviation from baseline to return. Calcium transient frequency is expressed as events/hour. Average values are expressed as mean ± SEM.

Immunocytochemistry

Imaged cultures were fixed and immunolabeled to compare calcium transient activity with immunocytochemical markers. To facilitate this comparison, cultures were plated on grid dishes and the grid coordinates captured for each recorded field. Following immunocytochemistry, labeled cultures were oriented to the captured grid coordinates and comparisons made. Standard immunocytochemical protocols used were described previously (Matsumoto, 1994a). Briefly, cultures were washed once in phosphate-buffered saline (PBS) and then fixed for 20 min at room temperature in 4% (w/v) paraformaldehyde (Sigma). After fixation, the cells were washed in PBS. Following a 1-h incubation with a blocking buffer (PBS, 0.1% Triton X (Sigma), and 5% goat serum (Gibco)), the cultures were incubated with a monoclonal antibody, 16A11 (mouse anti-Hu; Monoclonal Antibody Facility at the University of Oregon) at room temperature for 24 h. For Mg^{2+} culture experiments, the primary antibody was rabbit anti-neuron-specific enolase (Incstar). Unbound primary antibody was removed by repeated washing with blocking buffer. To visualize primaries with fluorescence, we incubated the cultures with anti-mouse Oregon green secondary antisera (Molecular Probes) for 40 min at 37°C. Many primary-labeled cultures were incubated with a biotinylated secondary followed by addition of avidin and biotin, using the protocol provided by a kit (Vectastain, Vector). This antibody complex was then visualized with a DAB reaction product. The cultures were viewed on an inverted microscope (Nikon Diaphot 200) with appropriate filters. Cells are scored as positive if the signal (fluorescence or dark product) exceeded background. Incubation with normal serum was used as a control.

Calcium Transient Activity and Lineage Analysis

In order to determine the fate of NC-derived cells that did or did not exhibit calcium transient activity, we loaded 1- to 3-day-old primary neural crest cultures, grown in standard culture medium, with OGB-1 and monitored the intracellular calcium levels of individual cells for 15 min (see Imaging Intracellular Calcium). For each recorded field (one per culture), an individual cell (one per field) was scored as active (exhibited one or more calcium spikes) or inactive and then intracellularly labeled using glass microelectrodes containing a solution of 6% lyseinated rhodamine dextran (10,000 MW; Molecular Probes) in 0.2 M KCl. The dye solution was injected ionophoretically. To ensure that we injected the cell of choice, we overlaid the calcium image with the labeled image and showed that they overlapped. Also, each injected culture was viewed within 4 h of the injection to determine survival and verify that a single cell was injected. Following the injection, the culture was incubated overnight in a sympathetic-inducing differentiation medium. This chemically defined medium is modified from Stemple and Anderson (1992), in which we complete the recipe without the addition of glycerol or nerve growth factor. The next day, the culture was switched back to the standard medium and cultured for 2 more days (3 days total), at which time the culture was fixed and immunolabeled for the neuronal marker, Hu. Lineage-tracked cells were identified and their phenotypes determined.

Pharmacological Agents and Their Application

Some pharmacological agents were diluted to the desired concentration in CaRec on the day of the experiment (thimerosal and caffeine). The 90 mM KCl and 20 mM $MgCl_2$ CaRec solutions were prepared by swapping equimolar concentrations of either KCl or $MgCl_2$ for NaCl. All chemicals were purchased from Sigma.

Statistical Analysis

Student's *t* tests were used to compare means between two populations. For comparisons across more than two populations, we used the analysis of variance to determine if there was a significant difference among the means compared (Sokal and Rohlf, 1969). If significance was established, an a posteriori test, the Student Newmann–Keuls was applied to do a multiple comparison of the mean populations tested to determine which were the same and which were different (Sokal and Rohlf, 1969). Results of statistical analysis were calculated using statistical software (PRISM) on a Macintosh G3 computer (Apple).

RESULTS

Cultured NC Cells Exhibit Spontaneous Calcium Transients

Using calcium imaging, we monitored intracellular calcium in primary NC cultures to determine if the NC-derived cells exhibited transient increases in intracellular calcium. To do this, we loaded NC-derived cells with the calcium indicator dye OGB and monitored their intracellular calcium levels for 1 h, capturing an image every 3 s (see Material and Methods). An example of a recorded field is shown in Fig. 1A. To demonstrate that the calcium fluctuations can be ascribed to individual cells, some cultures were fixed and labeled with propidium iodide to show that there was little overlap of individual cells (compare Figs. 1B and 1C). The generation of calcium transients appeared to be a property of individual cells. We did not observe synchronous activity among groups of cells, which would indicate a wave of activity traversing coupled cells. Furthermore, we did not detect the presence of dye coupling (Huang *et al.*, 1998; Lo, 1996), when we injected Lucifer yellow into active cells (not shown).

Many cells exhibited spontaneous increases in intracellular calcium. To analyze the data, the digitized pixel values were converted to percentage change in fluorescent intensity over the baseline fluorescence (% $\Delta F/F_0$) and plotted versus time (Fig. 1D). We observed two types of calcium waveforms (Gu *et al.*, 1994): spikes and waves (Fig. 2A). Calcium spikes reached their peak within 10 s of onset and then declined over a period of 25 to 60 s. The average duration of a calcium spike was 46.3 ± 2.6 s (mean ± SEM). Most calcium-spiking cells (77%) exhibited 1–4 calcium spikes/h (Fig. 3). However, many cells exhibited a higher frequency of calcium spiking (Fig. 2B), up to 96 spikes/h (Fig. 3). The other calcium waveform exhibited by NC-derived cells was the calcium wave, which had a relatively slow onset compared to the spike, >60 s, and a slow return to baseline, >60 s (Fig. 2A). The duration of these events was quite variable with a range of 120 to 360 s, much longer than the calcium spike. Some cells exhibited bursts of

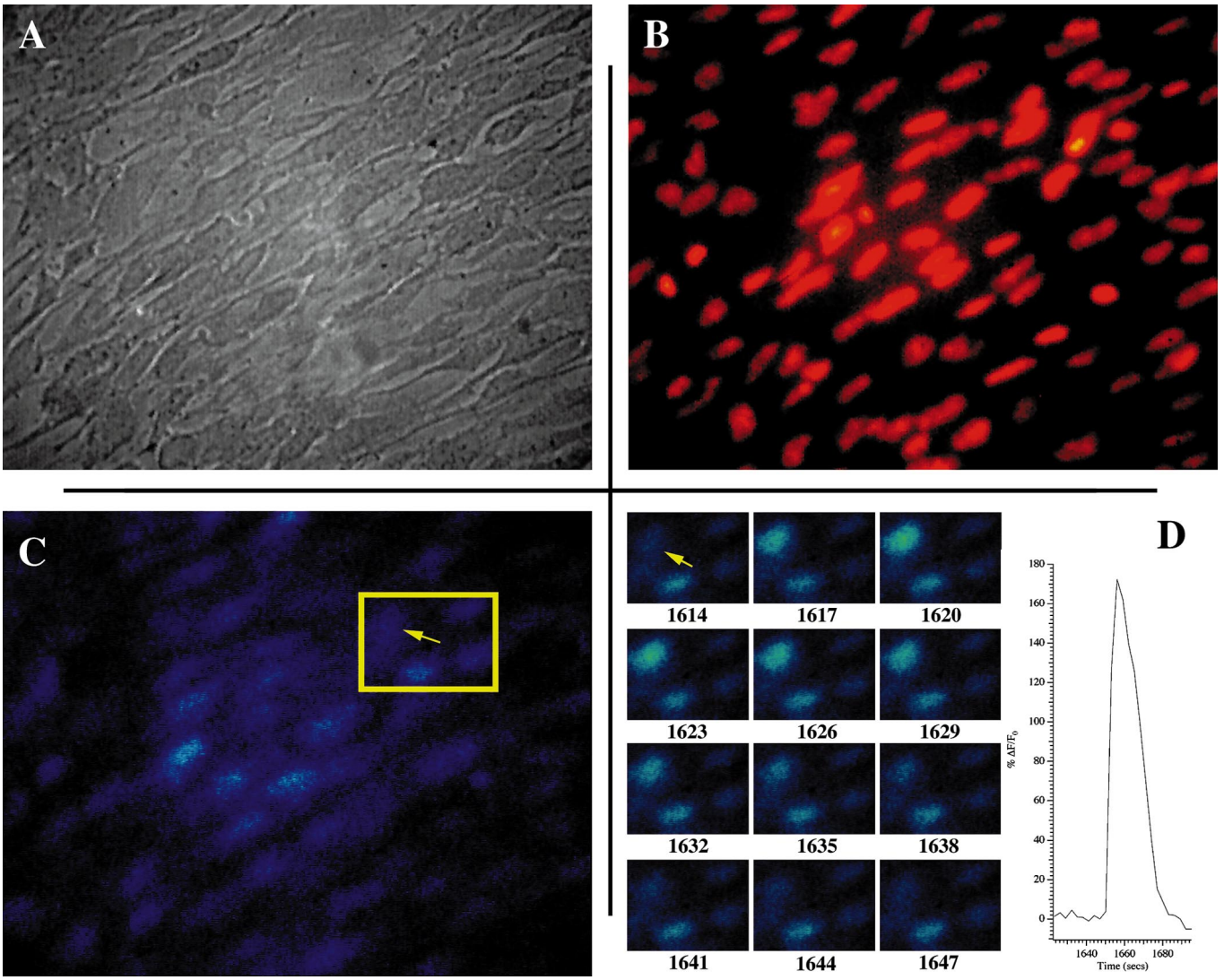

FIG. 1. NC-derived cells exhibited spontaneous calcium transients in culture. (A) 40× phase image of a calcium-imaged field. (B) Propidium iodide labeling of the same field as A. Calcium transients can be ascribed to individual cells since there was little overlap in nuclear labeling. (C) 40× fluorescent image of intracellular calcium using the calcium indicator dye OGB. (D) Single cell exhibiting a calcium transient. Sequence of calcium images, starting at time 1614, showing a cell (yellow arrow) from the field in C (yellow rectangle) exhibiting a single calcium transient event. The pixel values were converted to % $\Delta F/F_0$ (see Material and Methods) and plotted versus time.

calcium spikes that may or may not have been accompanied by calcium waves (Fig. 2C).

Cultured NC-Derived Cells Exhibit Spontaneous Calcium Transients during the Period of Neuronal Differentiation

NC-derived cells exhibited spontaneous calcium transients during the period that neurons are born in this culture system (Matsumoto, 1994). We have shown previously that NC cells differentiate into either sensory or sympathetic neurons, depending on the culture environment (Carey and Matsumoto, 1999). Their ability to differentiate into neurons is restricted to the first 6 days in culture with the greatest sensitivity to differentiation factors occurring in the first 4 days (S. G. Matsumoto, unpublished results). After 7 days, few neurons are produced.

NC-derived cells exhibited spontaneous calcium transient activity with the same time course as this neurogenic period. During the first 2 days in culture, 47.6 ± 3.5% of the cells in a recorded field exhibited calcium transient activity (Fig. 4A). The percentage of active cells decreased slightly during days 3 and 4 (38.4 ± 4.0%). The average percentage of active cells dropped significantly after 4 days in culture,

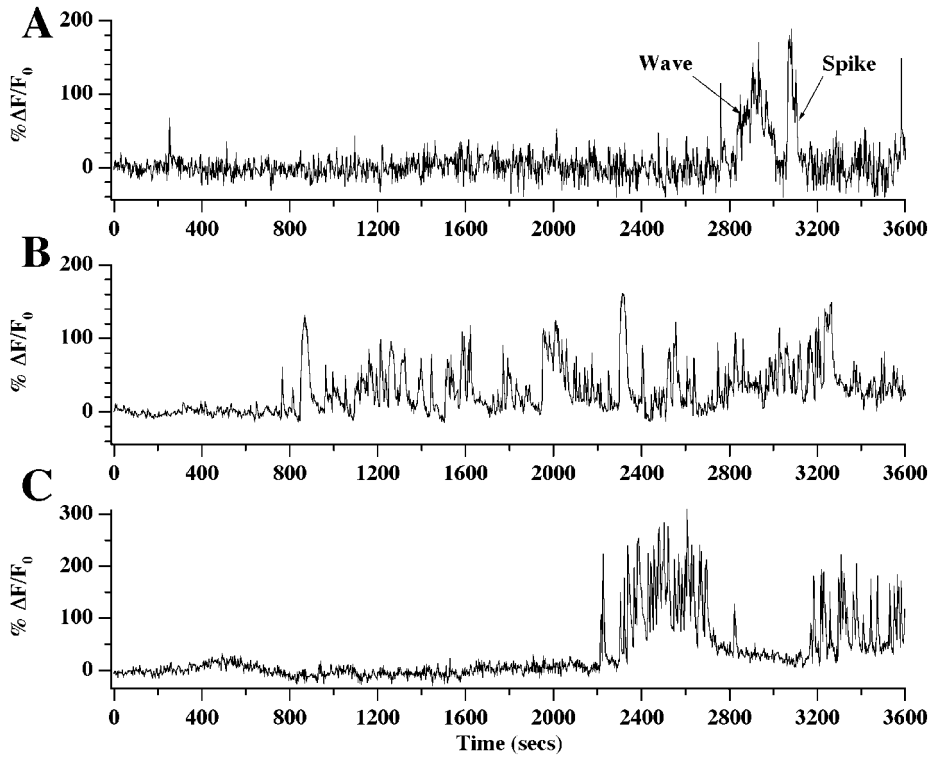

FIG. 2. NC-derived cells exhibited calcium spikes and/or calcium waves with varying frequencies and patterns. (A) A single cell exhibiting a calcium spike and a calcium wave. Calcium waves were defined as calcium influxes that reached their peak values with a time course greater than 60 s and returned to baseline with a similar time course, total duration >180 s. Calcium spikes were defined as calcium influxes that reached their peak values within 5–15 and returned to baseline in fewer than 60 s, total duration <75 s. (B) A single cell exhibiting a high frequency of calcium spiking activity. (C) A single cell exhibiting two bursts of calcium spike activity. Bursts of calcium spikes may (first burst) or may not (second burst) be accompanied by an underlying calcium wave.

falling to values below 20% ($P < 0.001$). Similarly, the frequency of calcium spikes in active cells was highest the first 6 days in culture (Fig. 4B). During the first 2 days in culture, NC-derived cells averaged 3.7 ± 0.1 spikes/h. The frequency of spikes in our sampled cells increased significantly to 4.8 ± 0.2 spikes/h the next 2 days ($P < 0.001$) and decreased to 3.5 ± 0.5 spikes/h on days 5 through 6 (Fig. 4B). Calcium spiking frequency declined significantly after 6 days to an average frequency of 2.5 spikes/h ($P < 0.001$). By day 10 in culture, most active cells exhibited only 1 calcium spike/h (not shown).

Thimerosal Induces Calcium Oscillations in NC-Derived Cells during the Period of Neuronal Differentiation

Less than 50% of the NC-derived cells in a recorded field exhibited calcium transients during days 1–2 in culture (Fig. 4A). To determine whether inactive NC-derived cells were capable of calcium transient activity, we used thimerosal to induce calcium oscillations. Thimerosal is a thiol reagent that sensitizes the inositol 1,4,5,-triphosphate (IP_3) receptor (IP_3R) inducing cytoplasmic calcium oscillations in those cells capable of IP_3-mediated calcium-induced calcium release (Bootman *et al.*, 1992; McDougall *et al.*, 1993). We have determined that the IP_3R channel is responsible for the primary influx of calcium during a calcium transient event (Carey and Matsumoto, submitted for publication). During days 1–2 in culture, almost all NC-derived cells, active and quiescent, responded to thimerosal (Fig. 5). This indicated that while fewer than 50% of recorded NC-derived cells exhibited spontaneous calcium transients at this stage most NC-derived cells had the potential to do so.

The ability to induce calcium oscillations with thimerosal was restricted to a subpopulation of NC-derived cells over time in culture (Fig. 3). Thus, while most NC-derived cells (active and inactive) responded to thimerosal initially, the percentage of inactive cells that responded to thimerosal dropped to 50% by day 3 (Fig. 5B). This percentage continued to decline with only 6% of the inactive cells sensitive to thimerosal by day 7. Active cells continued to respond to thimerosal through day 4, but dropped off significantly by day 7 (28.6 ± 10.3%). After day 7, all NC-derived cells failed to respond to thimerosal (not

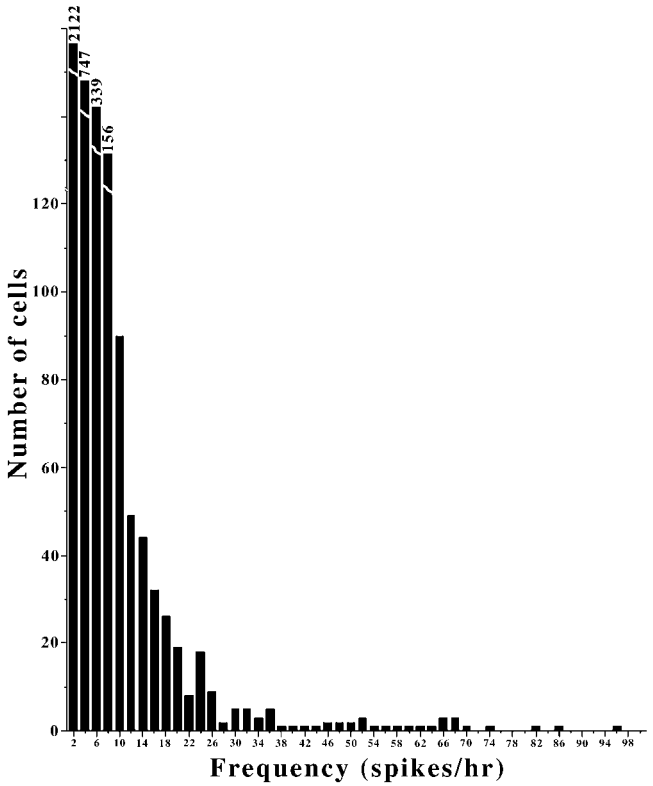

FIG. 3. Distribution of calcium transient frequencies for all NC-derived cells that exhibited calcium transients for all days in culture. Most active NC-derived cells exhibited <3 calcium spikes/h with some cells exhibiting up to 96 spikes/h.

shown). It is not clear why NC-derived cells lose their sensitivity to thimerosal. However, the decline in sensitivity corresponded to a loss of neurogenic potential within the population as a whole.

Neurons Differentiated in Culture Exhibited Spontaneous Ca^{2+} Transients in a Stage-Dependent Manner

As NC-derived cells undergo neuronal differentiation, they sequentially acquire many neuronal traits. For example, neurons begin to express the RNA binding protein, Hu, during the earliest stages of neurogenesis (Marusich *et al.*, 1994; Marusich and Weston, 1992). Within 12–48 h of their terminal mitosis, neurons begin to express voltage-dependent calcium currents (VDCC) (Bader *et al.*, 1983; Gottmann *et al.*, 1988). In our cultures, the first indication of neuronal differentiation is the expression of Hu immunoreactivity (Hu-IR) followed by acquisition of VDCCs and then sensitivity to caffeine (Fig. 6G). By day 10, all cultured neurons exhibit these three characteristics (not shown).

We can correlate calcium transient activity with the progression of neuronal maturation using these traits. We did this by monitoring intracellular calcium levels for 1 h and then perfusing, sequentially, 20 mM caffeine and 90 mM KCl, to determine the presence of ryanodine receptor (RyR)-dependent calcium release and VDCC, respectively. Subsequently, we fixed and immunolabeled these cultures with a neuronal marker, Hu (Marusich *et al.*, 1994; Marusich and Weston, 1992). Using a grid coordinate system, we correlated calcium transient activity with the expression of these markers, an example of which is shown in Fig. 6. The cell identified by the white arrow exhibited calcium transient activity (not shown), responded to caffeine (compare Figs. 6A and 6B), responded to high K^+ (compare Figs. 6A and 6C), and exhibited Hu-IR (compare Figs. 6D–6F).

Neurons exhibited calcium transient activity throughout the early stages of development and stopped soon after the acquisition of a caffeine response. During the first 4 days in

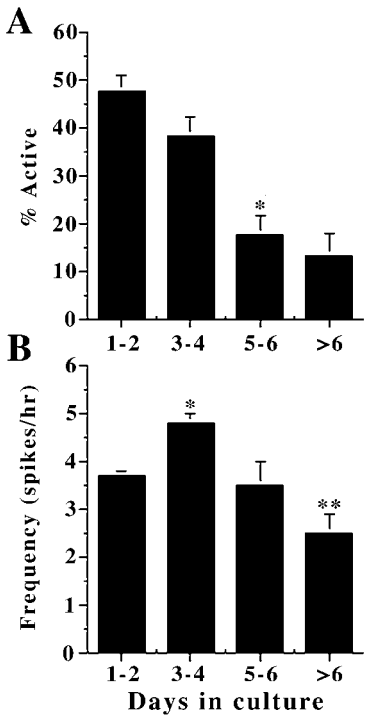

FIG. 4. Calcium transient activity decreases with time in culture. Intracellular calcium levels were monitored for 1 h (every 3 s), and we determined for every cell in the recorded field if it exhibited a calcium transient and the frequency at which it exhibited these calcium transients. This was repeated in cultures that were up to 12 days old. (A) Mean percentage (±SEM) of NC-derived cells that exhibited calcium transients (% Active) versus days in culture. Most calcium transient activity occurred in the first 4 days of culture (40–50%). After day 4, the percentage of active cells significantly decreased (*$P < 0.001$). (B) Mean calcium spike frequency (±SEM) for NC-derived cells that exhibited calcium transients versus days in culture. The mean calcium spike frequency peaked at days 3–4 (4.7 spikes/h, *$P < 0.001$) and declined after day 6 (2.5 spikes/h, **$P < 0.001$).

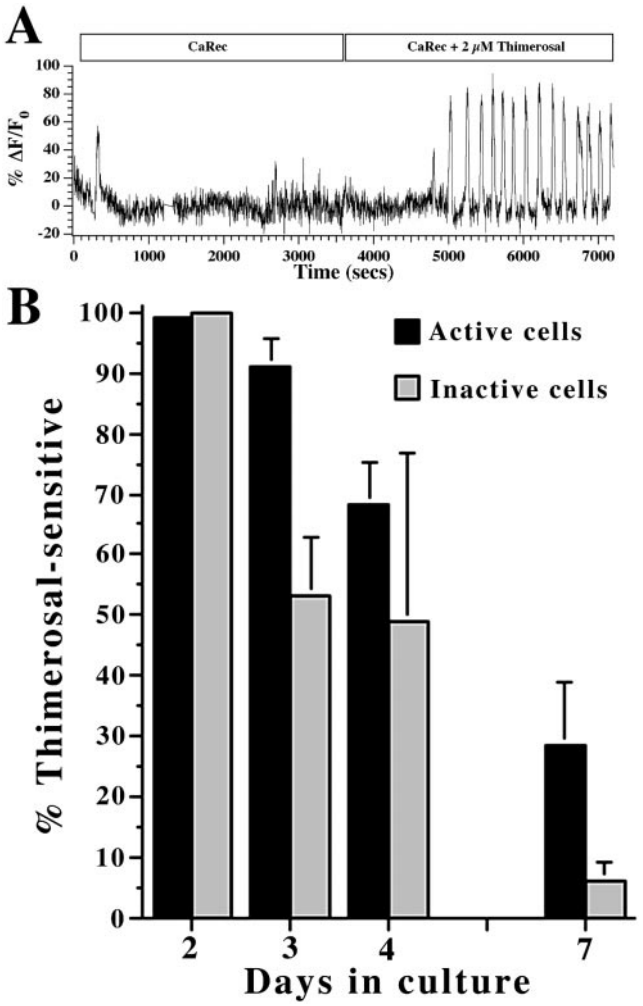

FIG. 5. Thimerosal induction of calcium oscillations in cultured NC-derived cells declined over time. (A) Intracellular calcium levels were monitored for 1 h in the presence of normal CaRec. In the second hour, cultures were perfused with CaRec + 2 μM thimerosal. Addition of thimerosal to the perfusion medium induced calcium oscillations in many NC-derived cells. (B) The mean percentage of NC-derived cells that responded to thimerosal declined over time in culture. At day 2 in culture, all NC-derived cells in a recorded field responded to thimerosal. By day 3, cells that exhibited calcium transient activity during the first hour (Active cells) continued to respond to thimerosal, while 50% of the cells that did not exhibit calcium transients the first hour (inactive) failed to respond to thimerosal. From day 4 to day 7, both populations declined in sensitivity to thimerosal. After Day 7, no NC-derived cells responded to thimerosal (data not shown).

culture, greater than 50% of the recorded neurons exhibited calcium transient activity (Table 1, All). This neuronal population was heterogeneous with respect to their stage of development: Hu-IR only (H only); Hu-IR and a high K^+ response (HK); and Hu-IR, a high K^+ response, and a caffeine response (HKC). During days 1–2 in culture, a greater percentage of HK and HKC neurons exhibited calcium transient activity than the earliest neurons (H only) (Table 1). After day 2, the percentages of neurons that exhibited calcium transients were similar for all stages of development tested. Calcium transient activity in the neuronal population dropped to 33.8 ± 12% after day 4 (Table 1, All). This was due to a shift in the composition of the neuronal population to a more mature developmental stage. The percentage of active H-only neurons remained high (75 ± 25%) while HK (36.5 ± 13%) and HKC neurons (32.7 ± 14%) declined. After day 6, few neurons (<10%) exhibited calcium transients (Table 1), with all activity ceased by day 10 (not shown).

The frequency of calcium transient activity increased soon after neurogenesis and declined as the neurons matured. During days 1–2, neurons exhibited 2.6 ± 0.3 spikes/h (Table 1, All). Calcium spiking frequency was similar for all neuronal developmental stages, ranging from 2.0 to 3.1 spikes/h. After day 2, calcium spiking frequency increased to 4.0 ± 0.6 spikes/h. This increase was due to an elevation in the calcium spiking frequency for H only (3.9 ± 0.7 spikes/h) and HK neurons (6.0 ± 1.8 spikes/h). HKC neurons did not exhibit an increased spiking frequency, since this population was primarily made up of early differentiating neurons (Matsumoto, 1994). During days 5–6, calcium spiking frequency increased further to 6.0 ± 1.5 spikes/h, because of the high calcium spiking frequencies of HK (5.7 ± 2.1 spikes/h) and HKC neurons (7.0 ± 2.3 spikes/h). After day 6, the calcium spiking frequency for all neuronal stages decreased to 2.3 ± 0.5 spikes/h. At this point, most neurons had matured (HKC; 1.6 ± 0.3 spikes/h). H-only and HK neurons continued to exhibit elevated calcium spiking frequencies (5.0 ± 2.1 and 3.5 ± 2.5 spikes/h, respectively). In summary, neurons exhibited calcium transient activity early in development that increased in frequency, as they acquired high K^+ and caffeine responses, and then stopped as they continued to mature.

Calcium Transient Activity in NC-Derived Cells Correlated with Neurogenic Potential in Culture

We have shown that cultured NC cells can give rise to either sensory or sympathetic neurons depending on the growth conditions (Carey and Matsumoto, 1999). For both neuronal populations, neurons are generated over the first 4 days in culture. The production of neurons then gradually declines until by day 7 neurogenesis ends. Although both sensory and sympathetic progenitors are present in these cultures, the growth conditions we used supported only sensory neuronal differentiation. The sympathetic progenitors do not differentiate unless exposed to a different culture medium (S. G. Matsumoto, unpublished results). However, sympathetic progenitors maintain their competence to differentiate for 6 days in this culture environment (Matsumoto, 1998, and unpublished results).

Calcium transient activity in non-Hu-IR (H(−)) NC-

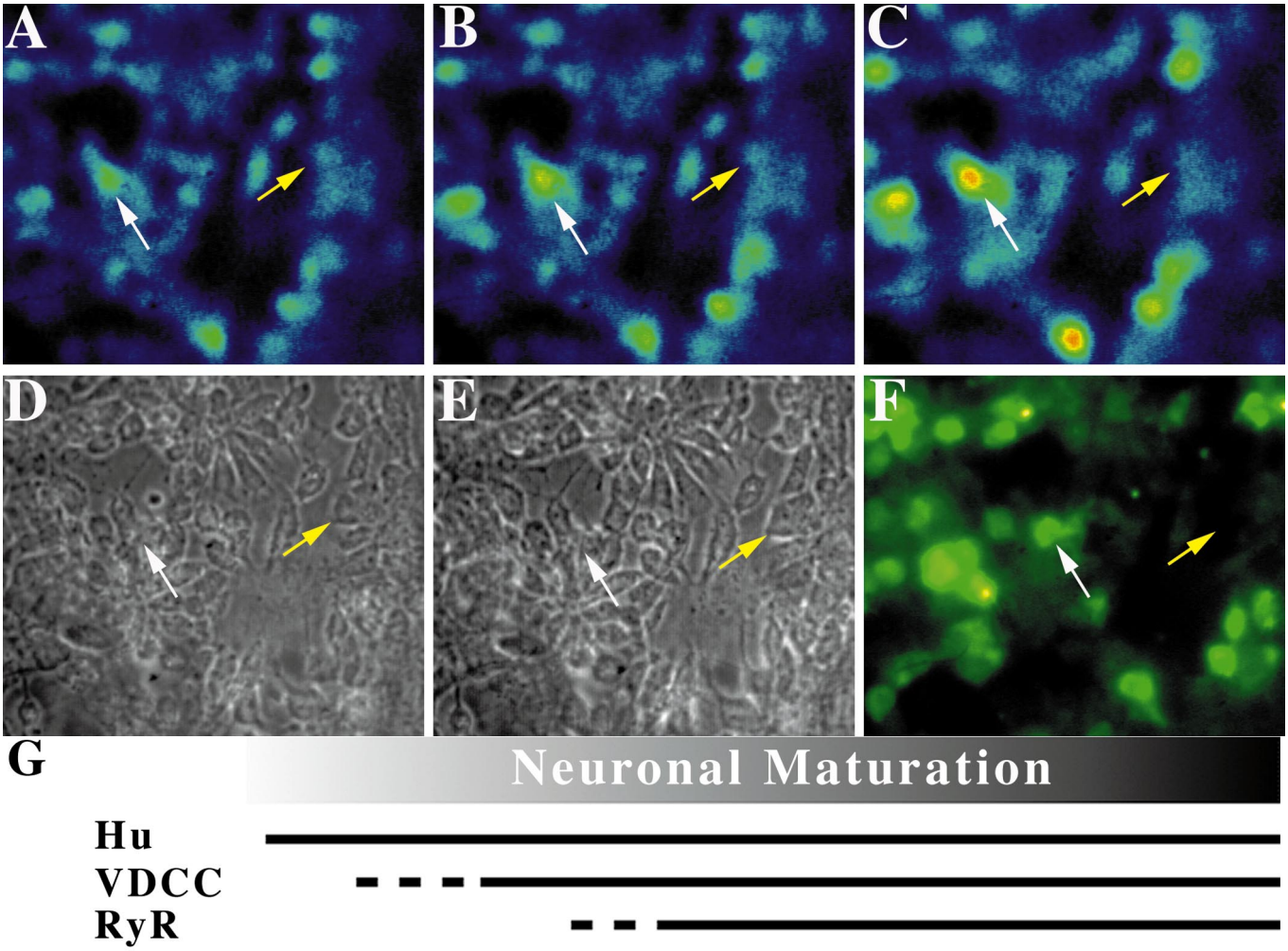

FIG. 6. Correlation of Hu-IR, calcium pharmacology, and calcium transient activity in cultured NC-derived cells. Intracellular calcium levels were monitored for 1 h. Baseline calcium levels are shown in (A). The cultures were then sequentially exposed to CaRec + 20 mM caffeine (B) and 90 mM KCl-modified CaRec solution (C) to determine if these cells had caffeine-sensitive calcium stores and/or high-voltage-activated calcium channels. After calcium imaging, the cultures were fixed and immunolabeled for the neuronal marker, Hu. Phase images before (D) and after (E) fixation were done to compare the effects of fixation. We then took a fluorescent image of the Hu labeling (F). We compared the calcium transient activity of each cell with the calcium pharmacology and Hu labeling, by orienting the culture using the grid coordinates (shadow of which may be seen in D and E). The white arrows identify a cell that exhibited calcium transient activity, responded to both caffeine and high KCl (compare A with B and C, respectively), and was a neuron (Hu+, F). The yellow arrows identify a cell that did not respond to either caffeine or high KCl and was not a neuron (Hu−). (G) Time course of calcium transient activity and acquisition of select neuronal characteristics during the course of maturation.

derived cells, i.e., NC-derived cells that are not neuronal, coincided with the culture's sensitivity to a "sympathetic differentiation" medium. Using the method described previously (Fig. 6), we monitored calcium transient activity in H(−)NC-derived cells over time in culture. Peak calcium transient activity for H(−)NC-derived cells occurred during days 1–4 with 35% of these cells exhibiting calcium transients (Table 2, All). A higher percentage of H(−)NC-derived cells that expressed excitable cell traits (responded to caffeine (C only), to high K^+ (K only), or both (KC)) exhibited calcium transient activity than cells that lacked either trait (U) (Table 2). After day 4, the percentage of active H(−)NC-derived cells declined to 14.9 ± 4.4% (Table 2, All). U H(−)NC-derived cells continued to exhibit a lower percentage of active cells (9.6 ± 3.0%) than cells that expressed excitable traits (28–33%; Table 2). By day 7, the percentage of all active H(−)NC-derived cells dropped below 10%. Calcium spiking frequency was highest on days

TABLE 1
Calcium Transient Activity in Neurons

Days in culture	Neurons: All	H-only	HK	HKC
	% Active			
1 to 2	56.4 ± 5.4 (16)	31.7 ± 8.1 (16)	73.9 ± 8.1 (15)	66.4 ± 7.3 (15)
3 to 4	52.1 ± 11 (9)	40.8 ± 16 (7)	55.0 ± 12 (8)	47.1 ± 13 (8)
5 to 6	33.8 ± 12 (6)	75 ± 25 (2)	36.5 ± 13 (6)	32.7 ± 14 (6)
>6	9.8 ± 5.4 (6)	25 ± 25 (4)	22.9 ± 20 (5)	6.7 ± 3.0 (6)
	Frequency			
1 to 2	2.6 ± 0.3 (303)	2.0 ± 0.2 (62)	2.2 ± 0.2 (88)	3.1 ± 0.5 (153)
3 to 4	4.0 ± 0.6 (70)	3.9 ± 0.7 (15)	6.0 ± 1.8 (21)	2.7 ± 0.6 (34)
5 to 6	6.0 ± 1.5 (74)	2.0 ± 0.6 (5)	5.7 ± 2.1 (38)	7.0 ± 2.3 (31)
>6	2.3 ± 0.5 (19)	5.0 ± 2.1 (3)	3.5 ± 2.5 (2)	1.6 ± 0.3 (14)

Note. Neurons (All) were analyzed based on calcium pharmacology: HK, responded to 90 mM KCl; HKC, responded to both caffeine and KCl; and H-only, did not respond to either caffeine or KCl. Data reported as means ± SEM. Numbers in parentheses are total numbers of cultures (% Active) or total numbers of cells (Frequency).

1–4 (3–4 spikes/h) with all H(−)NC-derived cells exhibiting similar frequencies (Table 2, All). After day 4, calcium spiking frequency declined slightly to 2.4 spikes/h and continued to decline to less than 2 spikes/h after day 6. This time course of calcium transient activity coincided with sympathetic differentiation potential with peak activity through day 4 and gradually declining until day 7, when both activities essentially end. Speculations on the identity of H(−)NC-derived cells that exhibited spontaneous calcium transient activity and those that exhibited excitable traits are reserved for the Discussion.

Elevated Mg^{2+} Blocks Calcium Transient Activity in NC-Derived Cells and Inhibits Neurogenesis in Culture

To determine if calcium transient activity is required for neuronal differentiation, we cultured NC cells in the presence of 20 mM Mg^{2+} to block the production of calcium transients. Studies have shown that elevated intracellular Mg^{2+} inhibits intracellular calcium release through the IP_3R by a noncompetitive interaction (Kasahara *et al.*, 1993; Volpe and Vezu, 1993). Perfusing CaRec plus 20 mM $MgCl_2$ on NC-derived cells inhibited calcium transient activity (compare Figs. 7A and 7B). This concentration of Mg^{2+} blocked responsiveness to thimerosal, as well (compare Figs. 7B and 7C). Overall, the addition of 20 mM $MgCl_2$ to the perfusion medium significantly reduced the mean percentage of active cells from 55.1 ± 12.1 to 5.4 ± 1.8% (Fig. 7D, $P < 0.001$).

Culturing NC cells with 20 mM Mg^{2+} reduced the number of neurons generated (Fig. 8). Under the current culture conditions, neurons differentiate in the first 6 days of culture from postmitotic NC cells (Matsumoto, 1994). The neurons can be identified at day 4 in culture by labeling with antisera against neuron-specific enolase (NSE) (Figs. 8A–8D). On average, NC cells cultured in control medium produced 2700 neurons/culture (Fig. 8E). Addition of 20 mM Mg^{2+} to the culture medium significantly reduced the number of neurons to fewer than 1000 neurons/culture (Fig. 9E, $P < 0.01$).

The effect of elevated Mg^{2+} on neuronal survival was assessed by culturing embryonic day 12 dorsal root ganglion (DRG) cells ± 20 mM Mg^{2+}. All of the neurons in these cultures are postmitotic at plating when grown under the culture conditions used in this study (Matsumoto, 1998). The survival of the embryonic DRG neurons in Mg^{2+} was not significantly different from that of the control cultures over 5 days in culture (87 ± 4% vs 89 ± 6%). We also pulsed cultures with BrdU (10 μM) for 12 h to determine if elevated Mg^{2+} inhibited cell proliferation. There were no obvious differences in the proportion of cells labeled with or without 20 mM Mg^{2+} (not shown). However, it is unlikely that we would detect a selective inhibition of a small number of cells, i.e., a select inhibition of neuronal progenitors.

Active NC-Derived Cells Can Give Rise to Neurons

To determine directly if cells that exhibit calcium transients are neuronal progenitors, we used lineage-tracing analysis to follow the fate of active and inactive NC-derived cells. Active and inactive cells were injected ionophoretically with a 10,000 MW lyseinated rhodamine dextran. Anode break was used to determine if the impaled cell was a neuron. Once injected, the culture was exposed to a sympathetic differentiation medium (see Material and Methods) overnight, switched to our standard medium for 2 days, and then fixed and immunolabeled for Hu-IR. Exposure to this medium optimizes the differentiation of sympathetic as well as sensory neuronal progenitors.

Comparing Hu-IR with the lineage marker, we found that active NC-derived cells gave rise to clones containing neurons, while inactive cells did not. In Fig. 9, a single active cell (Figs. 9A–9C) gave rise to three cells (Figs. 9D and 9E), two of which were Hu-IR (Figs. 9E–9G). Overall, 50% of lineage-traced active cells gave rise to clones containing neurons (Fig. 10). Inactive cells, however, were never observed to give rise to clones containing neurons (Fig. 10). The mean clone size for active (2.4 ± 0.4) and inactive

TABLE 2
Calcium Transient Activity in H(−)NC-Derived Cells

Days in culture	H(−)NC-derived cells				
	All	U	C-only	K-only	KC
			% Active		
1 to 2	33.9 ± 5.0 (20)	31.8 ± 5.0 (19)	84.0 ± 7.6 (9)	66.9 ± 12 (12)	78.8 ± 11 (8)
3 to 4	35.8 ± 5.6 (14)	27.7 ± 5.2 (14)	47.8 ± 3.8 (10)	49.5 ± 9.1 (11)	57.7 ± 6.1 (10)
5 to 6	14.9 ± 4.4 (8)	9.6 ± 3.0 (8)	30.7 ± 14 (5)	28.5 ± 12 (7)	32.9 ± 16 (5)
>6	6.4 ± 4.3 (6)	7.6 ± 5.8 (5)	14.3 ± 14 (4)	11.2 ± 7.2 (5)	0 (4)
			Frequency		
1 to 2	3.1 ± 0.1 (588)	3.1 ± 0.1 (412)	3.4 ± 0.3 (78)	2.9 ± 0.3 (62)	3.0 ± 0.4 (36)
3 to 4	3.2 ± 0.2 (469)	2.7 ± 0.3 (180)	2.9 ± 0.3 (82)	3.7 ± 0.4 (128)	4.3 ± 0.5 (79)
5 to 6	2.4 ± 0.2 (112)	2.5 ± 0.3 (42)	2.5 ± 0.7 (10)	2.3 ± 0.3 (39)	2.4 ± 0.3 (21)
>6	1.5 ± 0.2 (26)	2.2 ± 0.6 (14)	2.0 ± 1.0 (2)	1.5 ± 0.2 (10)	— —

Note. H(−)NC-derived cells (All) were analyzed based on calcium pharmacology: C-only, responded to 20 mM caffeine; K-only, responded to 90 mM KCl; KC, responded to both caffeine and KCl; and U, did not respond to either caffeine or KCl. Data reported as means ± SEM. Numbers in parentheses are total numbers of cultures (% Active) or total numbers of cells (Frequency).

(3.6 ± 0.6) labeled NC-derived cells was not significantly different ($P > 0.05$), indicating similar mitotic activity between the populations labeled. The clone sizes ranged from one to nine cells for both groups with neuron-containing clones having a clone size greater than 1.

DISCUSSION

Cultured NC-derived cells exhibited spontaneous calcium transient activity similar to what has been described in other models of neuronal differentiation. As with these other models, NC-derived cells exhibited calcium transients for a limited time during development, beginning with a low frequency (3 spikes/h), increasing as neurons mature (6 spikes/h), and then decreasing at the end of differentiation (Table 1). Similar patterns have been observed in the developing CNS. In the *Xenopus* cord, for example, immature neurons initially exhibit on average 1 calcium spike/h, increasing to an average of 3 spikes/h 5–10 h after neurogenesis, and then declining to 1 spike/h (Gu and Spitzer, 1995; Spitzer *et al.*, 1995). In the mammalian CNS, Owens and Kriegstein (1998) have shown that cells in the neocortical ventricular zone (neuronal precursors) exhibited spontaneous calcium transients, and as these cells progressed developmentally both the incidence of calcium transient activity and their frequency increased significantly.

In this study, we have shown that developing peripheral neurons exhibit calcium transient activity. Using the expression of time-dependent neuronal traits, we determined that neurons exhibited spontaneous calcium transients from the onset of expression of the earliest known neuronal characteristic (H-only cells) and continued through the acquisition of VDCCs (HK cells) and caffeine-sensitivity (HKC cells). As the neurons matured, they stopped exhibiting spontaneous calcium transients. The reason for the reduced calcium transient activity in neurons imaged during the first 2 days in culture (Table 1) was probably due to the degree of maturation of these neurons. We have previously shown that under these culture conditions two sets of sensory neurons differentiated: ED (early differentiating) and LD (late differentiating) (Matsumoto, 1994). The ED neurons arise within 24 h of plating, while the LD neurons differentiate 2 days to 6 days after plating. It is likely that the low level of calcium transient activity observed during days 1–2 reflects the more mature ED neurons, while more active neurons imaged from day 3 to 6 were the more recently differentiated LD neurons (Table 1). This modulation in calcium spiking frequency may be regulating neuronal maturation as seen in *Xenopus* spinal neurons (Gu and Spitzer, 1995).

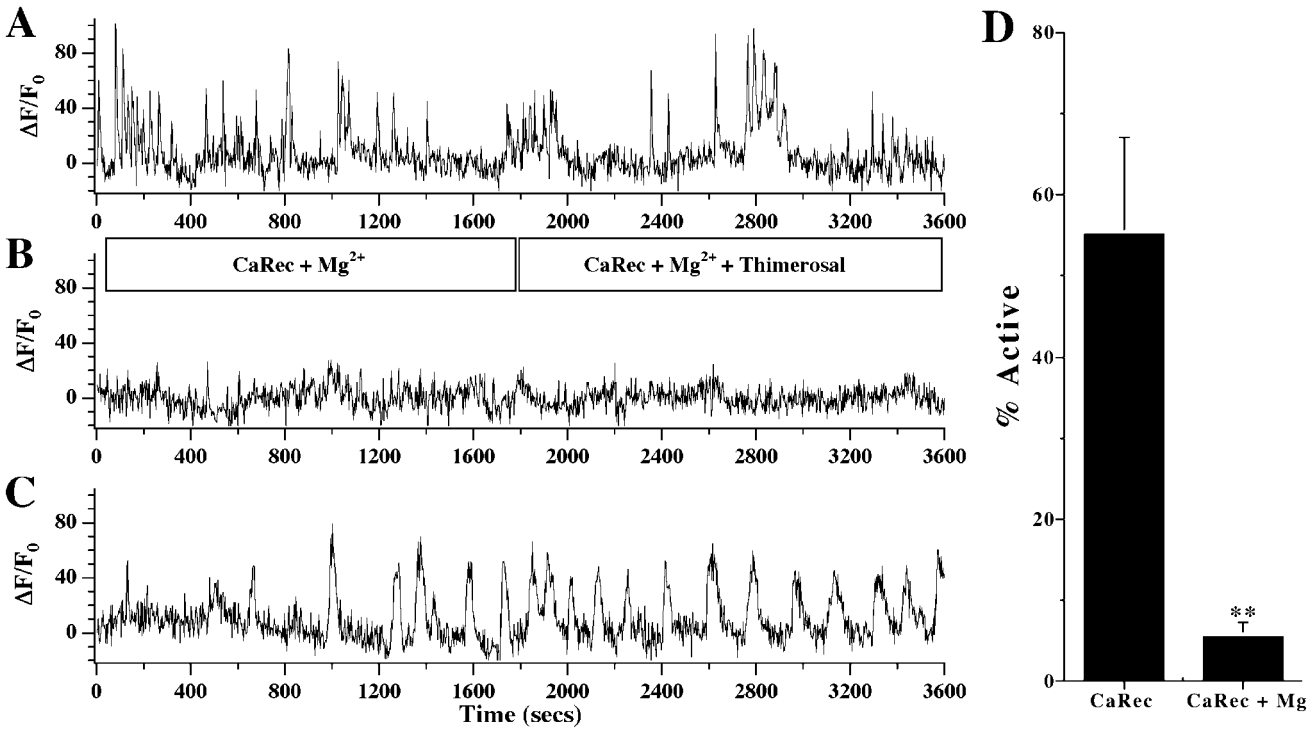

FIG. 7. 20 mM Mg^{2+} blocked spontaneous calcium transient activity and thimerosal-induced calcium oscillations in cultured NC-derived cells. Intracellular calcium levels were monitored for 1 h in the presence of normal CaRec (A). During the second hour, cultures were perfused with CaRec + 20 mM $MgCl_2$ for 30 min and then switched to CaRec + Mg^{2+} + 2 μM thimerosal for 30 min (B). For the third hour, cultures were perfused with CaRec + thimerosal, in the absence of Mg^{2+} (C). NC-derived cells that had exhibited calcium transients failed to exhibit these transients and respond to thimerosal in the presence of Mg^{2+}. Removal of Mg^{2+} relieves the inhibition on thimerosal-induced calcium oscillations. (D) Addition of 20 mM Mg^{2+} significantly reduced the mean percentage (±SEM) of active NC-derived cells (**$P < 0.01$).

In our study, it is important to note that only a subpopulation of NC-derived cells exhibited calcium transients. Our hypothesis is that the H(−)NC-derived cells that exhibit calcium transients are neuronal progenitors. In support of this, we found that H(−)NC-derived cells that exhibited calcium transients during the same time period as neuronal progenitors could be induced to differentiate into neurons (Matsumoto, 1998). In addition, neurogenesis and calcium transient activity (both % active and frequency) were highest the first 4 days in culture and sharply declined from day 5 to 6 with both activities ending after day 6 (Table 2). Similarly, the mechanism for generating calcium transients was lost with the same time course. This was indicated by the loss of sensitivity to thimerosal after day 6 (Fig. 5B). Thimerosal activates the same calcium oscillatory pathway used to generate spontaneous calcium transients (M. B. Carey and S. G. Matsumoto, in preparation). At this time, we cannot rule out that these active cells may represent other differentiating cell types, i.e., Schwann cell, melanocyte, or smooth muscle. We have, however, monitored calcium levels in sciatic nerve explants, which contain only glial progenitors (Walter, 1994), and found no calcium transient activity (S. G. Matsumoto, unpublished results).

NC-derived cells that expressed excitable traits had increased calcium transient activity (% active and frequency) over cells that did not. The expression of such traits may act to increase calcium transient activity by providing a means of calcium entry (VDCCs and RyR) that can increase the probability of a calcium transient event through a calcium-induced calcium-release mechanism. A similar mechanism for regulating calcium transient activity has been described in *Xenopus* spinal neurons (Gu and Spitzer, 1993). The function of this increased activity could modulate many cellular parameters from growth factor receptor activation (Rosen and Greenberg, 1996) to gene transcription (Buonanno and Fields, 1999). Both Hu+ and Hu− NC-derived cells were found to express excitable traits and increased calcium transient activity. The identity of the Hu− cells is unknown. However, it is possible that these cells are sympathetic neuronal progenitors that have begun to express some neuronal traits prior to overt differentiation (Rohrer and Thonen, 1987).

Blocking calcium transient activity, using 20 mM Mg^{2+}

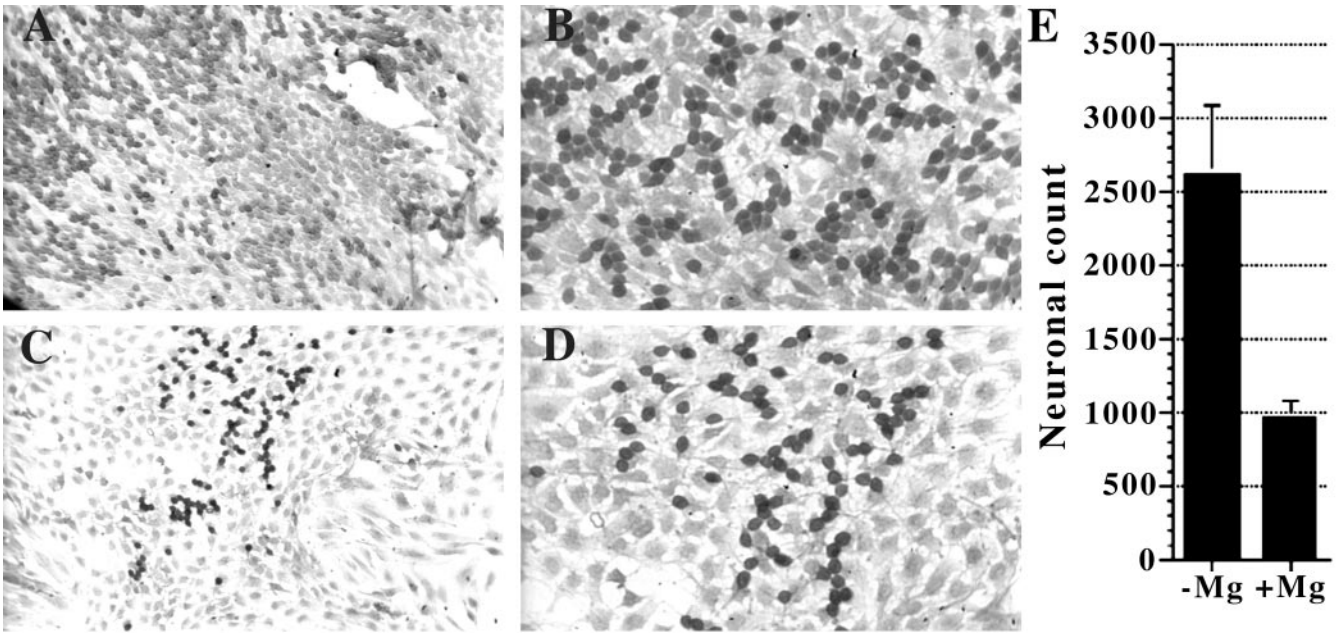

FIG. 8. 20 mM Mg^{2+} blocked neurogenesis in NC cultures. NC cells were cultured with normal culture medium ± 20 mM $MgCl_2$ for 4 days, starting at plating of the neural tubes. The cultures were then fixed and immunolabeled with a neuronal marker, NSE. The NSE labeling was visualized using a biotinylated secondary. (A) 20× phase image of a control NC culture ($-Mg^{2+}$). (B) 40× phase image of the same field as in (A). (C) 20× phase image of NC culture with Mg^{2+}. (D) 40× phase image of the same field as in (C). Note the presence of dark-stained neurons under both conditions, but a noticeable absence of the lighter stained neurons (compare A and C). (E) Mean (±SEM) number of neurons differentiated in the presence and absence of Mg^{2+}. Culturing NC cells in the presence of 20 mM Mg^{2+} significantly reduced the number of neurons differentiated (*$P < 0.01$).

(Fig. 7), inhibited neurogenesis (Fig. 8) with no effect on neuronal survival or overall cell proliferation. Elevated extracellular Mg^{2+} blocks calcium transient activity (Fig. 7). This blocking action seems to be selective for the mechanism for generating calcium transients in NC-derived cells (M. B. Carey and S. G. Matsumoto, in preparation). It has been shown, for example, that Mg^{2+} noncompetitively inhibits the release of intracellular calcium regulated by the IP_3R (Kasahara *et al.*, 1993; Volpe and Vezu, 1993). Although Mg^{2+} can affect other calcium channels (Neuhaus and Cachelin, 1990; Valdivia *et al.*, 1995), the concentration of Mg^{2+} we used had no effect on ryanodine receptors or VDCC (not shown), while it inhibited thimerosal sensitization of IP_3R (Fig. 7). It is possible, however, that elevated Mg^{2+} may be affecting neurogenesis by inhibiting the proliferation of a select population of NC-derived cells.

All of our experiments have consistently found that calcium transients are generated under conditions that support neurogenesis and are not observed when neuronal differentiation is absent. Thus a culture of NC-derived cells examined in the first week exhibits calcium transients as neurons are differentiating but not later, after neurogenesis is complete. This pattern holds true for other culture conditions as well. When NC-derived cells are cultured at low density, for example, they do not exhibit calcium transient activity and we do not observe the generation of neurons (M. B. Carey and S. G. Matsumoto, unpublished results). Other studies have also shown that calcium transient activity is required for neuronal differentiation. For example, inhibition of calcium signaling in *Xenopus* neuroblasts blocked neurogenesis (Jones *et al.*, 1995; Jones and Ribera, 1994). During neuronal maturation, inhibiting calcium spikes in *Xenopus* spinal neurons during their peak transient activity period inhibits expression of GABA-IR (Spitzer *et al.*, 1993), modification of K^+ conductance (Desarmenien and Spitzer, 1991), and regulation of axonal outgrowth (Holliday *et al.*, 1991) (Fig. 9).

It is unclear how neurogenesis is inhibited when calcium transients are blocked. Blocking calcium transient activity may prevent the NC-derived cells from responding to neuronal differentiation signals. A possible mechanism for this has been described for neuronal differentiation of PC12 cells. Studies have shown that calcium modulates the activity of the Ras–MAPK signaling cascade at various levels, increasing the responsiveness of this pathway to activating signals (Finkbeiner and Greenberg, 1996; Ghosh and Greenberg, 1995; Rosen *et al.*, 1994). One level described is at the growth factor-activated tyrosine kinase receptor. Rosen *et al.* (1996) has shown that calcium influx induces the dimerization of tyrosine kinase receptors, in-

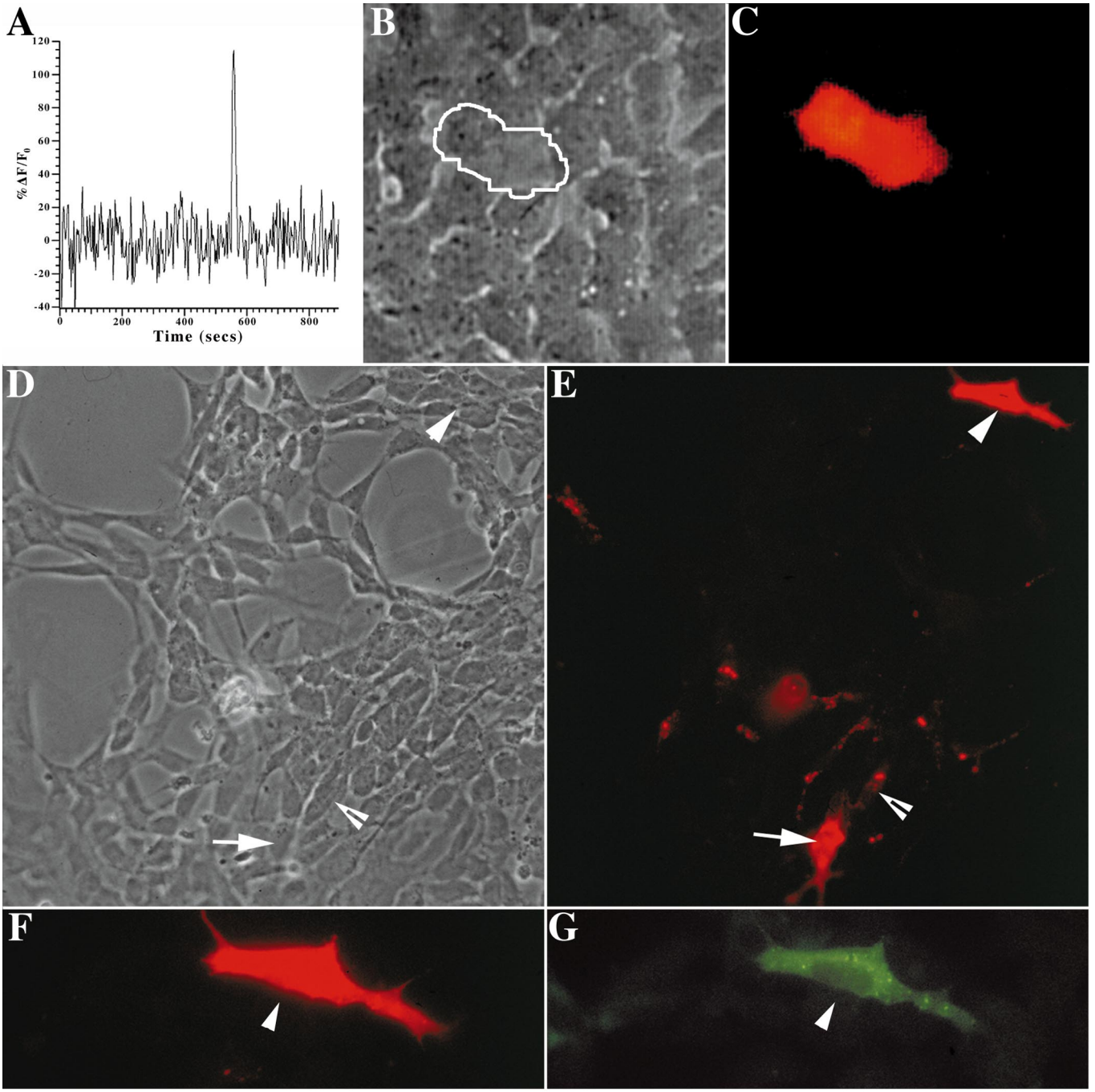

FIG. 9. NC-derived cells that exhibit calcium transients can give rise to neurons. Calcium levels in cultured NC-derived cells were monitored for 15 min. An active cell (A) was selected (B, outlined) and injected with a fixable lineage-tracking dye (C), lyseinated rhodamine dextran (10,000 MW). Culture was then exposed to sympathetic-inducing differentiation media overnight, switched back to the standard culture medium, and then fixed 3 days later. Fixed cultures were immunolabeled for the neuronal marker, Hu. (D) 20× phase image of NC-derived cells that retained the lineage label (arrowhead, inverted arrowhead, and arrow) (E). (F) 40× fluorescent image of labeled cell (arrowhead) expressing Hu-IR (G). Arrow-denoted cell in D + E also exhibited Hu-IR, while the inverted arrowhead-denoted cell was not Hu-IR.

creasing the sensitivity of the receptor to its ligand. Another potential mechanism has been described for regulating neuronal differentiation of neural crest stem cells. In a recent study (Lo *et al.*, 1999), it was shown that Phox2a regulates specification of neurotransmitter identity and was required for neurogenesis. Phox2a actions were dependent on signal transduction pathways that activate the transcription factor CREB, i.e., cAMP and intracellular Ca^{2+}. As seen in both PC12 cells and neural crest stem cells, calcium can regulate various signal transduction pathways to enhance growth factor signaling and transcription factor activation. In this way, calcium signaling may infer competence for neuronal differentiation. In our study, we blocked intracellular calcium release to inhibit calcium-dependent signaling during neuronal differentiation. The problem is that intracellular calcium release is required for a variety of cellular processes: cell proliferation, transcription, and motility. To better ascertain the role of calcium transient activity during development, pharmacological manipulations need to be directed at prospective downstream effectors of calcium, e.g., CAMKIV, CREB, and PKC (Hardingham *et al.*, 1998; Krebs, 1998; Santella and Carafoli, 1997; Tao *et al.*, 1998).

Finally, we provide direct evidence that calcium transient activity is part of the neuronal lineage. We found that a high percentage of active NC-derived cells gave rise to clones containing neurons, while inactive cells did not (Table 3). However, only 50% of the labeled active cells produced clones containing neurons. There are two possible explanations for why some active cells did not produce neurons. The first is that calcium transients may be necessary but not sufficient to induce neuronal differentiation. Other factors may be required in conjunction with calcium signaling to induce neuronal differentiation. Alternatively, it is possible that all of the active cells would have given rise to neurons in time. We allowed the culture to go for only 3 days after labeling before we analyzed the resultant progeny, while neuronal differentiation extends several days past this time point. This could result in labeled cells being fixed prior to an opportunity to differentiate. Although we cannot address the specific role calcium plays in regulating neuronal differentiation, the experiments in thus study suggest that its role is an important one.

TABLE 3
Summary of Injected Active and Inactive NC-Derived Cells

Calcium transient activity present:	No	Yes
No. of clones analyzed	9	12
Average (±SEM) No. of cells/clone	3.6 ± 0.6	2.4 ± 0.4
% of clones with neurons	0	50

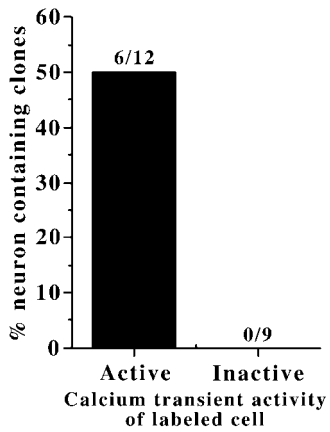

FIG. 10. Active and inactive NC-derived cells differ in their neuronal potential. This is a summary of the data described in Fig. 9. Each clone was determined to contain a neuron, if any of the dye-labeled progeny coexpressed Hu-IR. For each population (active or inactive), the % neuron-containing clones was determined as the number of Hu-IR-containing clones divided by the total number of clones analyzed times 100. A total of 12 active clones and 9 inactive clones were analyzed. The difference between the populations was significant ($P < 0.01$).

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