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Distribution of Multiple Types of Ca^{2+} Channels in Rat Sympathetic Neurons *In Vitro*

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SUMMARY

Ca^{2+} influx through voltage sensitive Ca^{2+} channels produces a rise in intracellular-free Ca^{2+}, $[Ca^{2+}]_i$, that serves as a trigger for the release of neurotransmitters. We measured $[Ca^{2+}]_i$ in primary cultures of superior cervical ganglion (SCG) neurons of the rat using 2-(6-(bis(carboxymethyl)amino)-5-methylphenoxy)ethoxy-2-benzofuranyl)5-oxazole carboxylic acid-based microfluorimetry. Recordings were obtained from either single or small bundles of neuronal processes and compared with recordings from single neuronal cell bodies. Depolarization with 50 mM K^+ produced a rapid increase in $[Ca^{2+}]_i$ consisting of both transient and sustained components. This response pattern was seen in recordings from both the soma and processes of SCG neurons. The entire response could be reversibly blocked by 30 μM La^{3+}. Nitrendipine, 1 μM, inhibited the response by 52 ± 7% and 49 ± 7 % in the soma and processes, respectively. The dihydropyridine (DHP) agonist 1,4-dihydro-2,-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylic acid methyl ester enhanced depolarization-induced increases in $[Ca^{2+}]_i$ in both regions of the neuron. The transient component of the response was greatly reduced by predepolarization, and the remaining sustained component was inhibited 77 ± 7% by nitrendipine (1 μM). These data demonstrate that both DHP-sensitive and -insensitive Ca^{2+} channels are present in processes as well as cell bodies of SCG neurons. The importance of these findings is discussed in relation to the insensitivity of neurotransmitter release from sympathetic neurons to DHP antagonists.

Multiple types of Ca^{2+} channels have been described in a variety of excitable tissues. One type of Ca^{2+} channel, designated L in the terminology of Nowycky *et al.* (1), is sensitive to DHP drugs. In sympathetic neurons, electrophysiological studies have revealed the presence of both DHP-sensitive (L-type) and -insensitive (N-type) Ca^{2+} channels (no T-type Ca^{2+} channels were found) (2). However, although L-type Ca^{2+} channels are present in these neurons, the depolarization-evoked release of neurotransmitter from these cells is not inhibited by DHP antagonists (3, 4). This situation typifies a discrepancy found in many types of neurons. For example, we have demonstrated the widespread distribution of functional DHP-sensitive Ca^{2+} channels in neurons throughout the brain (5). However, in most cases DHP antagonists do not block neurotransmitter release from central nervous system preparations (6–10). In general, neurotransmitters are released from terminal varicosities of neurons rather than from the cell soma. However, most previous studies on vertebrate neurons have only obtained direct information about Ca^{2+} channels in the cell soma. It is clearly important to obtain similar information about the properties of those Ca^{2+} channels that exist in nerve terminals in close proximity to the actual sites of neurotransmitter release. The small size of terminal varicosities and axons from vertebrate neurons has precluded the use of many standard electrophysiological techniques in such studies. However, optical methods based on the use of Ca^{2+}-sensitive fluorescent probes are an alternative (4, 11).

In this report, we have used an extremely sensitive microfluorometer and the fluorescent Ca^{2+} indicator fura-2 to measure $[Ca^{2+}]_i$ in both the processes and cell bodies of sympathetic neurons *in vitro* in an attempt to determine the distribution of Ca^{2+} channel types in these two locations. Interestingly, we have found that, although norepinephrine release from these cells is not blocked by DHP antagonists (3, 4), both the soma and processes of SCG neurons possess DHP-sensitive Ca^{2+} channels.

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Experimental Procedures

Cell culture. Sympathetic neurons were cultured from the SCG of neonatal rats by a modification of the method of

ABBREVIATIONS: DHP, dihydropyridine; fura-2, 2-(6-(bis(carboxymethyl)amino)-5-methylphenoxy)ethoxy-2-benzofuranyl)5-oxazole carboxylic acid; $[Ca^{2+}]_i$, intracellular-free Ca^{2+}; SCG, superior cervical ganglion; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Bay k 8644, 1,4-dihydro-2,-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylic acid methyl ester; NIT, nitrendipine (1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid 3-methyl-5-ethyl ester); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

Wakade *et al.* (12). Ganglia were removed from 1- to 3-day-old rat pups after decapitation and placed in Ca^{2+}/Mg^{2+}-free HEPES-based minimum essential medium. This medium contained (in mM) NaCl, 136; KCl, 5.4; KH_2PO_4, 0.44; Na_2HPO_4, 0.34; $NaHCO_3$, 2.63; glucose, 13.6; HEPES, 20; minimum essential media vitamins (GIBCO, Grand Island, NY), 10 ml/liter; minimum essential media amino acids (GIBCO), 20 ml/liter; phenol red, 10 mg/liter (GIBCO) with the pH adjusted to 7.4 with NaOH. The ganglia were enzymatically dissociated by incubation in media containing collagenase/dispase (Boehringer-Mannheim Biochemicals, Indianapolis, IN). After 1 hr incubation, trypsin (Worthington Diagnostic Systems, Freehold, NJ) was added and the ganglia incubated for an additional 15 min. Cells were dissociated further by mechanical trituration through a flame-constricted Pasteur pipet. The cells were plated on glass coverslips (no. 1, 25 mm round) coated with rat-tail collagen, prepared in our laboratory by the method of Bornstein (13). The growth medium was Leibowitz L-15 (GIBCO) supplemented with glucose, glutamine, and vitamins (12). The medium was further supplemented with 5% rat serum and 1 μg/ml nerve growth factor isolated from mouse saliva in our laboratory by the method of Burton *et al.* (14). Cultures were treated for 24 hr with cytosine arabinoside (10–100 μM) (Cytosar, Upjohn, Kalamazoo, MI) on the second day after plating to eliminate non-neuronal cells.

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was determined using a microfluorimeter to monitor the Ca^{2+}-sensitive fluorescent chelator, fura-2 (11). Neurons were loaded with the dye by incubation with 2 μM fura-2 acetoxymethyl ester (Molecular Probes Inc., Eugene, OR), which is membrane permeant, for 1 hr at 37° in HEPES-buffered Hanks' balanced salt solution, pH 7.45, containing 0.5% bovine serum albumin. The HEPES Hanks' solution was composed of (in mM) HEPES 20; NaCl, 137; $CaCl_2$, 1.3; $MgSO_4$, 0.4; $MgCl_2$, 0.5; KCl, 5.0; KH_2PO_4, 0.4; $NaHPO_4$, 0.6; $NaHCO_3$, 3.0; and glucose, 5.6. After the loading incubation, during which time the dye ester is hydrolyzed by cytosolic esterases to the membrane-impermeant polycarboxylate anion that is fura-2, the cells were washed twice in the HEPES-Hanks' solution and incubated for 30 min. It is difficult to measure precisely the amount of dye loaded into a cell. However, in cells loaded with a 100-μM fura-2 via internal dialysis with a patch clamp pipet, the fluorescence signal was similar to the very brightest cells used in this study. Thus, 100 μM would be an upper limit for the intracellular fura-2 concentration.

The coverslips containing the loaded and washed cells were then mounted in a flow-through chamber for viewing. The chamber is a modification of a previously described cell superfusion system (15). Briefly, the chamber consisted of a Plexiglas block machined to accommodate the coverslip as a bottom. Three reservoirs were cut into the block such that a thin sheet of buffer flowed out of the inlet reservoir, across the cells in the experimental chamber, and was drawn up across nylon mesh to the efflux reservoir to be evacuated by suction. Solutions in the chamber could be completely exchanged within 10 sec, including the delay between the large media reservoirs and the inlet to the chamber. Experiments were run at 22°.

The perfusion chamber was mounted on an inverted microscope (Diaphot, Nikon, Garden City, NY), and cells and processes were localized by standard phase contrast illumination. To excite the fura-2, the collimated light beam from a 200-W Hg arc lamp was passed through a dual beam spectrophotometer (Phoenix Instruments) which alternated wavelengths from 340 to 380 nm by means of a wheel spinning at a frequency of 60 Hz. In place of the original sample chamber, a collimating beam probe was placed to focus the light onto the end of a liquid light guide (3 mm × 1 m, Oriel, Stratford, CT). On the other end of the liquid light guide, a similar probe was positioned to direct light through the epifluorescence illuminator of the microscope. The light was reflected off a dicroic mirror (Nikon, DM 400) and through a ×70 phase contrast oil immersion objective (E. Leitz Inc., Rockleigh, NJ, numerical aperature 1.15). The emission fluorescence was selected for wavelength with a 480-nm barrier filter, and recordings were defined spatially with a rectangular diaphragm. The fluorescence emission was analyzed with a photomultiplier tube and discriminator (Thorn EMI Gencom Inc., Plainview, NY). The discriminator output was converted to pulses, which were then integrated by passing the signal through an 8-pole low pass Bessel filter at 500 Hz. The photomultiplier signal was fed into one channel of an analog to a digital converter PDP-11/73 computer system (Indec Systems, Sunnyvale, CA). The signal from two photodiodes, each placed in a small portion of the light beam directed to the monochromators, were fed into two additional channels of the analog to digital converter.

The photomultiplier output was sorted into signals from 340 and 380 nm excitation by using the photodiode output as syncronizing signals. In the typical traces described here, 30 ratios were determined in 1.1 sec, the average ratio was displayed on-line, and the average intensity values for each wavelength were stored. After completion of a given experiment, the microscope stage was adjusted so that no cells or debris occupied the field of view defined by the diaphragm, and background light levels were determined. Background light levels ranged from less than 5% for large cell bodies to close to 50% for very fine processes. Autofluorescence from cells that had not been loaded with fura-2 was not detectable. Records were later corrected for background and the ratios recalculated. Ratios were converted to free $[Ca^{2+}]$ by using the equation $[Ca^{2+}] = K(R\text{-}R_{min})/(R_{max}\text{-}R)$, in which R is the 340/380-nm fluorescence ratio (11). The maximal ratio (R_{max}), the minimal ratio (R_{min}), and the constant K (K is the the product of the dissociation constant for fura-2 and the ratio of the free and bound forms of the dye at 380 nm) were determined from a standard curve to which the above equation was fit using a nonlinear least squares fit computer program. The system was calibrated after any adjustment in the apparatus. Values for the constants R_{min}, R_{max}, and K ranged from 0.121 to 0.334, 4.02 to 5.06, and 2034 to 2373, respectively. The standard curve was determined for the fura-2 pentapotassium salt in calibration buffer (which contains, in mM: HEPES, 20; KCl, 120; NaCl, 5; KH_2PO_4, 1; $NaHCO_3$, 5; pH 7.1) containing 10 mM EGTA ($K_s = 3.969 \times 10^6$ M) (16) with varying amounts of Ca^{2+} added, which were calculated to give free Ca^{2+} concentrations ranging from 0–2000 nM. Records were digitally filtered with an algorithm, which added ½ of the value of each data point with ¼ of the value of each of the two neighboring points. The data were cycled through this routine five times. Results are presented as means ± SE.

All experiments were performed on cells continuously perfused with HEPES Hanks' solution. Depolarization-induced Ca^{2+} influx was produced by changing the perfusing solution

from low K^+ (5 mM) to high K^+ (50 mM), with K^+ exchanged for Na^+ reciprocally. Nominally Ca^{2+}-free solutions were prepared by simply omitting Ca^{2+}. For experiments with La^{3+}, all anions were replaced with Cl^- to prevent precipitation.

Results

Neurons from the SCG of the rat were grown in primary culture for 3–7 days before experimentation. Fig. 1A shows a photomicrograph of a typical field of these neurons. Note the thick bundle of processes (Fig. 1A, *panel 1*) emanating from the small cluster of cell bodies. Although these structures provided a somewhat better signal in our experiments than the very fine (<1 μm) processes emanating from a single cell body (Fig. 1A, *panel 2*), we were able to reproducibly measure signals from single processes. In the present studies, experiments were performed on both single and bundles of processes. All soma recordings were obtained from single cell bodies (Fig. 1A, *panel 3*). The rectangles define representative areas from which recordings would be made for each of these structures.

Fig. 1B shows the typical response of a cell body to depolarization with 50 mM K^+. The upper traces represent background corrected fluorescence signals from excitation at 340 and 380 nm. Below is the $[Ca^{2+}]_i$ calculated from a standard curve in which the ratio of these two signals is plotted versus known concentrations of Ca^{2+} (see Experimental Procedures). Application of 50 mM K^+ for 2 min induced a large rise in $[Ca^{2+}]_i$ to a level of 1313 ± 97 nM ($N = 10$) from a basal level of 99 ± 7 ($N = 10$). After removal of the depolarizing stimulus and relaxation of the Ca^{2+} signal to basal levels, a second, identical stimulation produced a response that was 90 ± 5% of the initial response. Indeed, as shown in this example, sequential depolarizing stimuli repeatedly produced essentially the same response. $[Ca^{2+}]_i$ did not rise to quite the same level in SCG processes, increasing to 914 ± 168 nM ($N = 12$) from a similar basal level of 109 ± 13 nM ($N = 12$). Thus, using this paradigm, the peak $[Ca^{2+}]_i$ seen in processes corresponded to a level 30 ± 13% lower than that recorded from the neuronal soma. Multiple responses to depolarization could also be elicited from SCG processes, as shown in Fig. 1D. The second response recorded from processes, like those from cell bodies, decreased little from the initial response (1 ± 7%).

In our initial experiments, we determined the source of the Ca^{2+} involved in the observed depolarization-induced rise in $[Ca^{2+}]_i$. Depolarization in nominally Ca^{2+}-free media failed to elicit any rise in $[Ca^{2+}]_i$ in either the soma or processes (see below), suggesting that influx of extracellular Ca^{2+} is necessary. Furthermore, as shown in Fig. 1C, depolarization in the presence of the inorganic Ca^{2+} channel blocker, La^{3+}, failed to produce a significant rise in $[Ca^{2+}]_i$. Depolarization-induced responses recorded from cell bodies were only 1 ± 1% ($N = 4$) of control values in the presence of 30 μM La^{3+}. Similarly, in processes of these cells, depolarization-induced rises in $[Ca^{2+}]_i$ were only 11 ± 7% ($N = 4$) of control in the presence of 30 μM La^{3+}. Fig. 1, D and E, shows responses from control and La^{3+}-treated processes, respectively. The increase in basal $[Ca^{2+}]_i$ observed in this La^{3+} treated process (Fig. 1E) was occasionally seen, although most other La^{3+}-treated processes did not exhibit this type of response. This particular recording is shown because it displays an especially good recovery from the La^{3+} treatment. Concentrations of La^{3+} below 100 μM have been shown to inhibit Ca^{2+} influx through voltage-sensitive calcium channels without a significant effect on efflux mechanisms such as the Na^+/Ca^{2+} exchanger or the Ca^{2+} ATPase in squid axon (17). Thus, the increase in $[Ca^{2+}]_i$ produced by K^+ depolarization is dependent on Ca^{2+} influx through voltage sensitive calcium channels. However, we cannot rule out the possibility that the initial influx of Ca^{2+} is amplified by release of Ca^{2+} from intracellular stores.

DHPs are specific modulators of L-type calcium channels. In the experiments shown in Fig. 2, we used these drugs as a probe for the presence of L-type channels in the soma and processes of SCG neurons. DHP agonist effects were studied by using a previously used paradigm (5). Cells were slightly depolarized by perfusion with 15 mM K^+, which increased basal $[Ca^{2+}]_i$ by 52% to 152 ± 20 nM ($N = 5$). In processes, basal $[Ca^{2+}]_i$ increased by 50% to 165 ± 32 nM ($N = 6$). The addition of 1 μM Bay k 8644, a DHP agonist, produced a significant rise in $[Ca^{2+}]_i$ in both the soma and processes of these cells, as shown in Figs. 2A and B. Bay k 8644 increased $[Ca^{2+}]_i$ relative to basal (15 mM K^+) levels by 100 ± 21% ($p < 0.01$, paired t test) in the soma and 51 ± 6% ($p < 0.001$, paired t test) in the processes. These responses were small relative to those elicited by depolarization with 50 mM K^+, but they occurred in every cell soma ($N = 14$) and process ($N = 11$) tested. The rise in $[Ca^{2+}]_i$ produced by Bay k 8644 in these cells was similar to increases reported previously for central neurons *in vitro* under similar experimental conditions (5).

The contribution of L-type channels to the Ca^{2+} influx induced by 50 mM K^+ was studied by determining the portion of the response blocked by a high, maximally effective concentration of NIT, a potent DHP Ca^{2+} channel blocker. Multiple responses could be elicited from both the soma and processes of SCG neurons with only slight attenuation, as shown in Fig. 1, B and D. However, in the presence of 1 μM NIT, as shown in Fig. 2, C and D, the second response was inhibited, relative to control, in both somata (53 ± 7%, $N = 6$) and processes (50 ± 7%, $N = 7$). Subsequent perfusion with drug-free media produced a gradual washout of the drug and recovery of the response. The slow rate of washout is not surprising considering the highly lipophilic nature of the drug. The effects of DHP antagonists on depolarization-induced Ca^{2+} influx into SCG neurons are summarized in Fig. 3.

The depolarization-induced rise in $[Ca^{2+}]_i$ appeared to be composed of two components. To clarify this observation, we expanded the time scale of the response by calculating a ratio every 100 msec. The result of this experiment (Fig. 4A) clearly demonstrates the biphasic nature of the response. There was an initial transient phase followed by a long-lasting plateau phase that declined very slowly and then relaxed rapidly after removal of the depolarizing stimulus. This biphasic response was seen in cell processes as well.

In an attempt to determine which Ca^{2+} channels contributed to the different portions of the response, we performed the experiments shown in Fig. 4, B–D. In Fig. 4B, a control response to 50 mM K^+ was produced subsequent to incubation in Ca^{2+}-free media and then readdition of Ca^{2+}. It can be seen that this response was identical to the normal control responses shown in Figs. 1 and 2, indicating that pretreatment with Ca^{2+}-free medium *per se* did not alter the characteristics of the response. The transient and sustained plateau phases are especially visible in this example because the cell was depolarized for a long (15-min) period of time. The control response was then followed

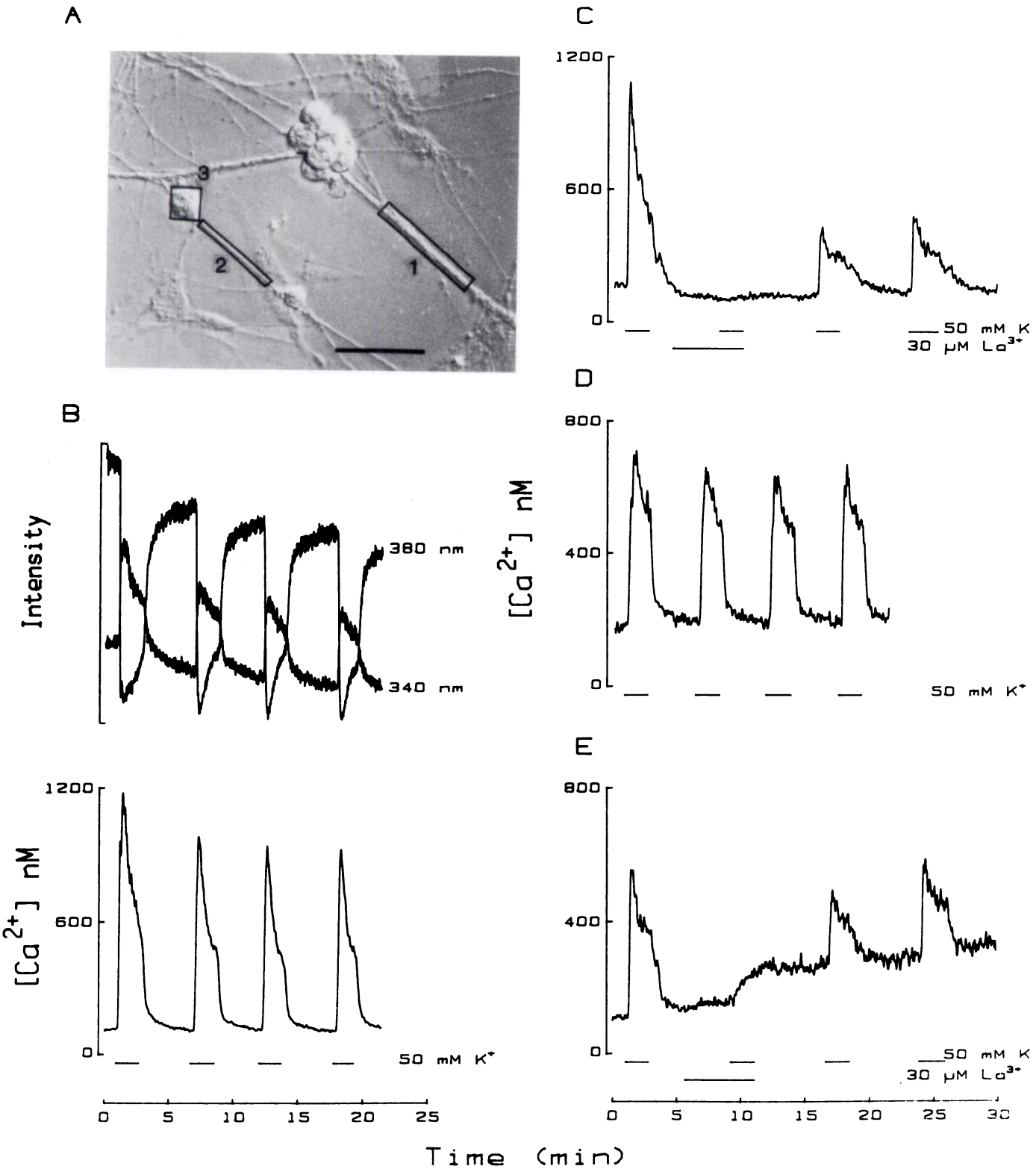

Fig. 1. Photomicrograph of cultured sympathetic neurons and depolarization-induced increases in $[Ca^{2+}]_i$ in the absence and presence of La^{3+}. A. Hoffman modulation contrast photomicrograph of neurons cultured from the SCG (×400) illustrating typical photomultiplier tube diaphragm settings for: (1) a bundle of processes, (2) a single process, and (3) a cell body. Calibration bar is 200 μm. A cell soma (B) and process (D) were alternately perfused with 5 mM K^+ and 50 mM K^+ media. The upper panel in B shows background corrected fluorescence intensity recordings from excitation at 340 and 380 nm. These values were then converted to ratios, and the calculated $[Ca^{2+}]_i$ values are shown in the lower panel (see Experimental Procedures for calculations) Depolarization-induced responses were determined in the presence of 30 μM La^{3+} (during second stimulus) for a cell soma C and process E. Horizontal bars mark additions to the media and are labeled at right.

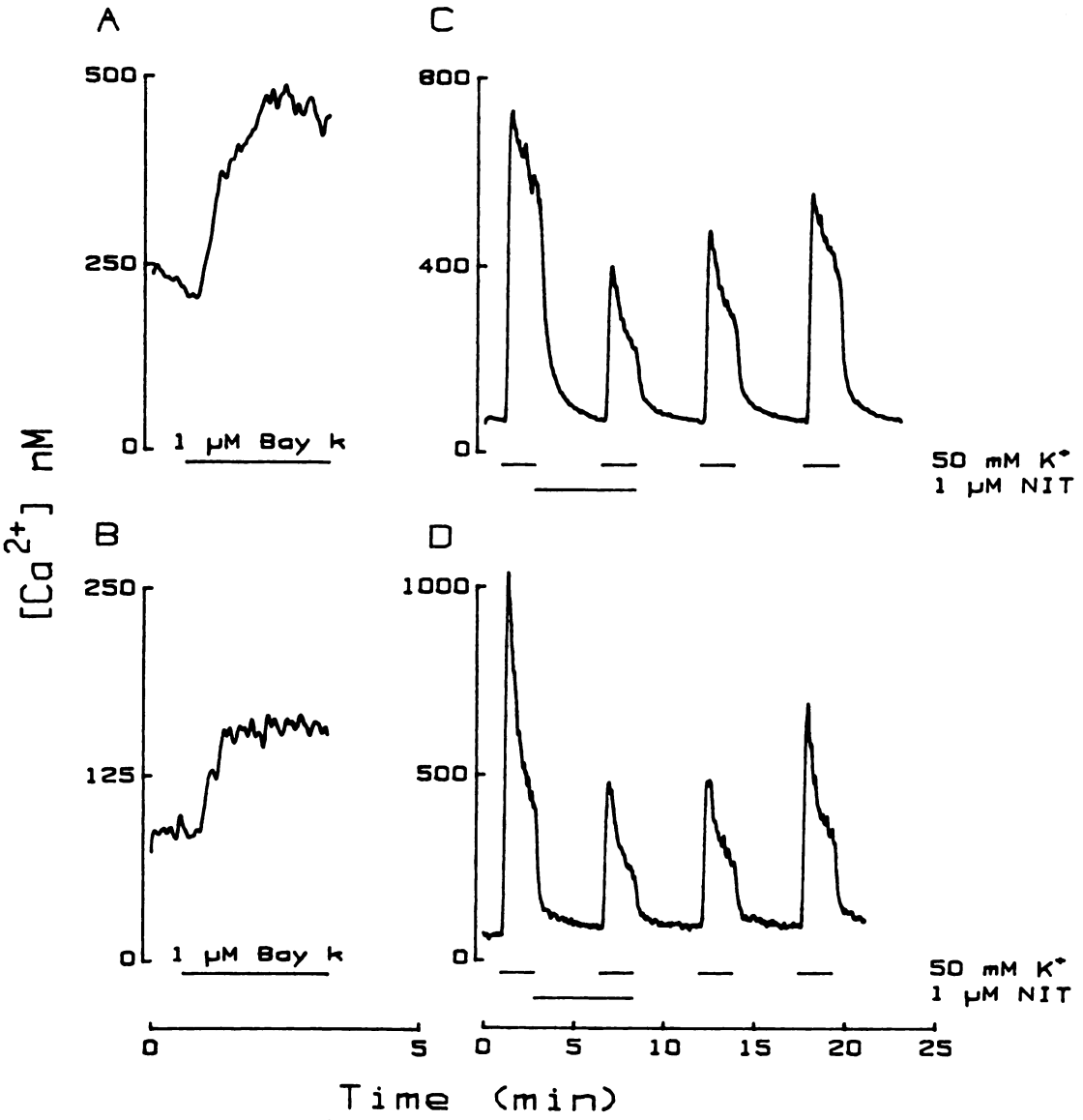

Fig. 2. DHP agonist and antagonist effects on $[Ca^{2+}]_i$ in SCG somata and processes. A cell soma A and process B were perfused with 15 mM K^+, and 1 μM Bay k 8644 was added as indicated by the underlying bar. A cell soma C and process D were perfused with 50 mM K^+ in the presence (second stimulus) and absence of 1 μM NIT. Horizontal bars mark additions to the media and are labeled at right.

by depolarization in the absence of Ca^{2+}, followed by readdition of extracellular Ca^{2+}. Predepolarization abolished the transient portion of the rise in $[Ca^{2+}]_i$, indicating a voltage dependent inactivation of this first phase of Ca^{2+} influx. The remaining, sustained plateau portion of the response was equivalent in size to that reached after relaxation from the transient peak of a normal biphasic response. The inactivation of the transient phase by predepolarization was readily reversed, as shown in Fig. 4 C. In this experiment a control response was elicited by perfusion with 50 mM K^+, followed by a second response in which the transient phase of the rise in $[Ca^{2+}]_i$ was allowed to voltage inactivate by predepolarization in Ca^{2+}-free media. Thus, only the slow plateau phase of the response was seen. Finally, the cell was perfused with low K^+ media allowing it to repolarize so that when the third response was elicited, a biphasic rise in $[Ca^{2+}]_i$ was once again seen. Reproducible multiple responses could be generated in cell bodies and processes after predepolarization as shown in Figure 4D. Therefore, the DHP-sensitive portion of the sustained increase in $[Ca^{2+}]_i$ could be determined by using a NIT treatment protocol similar to that used in Fig. 2. The result can be observed in Fig. 4E. Subsequent to a normal biphasic response and a control predepolarized sustained response, the cells were perfused with 1 μM NIT during a second predepolarization and Ca^{2+} response. This second, NIT-treated response was inhibited by 77 ± 7% (N = 5). Thus, the long-lasting elevation in $[Ca^{2+}]_i$ produced by perfusion with 50 mM K^+ is primarily, although not entirely, the result of sustained Ca^{2+} influx through DHP-sensitive Ca^{2+} channels.

Discussion

It has previously only been possible to obtain limited direct information about the properties of Ca^{2+} channels existing in the axons and terminals of vertebrate neurons (reviewed in Refs. 8 and 9). For example, radiotracer flux studies using synaptosomes have provided some information about the na-

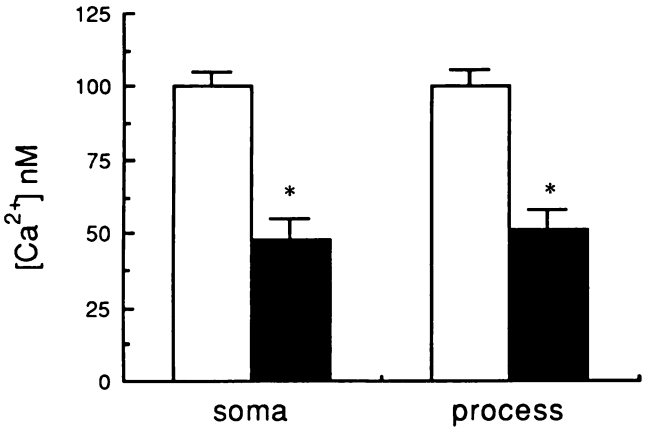

Fig. 3. Effect of 1 μM NIT on the depolarization-induced increase in $[Ca^{2+}]_i$ in SCG somata and processes. Peak increases in $[Ca^{2+}]_i$ in response to the second 2-min application of 50 mM K^+ were determined in the absence (*open bars*) and presence (*solid bars*) of 1 μM NIT. Experiments were performed as shown in Fig. 2 C–D and normalized to the initial, drug-free response. *, $p < 0.005$, Student's *t* test.

ture of such Ca^{2+} channels (6, 7). These portions of vertebrate neurons are generally not amenable to electrophysiological recording techniques, although recent studies reported by Lipscomb et al. have described patch-clamp recordings from the processes and growth cones of sympathetic neurons (18). Consistent with our results, they report the presence of both N- and L-type Ca^{2+} channels in the processes of these neurons. The use of the sensitive dye fura-2 provides an alternative method, as demonstrated in the studies described here.

Our results provide evidence for the relatively uniform distribution of DHP-sensitive (L-type) and insensitive (N-type) Ca^{2+} channels throughout the soma and processes of rat sympathetic neurons grown in primary culture. It is important to recognize that photon counting, the technique used here, essentially integrates the $[Ca^{2+}]_i$ over the entire area from which recordings are made. Thus, the data do not provide precise insights regarding the possible microdistribution of Ca^{2+} channels. These results do, however, indicate that the channel types are not segregated significantly in the cell bodies versus the processes of these neurons.

The presence of L-channels in both the soma and processes of sympathetic neurons is particularly interesting when one considers the insensitivity of neurotransmitter release from these cells to DHP antagonists[1] (4). Norepinephrine release evoked by depolarization with elevated K^+, using a paradigm virtually identical to that used here, was not blocked by DHP antagonists. Neurotransmitter release from sympathetic neurons is thought to occur predominantly from varicosities that are scattered along the axons emanating from sympathetic neuron cell bodies. Our results indicate that these regions of the neuron do indeed contain L-channels that are activated by the same depolarizing stimuli that produce NIT-insensitive norepinephrine release. It would seem therefore, that some further level of microheterogeneity in the arrangement of L and N channels may exist with respect to sites of norepenephrine release. Indeed, as the influx of Ca^{2+} via a particular Ca^{2+} channel will have a restricted sphere of influence because of internal buffering mechanisms (19, 20), such a microarrangement of different types of Ca^{2+} channels may be an important way of restricting their functions. Thus, it may be that N-channels are found clustered in close proximity to zones of norepinephrine release in these neurons. Some electronmicroscopic and electrophysiological evidence also suggests the clustering of neuronal Ca^{2+} channels (21–23). It is interesting to note that the DHP agonist Bay k 8644 will enhance voltage sensitive Ca^{2+} influx into sympathetic neuronal processes and will also enhance depolarization induced norepinephrine release (3, 4). Thus, Bay k 8644 increased the maximal amount of neurotransmitter released in response to 70 mM K^+, presumably by producing an especially large influx of Ca^{2+} through DHP-sensitive channels. Only when such a supramaximal Ca^{2+} influx was generated were DHP-sensitive Ca^{2+} channels found to contribute to neurotransmitter release. Such experiments further indicate that functional L-channels are indeed found in processes and can provide Ca^{2+} for neurotransmitter release under extreme conditions. As Bay k 8644 selectively enhances Ca^{2+} influx through L-channels, it may be that under such conditions the normal local buffering capacity is overwhelmed and that now Ca^{2+} entering via this route may also contribute to transmitter release. This model has been discussed in detail elswhere (8).

The biphasic nature of the Ca^{2+} influx we have observed is also of interest. There is a rapidly inactivating phase that appears to be partially inhibited by NIT, suggesting a contribution from both DHP-sensitive and insensitive Ca^{2+} channels to this phase of Ca^{2+} influx. Furthermore, it is clear that this transient portion of the response can be blocked by predepolarization. After such treatment there is a sustained plateau phase of the response, which appears to be caused by maintained Ca^{2+} influx through both L and N channels. In chick dorsal root ganglion cells, which also possess both L and N channels, electrophysiological studies have shown that there is a region in which their activation and inactivation curves cross (22–23). This suggests that at some membrane potentials, Ca^{2+} currents may reach a steady state in which their activation and inactivation rates are equal. It is likely that these so-called "window currents" are responsible for the sustained influx of Ca^{2+} observed after predepolarization and possibly for a portion of norepinephrine release from these neurons also observed after predepolarization (4). The realization that such a sustained phase of Ca^{2+} influx can occur may be of physiological significance. Thus, depolarization of a cell to this membrane potential may produce neurotransmitter release with different temporal characteristics from that produced by stronger depolarizations. The absolute levels of $[Ca^{2+}]_i$ reached and the shape of the two phases of the depolarization-induced response are the results of not only Ca^{2+} influx but also of buffering and efflux mechanisms.

We have observed the presence of L channels in a number of types of neurons, including those from the rat SCG (this study), the rat dorsal root ganglion[2] and the myenteric plexus.[3] Furthermore, virtually every neuron cultured from the central nervous system was found to be sensitive to DHP drugs (5). We have noted, however, that the relative distribution of DHP sensitive and insensitive channels seems to vary between cell

[1] L. D. Hirning, A. P. Fox, E. W. McClesky, B. M. Olviera, S. A. Thayer, R. J. Miller, and R. W. Tsien, submitted for publication.

[2] S. A. Thayer, T. M. Perney, and R. J. Miller, unpublished observations.

[3] L. D. Hirning, R. J. Miller, and M. S. Sturek, submitted for publication.

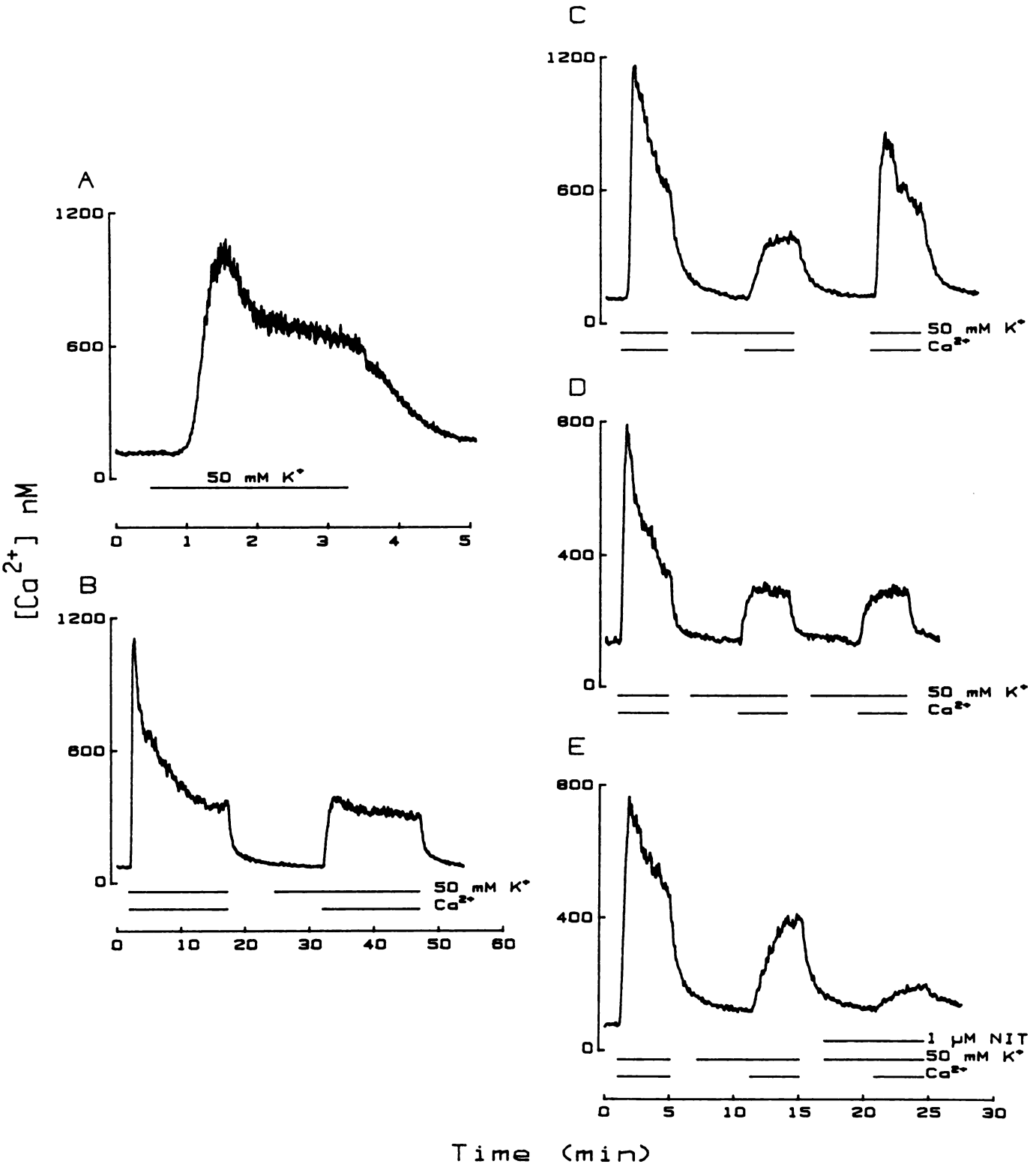

Fig. 4. Evaluation of the biphasic $[Ca^{2+}]_i$ response by rapid time course, predepolarization, and NIT treatment experiments. A. The $[Ca^{2+}]_i$ response to perfusion with 50 mM K^+ was measured at a 100-msec sampling rate. B. A typical depolarization-induced rise in $[Ca^{2+}]_i$ was produced subsequent to incubation in Ca^{2+}-free media. After recovery the cell was perfused with 50 mM K^+ in Ca^{2+}-free media, followed by readdition of Ca^{2+}. Similar protocols were followed to generate biphasic responses before and after a predepolarized response (C), a biphasic response followed by two predepolarized responses (D), and a biphasic response followed by a predepolarized response, which was then followed by a predepolarized response in the presence of 1 μM NIT. B–E. 1.3 mM Ca^{2+}, 50 mM K^+, and 1 μM NIT were added as indicated by underlying bars.

types. Collectively, these results suggest that L-channels are commonly used to regulate neuronal $[Ca^{2+}]_i$.

In conclusion, this report establishes the presence of both DHP-sensitive and -insensitive Ca^{2+} channels in the processes and cell bodies of SCG neurons. Determination of the physiological function of L-channels in these neurons is an interesting area for additional study because they do not appear to play a significant role in the release of classic neurotransmitters.

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