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INCREASES IN INTRACELLULAR CALCIUM ION CONCENTRATION DURING DEPOLARIZATION OF CULTURED EMBRYONIC *XENOPUS* SPINAL NEURONES

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SUMMARY

1. Changes in intracellular Ca^{2+} ion concentrations ($[Ca^{2+}]_i$) during potassium-induced depolarizations were studied in cultured embryonic *Xenopus* spinal neurones using the Ca^{2+}-sensitive dye Fura-2 and quantitative fluorescence microscopy.

2. Membrane voltages attained during exposure to bath solutions containing 3, 10, 20, 30, 40 and 50 mM-K^+ were determined under current clamp. In 3 mM-K^+-containing solution (normal saline), the resting potential was −65 mV. The threshold voltage required to observe a measurable rise in $[Ca^{2+}]_i$ was −40 mV (external potassium concentration $[K^+]_o$ = 20 mM). The depolarization-induced $[Ca^{2+}]_i$ signal had two components: a non-relaxing component, and, at voltages positive to −40 mV, an additional transient component on the rising phase that decayed over tens of seconds. There was substantial variability in the magnitudes of resting and voltage-induced changes in $[Ca^{2+}]_i$, but $[Ca^{2+}]_i$ responses were qualitatively consistent between neurones of similar ages.

3. External potassium (K_o^+)-induced increases in $[Ca^{2+}]_i$ were spatially non-homogeneous. The largest increases were seen in the nucleus, near the base of a major neurite, and in growth cones. Increases occurred more rapidly in neurites and growth cones than in somas. T-type and high-voltage-activated (HVA) channels appeared to be present in all cell regions.

4. Increases in $[Ca^{2+}]_i$ evoked by 50 mM-K^+ (depolarization to approximately −15 mV) were sensitive to treatments demonstrated to inhibit Ca^{2+} currents in these cells (T-type, HVA–relaxing and HVA–sustained), including Ni^{2+} (200 μM), Met-enkephalin (17·5 μM), and ω-conotoxin (ω-CgTx; 5·5 μM). $[Ca^{2+}]_i$ increases were reduced by caffeine (10 mM) and ryanodine (10–100 μM), agents that affect Ca^{2+} release from intracellular stores.

5. A sustained increase in $[Ca^{2+}]_i$ observed at approximately −40 mV ($[K^+]_o$ = 20 mM) was investigated in greater detail. Concentrations of Ni^{2+} sufficient to block T-type Ca^{2+} current slowed but did not block the rise in $[Ca^{2+}]_i$ induced by 20 mM-K^+. Met-enkephalin did not affect the $[Ca^{2+}]_i$ response. ω-CgTx reduced the amplitude of the $[Ca^{2+}]_i$ response, but did not eliminate the sustained component.

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Verapamil (100 μM), caffeine and ryanodine differentially reduced the sustained component as compared to the initial rising phase. These observations suggest that the rising phase was due to Ca^{2+} influx through T-type and other Ca^{2+} channels, and that the sustained phase was differentially sensitive to inhibition of internal Ca^{2+} release.

6. The data indicate that slow subthreshold depolarizations can cause long-lasting changes in $[Ca^{2+}]_i$, and that at subthreshold voltages $[Ca^{2+}]_i$ is influenced by interactions between Ca^{2+} entry through Ni^{2+}- and ω-CgTx-sensitive pathways (including T-type Ca^{2+} channels), and ryanodine-sensitive Ca^{2+} release from intracellular stores. Thus Ca^{2+}-dependent developmental processes promoted by subthreshold depolarizations may be modulated by both Ca^{2+} entry and intracellular Ca^{2+} release.

INTRODUCTION

In developing neurones, changes in intracellular Ca^{2+} ion concentration ($[Ca^{2+}]_i$) are thought to influence not only neurotransmitter release (as in adult neurones), but numerous other processes including neurite outgrowth (Cohan, Connor & Kater, 1987), biochemical differentiation (e.g. neurotransmitter phenotype; Walicke & Patterson, 1981) and survival (Collins & Lile, 1989). Voltage-gated Ca^{2+} channels are one class of membrane macromolecule that mediates passage of Ca^{2+} ions across the cell membrane, and the electrophysiological characteristics of Ca^{2+} currents present in developing cultured *Xenopus* spinal neurones were presented in the preceding paper. While activation of Ca^{2+} currents by depolarization is followed by increase in $[Ca^{2+}]_i$ (Baker, Hodgkin & Ridgway, 1971; Smith & Zucker, 1980; Bolosover, 1986, 1987; Thayer & Miller, 1990), the relationship between these two processes is quite complex, and is influenced by cytoplasmic Ca^{2+} buffering, sequestration and extrusion mechanisms in addition to the gating characteristics of the Ca^{2+} currents involved.

In the present study, the Ca^{2+}-sensitive dye Fura-2 has been used to analyse the influence of extracellular K^+ (K_o^+)-induced depolarizations to sub- and suprathreshold voltages on $[Ca^{2+}]_i$ in cultured embryonic *Xenopus* spinal neurones, cells whose differentiation and early development has been extensively investigated (see, for reviews, Spitzer, 1985; Warner, 1985). These experiments focused on three issues: the intracellular distribution of the increases in $[Ca^{2+}]_i$ induced by depolarization, the relationship of these increases to activation of the voltage-gated Ca^{2+} channels described in the preceding paper (Barish, 1991), and modulation of these increases in $[Ca^{2+}]_i$ by Ca^{2+} release from intracellular stores. As with the preceding investigation of voltage-gated Ca^{2+} channels (Barish, 1991), $[Ca^{2+}]_i$ increases at subthreshold voltages were investigated in particular detail because of their potential importance in activity-dependent modulation of development.

The data obtained indicate that within a voltage range bounded by the resting potential and the action potential threshold (i.e. between -65 and -30 mV), depolarization initiates increases in $[Ca^{2+}]_i$ that are maintained for the duration of minutes-long shifts in membrane potential. At more positive voltages, increases in $[Ca^{2+}]_i$ are larger and display a prominent transient component. Depolarization-induced increases in $[Ca^{2+}]_i$ display pharmacological profiles indicative of the

involvement of T-type Ca^{2+} current at subthreshold voltages, and T-type and high-voltage-activated (HVA) Ca^{2+} currents at more positive voltages. Further, at this early developmental stage, release of Ca^{2+} from intracellular stores significantly amplifies $[Ca^{2+}]_i$ signals resulting from depolarization. Of particular interest are the long-lasting changes in $[Ca^{2+}]_i$ observed at subthreshold voltages, which involve interactions between Ca^{2+} entry and release of Ca^{2+} from intracellular stores. It may be more appropriate to consider these increases to be modulation of resting $[Ca^{2+}]_i$ by membrane voltage.

A preliminary report of some of these results has appeared (Barish, 1989).

METHODS

Preparation of cells

Xenopus neural plate cells were grown in culture chambers consisting of 25 mm diameter cover-slips onto which Teflon O-rings (diameter 9 mm) were glued with heat-cured Sylgard 182 (Dow Corning, Midland, MI, USA) to make a well with a volume of approximately 0·13 ml. Cells were dissociated and cultured as described in the preceding paper (Barish, 1991), except that in these experiments either poly-L-lysine/laminin (3:1 w/v from 100 μg/ml stocks in sterile water; Sands & Barish, 1989) or newborn calf serum (HyClone, Logan, UT, USA) were used to coat glass cover-slips. Cover-slips were inserted into a milled holder on the stage of an inverted microscope; this arrangement permitted cells to be grown and viewed in the same cover-slip chamber.

$[Ca^{2+}]_i$ *imaging techniques*

Fura-2 AM was purchased from Molecular Probes (Eugene, OR, USA). For loading, 50 μg aliquots of dye were reconstituted in 50 μl dimethyl sulphoxide plus 8% Pluronic F-127 detergent (Molecular Probes), and this mixture was added to loading solution (Ca^{2+}, Mg^{2+}-free Hank's basal salt solution diluted to 60% ionic strength with tissue culture-grade sterile water and supplemented with Ca^{2+} to 10 μM) to give a final dye concentration of 1·5 μM. Cells were loaded with Fura-2 at room temperature in the dark for 1 h, and then returned to physiological saline for 15–30 min before transfer to the microscope.

Measurements of $[Ca^{2+}]_i$ were performed using modified video-interfaced Zeiss inverted microscopes (IM-35 or ICM-405) at UC Irvine and at the City of Hope. The two instruments employed were similar except in details that are indicated in the description below. When two items are listed, the first reflects usage at UC Irvine and the second at the City of Hope; the changes reflect the evolution of imaging technology during the period separating assembly of the two systems. No systematic differences in the data obtained at the two sites were noted. Illumination was provided by 75 W xenon bulbs, passed through either a Deltascan I (Photon Technology International, Princeton, NJ, USA) dual wavelength monochrometer, or a Sutter Instruments (Novato, CA, USA) Lambda-10 filter changer. Excitation wavelength pairs used were either 345 and 375 nm (slit widths 12 nm) or 350 and 380 nm (bandwidths 10 and 13 nm). Dichroic mirrors were 405 nm or 400 nm. Fluorescence emission was measured at 510 nm (180 or 40 nm bandwidths). Optical filters at both sites were purchased from Omega Optical (Brattleboro, VT, USA). Objectives used were Zeiss 40× Plan-Neofluor (0·9 NA) or Olympus 40× UV APO (1·4 NA). Cameras used were Hamamatsu (Photonic Microscopy, Oak Brook, IL, USA) SIT or intensified CCD. To accommodate for lag in the SIT camera, image acquisition was delayed for 400 ms at each wavelength; this value was determined empirically from tests of image decay after opening and closing the illuminator shutter. For the intensified CCD camera, 60 ms were allowed for camera lag. The VideoProbe image processing system (ETM Systems, Mission Viejo, CA, USA) is highly optimized for dye measurements of intracellular ion concentrations. Calculations were made on a pixel-by-pixel basis in frames of 512 × 512 pixels. The plane of focus was normally placed at just above the surface of the cover-glass, so that neurites and as much of the soma as possible were imaged. No masking of portions of the cell outside its perimeter was employed.

The imaging and calibration procedures followed were based on those described by Grynkiewicz, Poenie & Tsien (1985). Pairs of fluorescence images (averages of 16 frames) at short and long excitation wavelengths (F_{short} and F_{long}) were collected, background subtracted, divided to give the

ratio image R (F_{short}/F_{long}), and stored digitally. Typically, ratios were determined at 10 s intervals. Intracellular Ca^{2+} ion levels were computed from the relation $[Ca^{2+}]_i = K_d\ (F_{min/long}/F_{max/long})\ (R-R_{min})/(R_{max}-R)$, where the dissociation constant K_d = 135 nM at 20 °C (Grynkiewicz *et al.* 1985), $F_{min/long}$ and $F_{max/long}$ are the fluorescence emission intensity for long wavelength excitation at minimal and saturating $[Ca^{2+}]_i$, and R_{min} and R_{max} are the minimum and maximum ratios F_{short}/F_{long} at minimal and saturating $[Ca^{2+}]_i$.

R_{min} and R_{max} were determined from images of Fura-2-loaded neurones. The R_{min} image was acquired after incubating neurones in Ca^{2+}-free (2 mM-EGTA) external solution containing 5 μg/ml ionomycin for 20–30 min. A small increase in $[Ca^{2+}]_i$ was often noted when this solution was added, suggesting release of intracellular Ca^{2+}. The R_{max} image was acquired after adding solution containing 2 mM-Ca^{2+} and 5 μg/ml ionomycin. The values of R_{min}, R_{max} and ($F_{min/long}/F_{max/long}$) used in calculations were determined from regions of cells free of yolk granules, which could be readily identified (see for example the images shown in Fig. 2).

In the experiments presented here each neurone served as its own control, and in this way consequences of quantitative uncertainties related to dye artifacts were minimized. Those artifacts that are well known include uncleaved and Ca^{2+}-insensitive Fura-2 AM within certain compartments (which will lead to underestimates of $[Ca^{2+}]_i$; Scalon, Williams & Fay, 1987), sequestration of Fura-2 into organelles protected from changes in $[Ca^{2+}]_i$ (Almers & Neher, 1985; Connor & Tseng, 1988), and binding of Fura-2 to intracellular membranes (which may change its K_d and other properties; Konishi, Olson, Hollingworth & Baylor, 1988). In these cultured embryonic *Xenopus* neurones, the interiors of yolk granules may be an environment protected from changes in $[Ca^{2+}]_i$. Thus the presence of yolk granules may cause underestimation of cytoplasmic $[Ca^{2+}]$ during averaging of signals derived from an entire soma. However, Fura-2 reported qualitatively similar patterns of changes in $[Ca^{2+}]_i$ in granule-free and granule-containing regions of cells, suggesting that the yolk granules were not actively distorting the Ca^{2+} dynamics of the cell interior.

Solutions

The standard external solution was composed of (in mM): 96 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 5 HEPES; pH 7·4 (adjusted with NaOH). In practice, external solutions containing different K^+ concentrations were prepared from two stocks (0 and 50 mM-K^+) with equimolar substitution of K^+ for Na^+. All external solutions routinely contained 10^{-7} M-tetrodotoxin (TTX) to block generation of Na^+-dependent action potentials. Ryanodine (lot no. 8E09) was purchased from AgriSystems International (Wind Gap, PA, USA). ω-Conotoxin (lot no. 801074) and ionomycin were purchased from Calbiochem (La Jolla, CA, USA). All other reagents were from Sigma.

Solutions were added to the culture chamber using a syringe connected to an inlet tube, and were removed by aspiration from the top of the Teflon O-ring that formed the chamber wall. Complete exchange using 3 ml of solution could be effected in 15–20 s. At the beginning of each experiment normal external solution was passed though the chamber to ensure the absence of cell movement that could give rise to artifactual $[Ca^{2+}]_i$ signals.

RESULTS

Calibration of K^+-induced depolarizations

In this investigation into the relationship between membrane voltage and $[Ca^{2+}]_i$, neurones were depolarized by increasing the extracellular K^+ ion concentration. As a preliminary to these experiments, the quantitative relationship between the extracellular K^+ ion concentration ($[K^+]_o$) and membrane voltage (V_m) was determined by recording V_m under current clamp while the extracellular K^+ ion concentration was varied between 3 and 50 mM. The results of this exercise are shown in Fig. 1*B*, where the steady-state voltages achieved during exposure to $[K^+]_o$ between 3 and 50 mM (in TTX-containing solutions) are plotted alongside action potentials recorded from similar cultured neurones in response to depolarizing current injections. Not unexpectedly, for K^+ concentrations greater than about

10 mM, V_m agreed well with the prediction from the Nernst relation (continuous line in Fig. 1*B*) assuming a purely K^+-selective membrane. More importantly, comparison of panels *A* and *B* in Fig. 1 indicates that 20 mM-extracellular K^+ depolarized the membrane to approximately −40 mV, a value just negative to the action

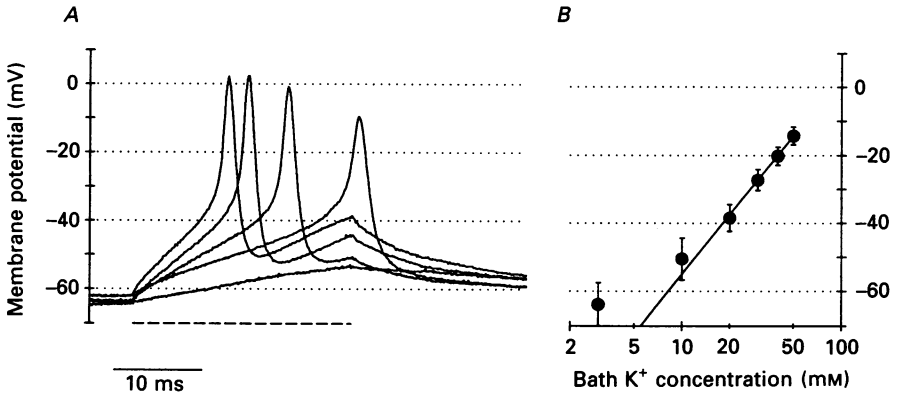

Fig. 1. Action potentials recorded from a cultured embryonic *Xenopus* spinal neurone after 24 h in culture (*A*) and steady-state voltages attained during exposure to external solutions containing the K^+ concentrations indicated (*B*; mean ± S.D., $n = 11$). At 20 mM K^+ depolarized neurones from a resting potential of approximately −65 mV to approximately −40 mV, a voltage just negative to the action potential threshold. The membrane potential reached approximately −15 mV during exposure to 50 mM-K^+-containing external solutions. Recordings in *B* were made in the presence of 10^{-7} M-TTX.

potential threshold and within the activation range for T-type Ca^{2+} current (Barish, 1991). Membrane voltage reached approximately −15 mV in 50 mM-K^+-containing solution, a value within the activation ranges of HVA–relaxing and HVA–sustained Ca^{2+} currents.

Voltage dependence of changes in intracellular Ca^{2+} ion concentrations

A Nomarski DIC image of Fura-2-loaded neurone (24 h in culture) is shown in Fig. 2*A*. This neurone had one large process and growth cone on the upper right of the soma, and a smaller process as well. The nucleus is located in the centre left of the soma. The large bright structures are yolk granules that persist in all cells for several days.

Images of this cell showing the magnitude and distribution of $[Ca^{2+}]_i$ during exposure to increasing concentrations of extracellular K^+ are shown in panel *B*. Increased brightness denotes increased $[Ca^{2+}]_i$ (scale bar). The dark round inclusions are the yolk granules (compare with panel *A*). During K^+-induced depolarizations, the largest changes in $[Ca^{2+}]_i$ were generally seen in the vicinity of the nucleus, near the base of a major neurite (but not both in the case of bipolar neurones), and in the growth cone. Smaller changes were typically seen in the neurites separating somas and growth cones, and the smallest changes in a region opposite the nucleus near the edge of the cell. Regions of lowest reported $[Ca^{2+}]_i$ were typically associated with yolk granules, a pattern suggesting that the granules enclose volumes protected from increases in $[Ca^{2+}]_i$ observed elsewhere in the cytoplasm. In regions of the soma free

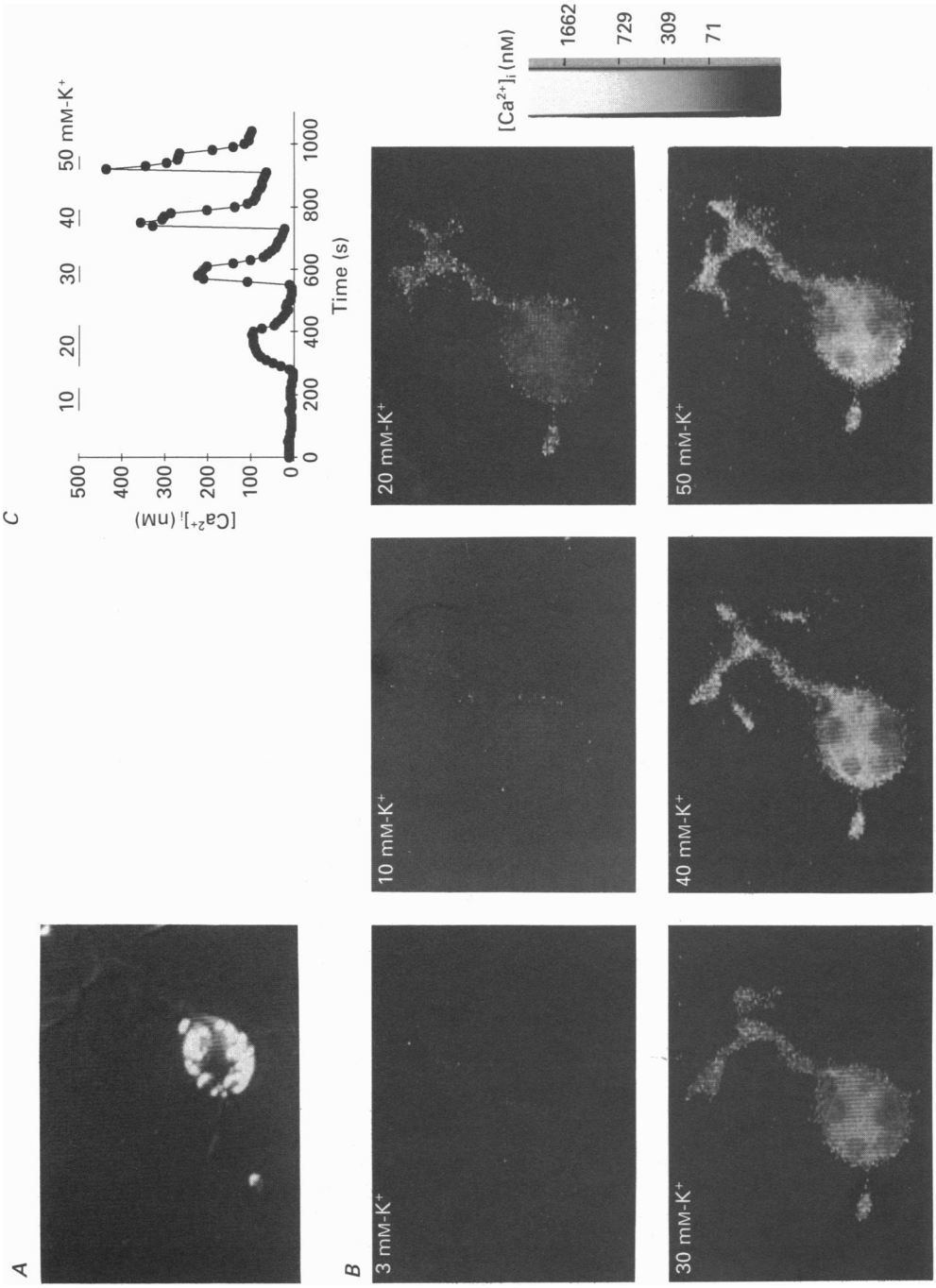

Fig. 2. For legend see facing page.

of yolk granules, higher values of $[Ca^{2+}]_i$ were seen in the nuclear region than in other granule-free regions of the soma or in the process. This is especially evident in the image collected in the presence of 50 mM-K^+. Despite these quantitative differences between yolk granule-containing and -free regions, and nuclear and cytoplasmic portions of yolk-free regions, qualitatively similar patterns of changes in $[Ca^{2+}]_i$ were seen in all areas during depolarizations.

Not all cells grew as thin as the example presented in Fig. 2, and regional distributions of $[Ca^{2+}]_i$ were not always as obvious. For the analysis reported here $[Ca^{2+}]_i$ was computed as the average free-Ca^{2+} level throughout the neurone soma; processes and growth cones were excluded from measurements except as noted.

Typical time courses of spatially averaged increases in somatic $[Ca^{2+}]_i$ during elevations of extracellular K^+ are illustrated in panel *C*. The resting $[Ca^{2+}]_i$ in this cell was estimated to be 11 nM (see below). Elevating the $[K^+]_o$ from 3 to 10 mM, which will depolarize the cell from the resting potential of approximately −65 mV to approximately −50 mV, did not affect $[Ca^{2+}]_i$. Raising $[K^+]_o$ to 20 mM caused a gradual (over 1–2 min) increase in $[Ca^{2+}]_i$ to 97 mM. During K_o^+-induced depolarizations to voltages more positive than about −30 mV, $[Ca^{2+}]_i$ waveforms exhibited transient peaks whose rising and falling phases were more rapid at more positive voltages. In this cell application of 50 mM-K^+ rapidly raised $[Ca^{2+}]_i$ to a transient peak of 437 nM.

In twenty-five cells similarly investigated, resting $[Ca^{2+}]_i$ was 48 ± 39 nM (mean ± S.D.), steady-state $[Ca^{2+}]_i$ in 20 mM-extracellular K^+ (estimated V_m approximately −40 mV) was 87 ± 56 nM, and peak $[Ca^{2+}]_i$ in 50 mM-extracellular K^+ (estimated V_m approximately −15 mV) reached 550 ± 324 nM. These increases in $[Ca^{2+}]_i$ did not occur when Ca^{2+} was omitted from the external solution. The relatively low value of resting $[Ca^{2+}]_i$ reported by Fura-2 in these cells as compared to the somewhat higher values (approximately 100 nM) obtained from cultured mammalian neurones using similar dye-loading techniques (see, for example, Connor, 1986) may reflect the contribution of volumes enclosed by yolk granules to the averaged $[Ca^{2+}]_i$.

Changes in $[Ca^{2+}]_i$ with voltage occurred more rapidly in peripheral neurites than in somas, although changes were qualitatively similar. Figure 3*A* shows ratio images

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Fig. 2. *A*, Nomarski DIC image of a cultured embryonic *Xenopus* spinal neurone at 8 h in culture. The cell nucleus is in the left central portion of the soma, and a major neurite and growth cone is emerging to the upper right. The very bright white structures are yolk granules. In a monopolar cell such as this one the major neurite typically emerges opposite the nucleus. The soma diameter is approximately 25 μm. *B*, images of the cell in panel *A* showing increases in $[Ca^{2+}]_i$ during depolarizations elicited by elevating the extracellular K^+ concentration to the values indicated. In these panels $[Ca^{2+}]_i$ is indicated by brightness, as denoted by the scale bar. The voltages attained during K^+ depolarizations, determined in current clamp experiments, are indicated in Fig. 1*B*; the K^+ concentration of normal extracellular solution is 3 mM. The panels corresponding to 3 and 10 mM-K^+ were printed somewhat lighter so that the cell outline could be seen. TTX (10^{-7} M) was present in all depolarizing solutions used in this study. *C*, plot of $[Ca^{2+}]_i$ against time for this cell; elevated concentrations of K^+ were applied during the periods indicated. Pairs of images at short and long excitation wavelengths (see Methods) were acquired at one pair every 10 s. For construction of plots such as this, average $[Ca^{2+}]_i$ was determined only for the soma region, and neurites were excluded except as noted.

of the soma and process of a neurone 10 and 20 s after application of 50 mM-K^+ solution. During the depolarization, $[Ca^{2+}]_i$ in the process increased more rapidly and reached a higher average level. Before elevation of extracellular K^+, $[Ca^{2+}]_i$ was approximately equal in the soma and process, but on a grey scale the cell image was

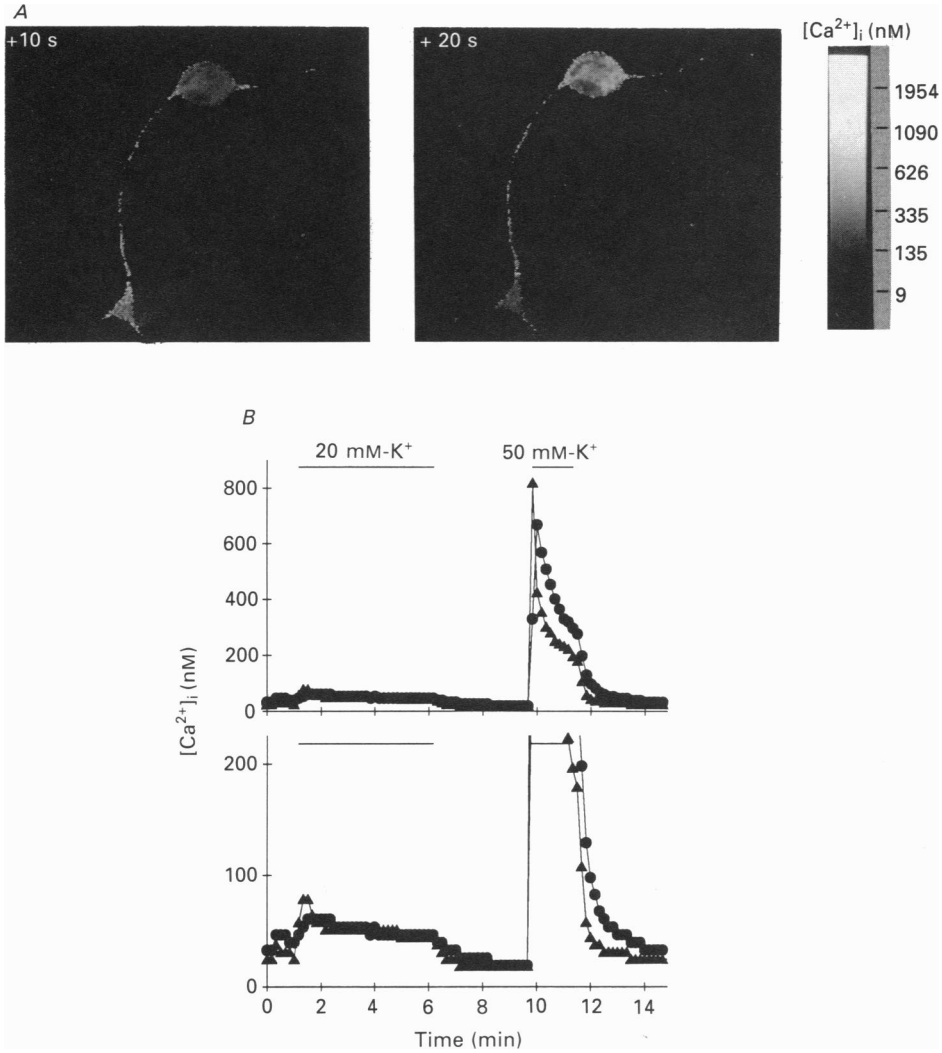

Fig. 3. *A*, grey-scale $[Ca^{2+}]_i$ images of cultured neurone taken 10 and 20 s after beginning addition of 50 mM-K^+-containing solution to the bath. Complete bath exchange took approximately 20 s, and these images thus show the neurone during the period of depolarization. *B*, increases in $[Ca^{2+}]_i$ recorded from the soma (●) and process (▲) of the cell in panel *A*. For increased clarity the same data are plotted on two different vertical scales above and below. Responses in these two regions were qualitatively similar but quantitatively different during times when membrane voltage was changing rapidly.

too dark to reproduce well. The waveforms of changes in $[Ca^{2+}]_i$ during sub- and suprathreshold depolarizations are shown in panel *B*. For both depolarizations $[Ca^{2+}]_i$ in the neurite (▲) increased and declined more rapidly than in the soma (●).

Ca^{2+} entry during sustained $[Ca^{2+}]_i$ responses to subthreshold depolarizations

A series of experiments (not shown) established that in the absence of extracellular Ca^{2+}, K_o^+ depolarization did not elicit increases in $[Ca^{2+}]_i$. However, because of interest in the long-lasting responses to 20 mM-K^+, further experiments dem-

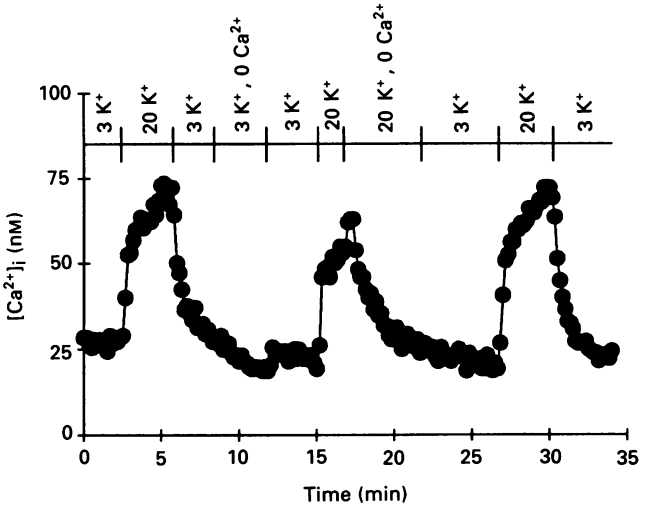

Fig. 4. Sustained increases in $[Ca^{2+}]_i$ induced by 20 mM-K^+ were not observed in nominally Ca^{2+}-free (Mg^{2+}-substituted) external solutions. In this cell, 3 mM-K^+, 0 Ca^{2+} solution reduced resting $[Ca^{2+}]_i$ slightly. Removal of external Ca^{2+} during application of 20 mM-K^+ truncated the increase in $[Ca^{2+}]_i$, and returned it to the baseline level.

onstrating Ca^{2+} entry during the sustained phase of the $[Ca^{2+}]_i$ response were performed. The experiment shown in Fig. 4 demonstrates that the sustained phase required Ca^{2+} entry. After a control application of 20 mM-K^+ and wash with normal Ca^{2+}-containing 3 mM-K^+ solution, addition of nominally Ca^{2+}-free (substitution with Mg^{2+}) 3 mM-K^+ solution reduced the resting $[Ca^{2+}]_i$ slightly, an effect that was seen in approximately 50% of cases ($n = 5$). After return to normal 3 mM-K^+ solution, Ca^{2+}-containing 20 mM-K^+ solution was applied, followed by Ca^{2+}-free 20 mM-K^+ solution. Removal of extracellular Ca^{2+} during the depolarization caused $[Ca^{2+}]_i$ to decline to resting levels with a time course similar to that usually seen after removal of elevated K^+ solutions.

Further evidence that Ca^{2+} influx is involved in generation of the sustained component was obtained from experiments in which external Mn^{2+} was used to quench Fura-2 fluorescence. Figure 5 shows the intensity of fluorescence emission (in arbitrary units) of a Fura-2-loaded soma under 380 nm excitation (F_{380}). The experiment was performed in Ca^{2+}-free external solution, a condition under which, as shown in Fig. 4, depolarization-induced increases in $[Ca^{2+}]_i$ (and thus reductions in F_{380}) are not seen, and under which decreases in F_{380} can be expected to reflect Mn^{2+} entry. As shown, in Ca^{2+}-free 3 mM-K^+ solution, addition of 250 μM-Mn^{2+} caused a decrease in F_{380} consistent with the tonic activation of a Mn^{2+}-permeant pathway at the resting potential. After removal of Mn^{2+}, application of Ca^{2+}-free 20 mM-K^+ solution did not change F_{380}. Subsequent addition of Mn^{2+} resulted in a sharp

reduction in F_{380} indicative of minutes-long activation of a Ca^{2+} entry pathway. The slope of the decline in F_{380} did not change during the period of depolarization, an observation consistent with non-relaxing Mn^{2+} entry.

Pharmacology of depolarization-induced increases in intracellular Ca^{2+} ion concentrations

A series of experiments examined the extent to which the pharmacological sensitivities of depolarization-induced increases in $[Ca^{2+}]_i$ reflected those of the Ca^{2+} currents described in the preceding paper (Barish, 1991).

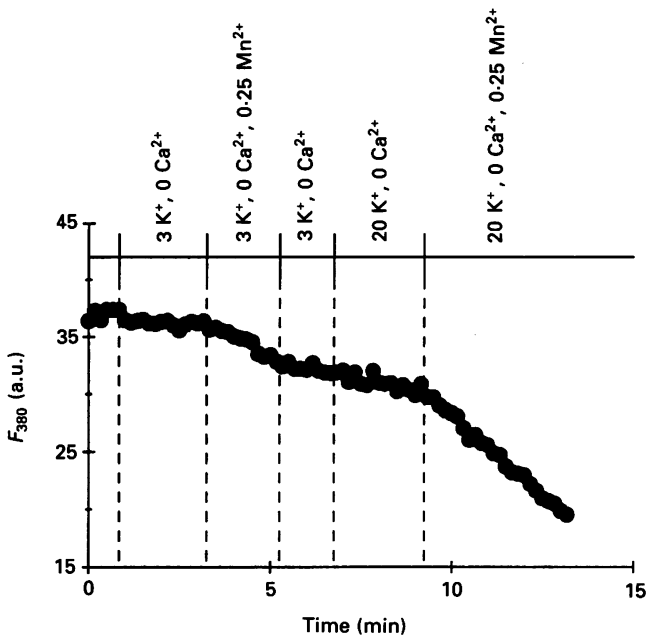

Fig. 5. Quench of Fura-2 fluorescence at 380 nm excitation (F_{380}, in arbitrary units, a.u.) under Ca^{2+}-free conditions by external Mn^{2+} (250 μM; ion concentrations noted in millimolar units) to show that a divalent cation-permeable pathway is activated during the sustained phase of $[Ca^{2+}]_i$ responses to 20 mM-K^+ depolarization.

Ni^{2+}, Met-enkephalin and verapamil

Inhibition of T-type and HVA–relaxing Ca^{2+} currents with Ni^{2+} and methionine-enkephalin (Tyr-Gly-Gly-Phe-Met; Met-enkephalin) reduced changes in $[Ca^{2+}]_i$ induced by exposure to 50 mM-K^+ (depolarization to approximately -15 mV). At this voltage, T-type, HVA–relaxing and HVA–sustained components of Ca^{2+} current will be activated (see Fig. 10 of the preceding paper). As illustrated in Fig. 6*A*, both 200 μM-Ni^{2+} and 17·5 μM-Met-enkephalin reduced the initial large increase in $[Ca^{2+}]_i$ seen with suprathreshold depolarizations, Ni^{2+}, by $33 \pm 9\%$ (mean $\pm$ S.D., $n = 5$) and Met-enkephalin by $26 \pm 16\%$ ($n = 3$). The transient peak was almost completely eliminated by simultaneous application of Ni^{2+} and Met-enkephalin ($n = 3$).

In contrast, only Ni^{2+} but not Met-enkephalin was effective on $[Ca^{2+}]_i$ responses to subthreshold depolarizations. Figure 6*B* shows the results of an experiment in which

the effects of Ni^{2+}, Met-enkephalin and verapamil on increases in $[Ca^{2+}]_i$ induced by 20 mM-K^+ were compared. Ni^{2+} (200 μM) slowed the rise in $[Ca^{2+}]_i$, and reduced the steady-state increase by 44 ± 11 % ($n = 3$). Met-enkephalin (17·5 μM), which reduced the $[Ca^{2+}]_i$ response to 50 mM-K^+, had minimal effect on the increase in $[Ca^{2+}]_i$ at this

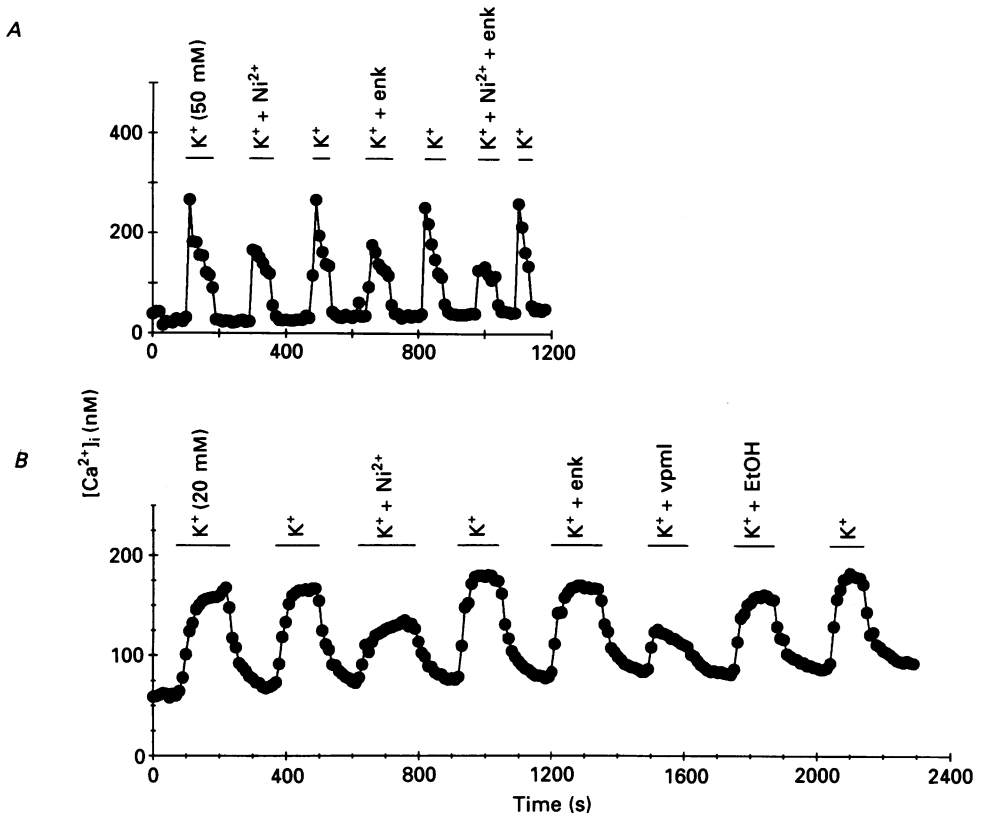

Fig. 6. Effects of Ni^{2+} and Met-enkephalin (enk), inhibitors of T-type and HVA–relaxing Ca^{2+} currents, on increase in $[Ca^{2+}]_i$. *A*, increases in $[Ca^{2+}]_i$ induced by application of 50 mM-K^+-containing solution (depolarization to approximately −15 mV). Note that inhibition of T-type current (by Ni^{2+}; 200 μM) and HVA–relaxing current (by Met-enkephalin; 17·5 μM) individually reduced but did not block the transient component of the $[Ca^{2+}]_i$ increase, but that the peak was eliminated by simultaneous application of both inhibitors. *B*, increases in $[Ca^{2+}]_i$ induced by application of 20 mM-K^+. The rising phase of the increase in $[Ca^{2+}]_i$ induced by 20 mM-K^+ was reduced by 200 μM-Ni^{2+}, but was minimally affected by Met-enkephalin (17·5 μM; compare with panel *A*). Verapamil (vpml; 100 μM) reduced the sustained component of $[Ca^{2+}]_i$ increase, while sparing the rising phase. The ethanol (EtOH) control for verapamil vehicle had minimal effect.

more negative voltage ($n = 3$). Verapamil (100 μM), which reduced HVA Ca^{2+} currents when assayed electrophysiologically (Barish, 1991), did not affect the initial rate of rise in $[Ca^{2+}]_i$, but reduced the maximum increase in $[Ca^{2+}]_i$ by 60 ± 10 % ($n = 3$), and truncated the sustained phase of the response.

The effects of Ni^{2+} are shown in more detail in Fig. 7. At the dose selective for T-type Ca^{2+} current (200 μM), Ni^{2+} partially inhibited subthreshold increases in $[Ca^{2+}]_i$.

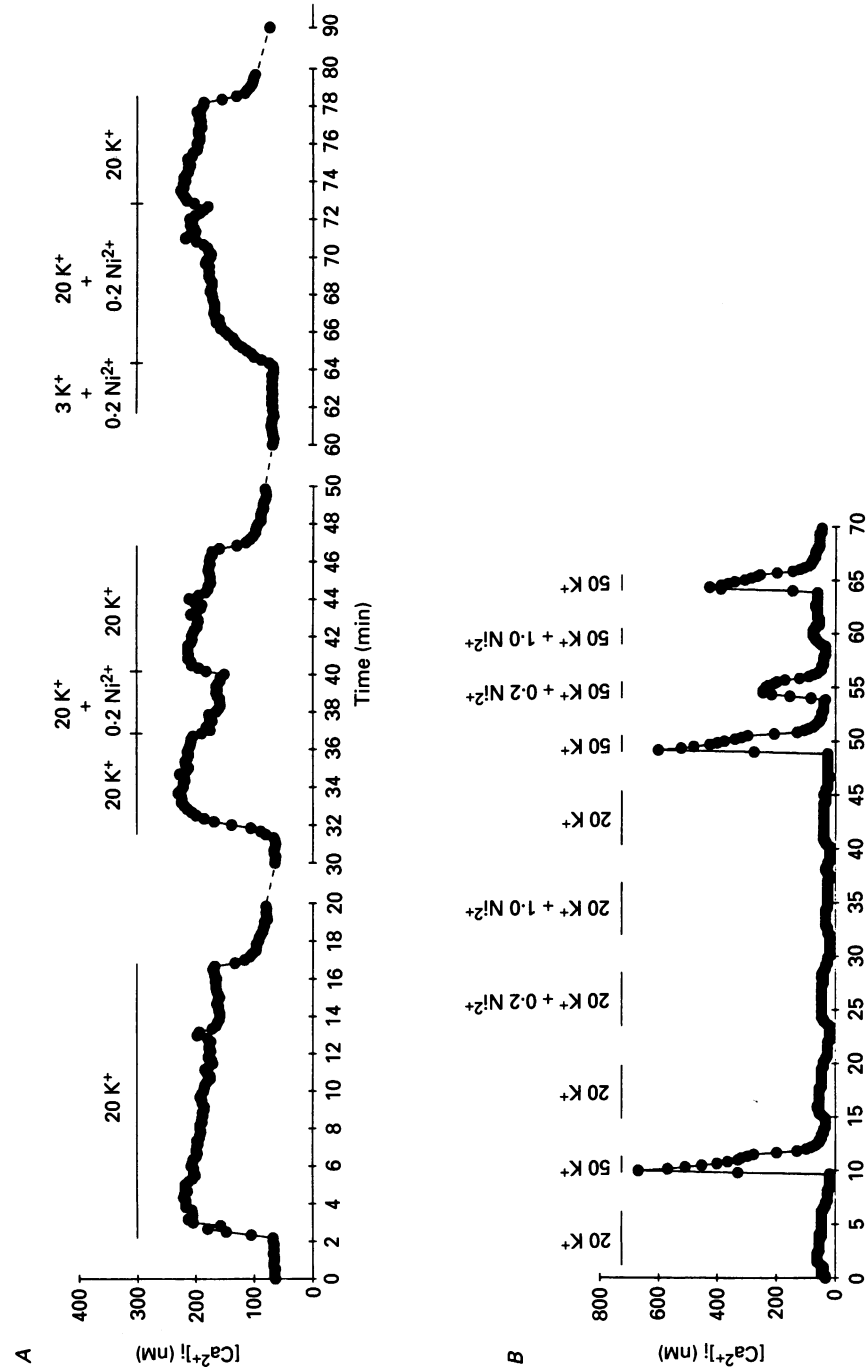

Fig. 7. *A*, effects of Ni^{2+} on increases in $[Ca^{2+}]_i$ induced by subthreshold depolarization. Ni^{2+} at a dose sufficient to selectively inhibit T-type Ca^{2+} current (200 μM, ion concentrations noted in millimolar units) slowed the rise in $[Ca^{2+}]_i$ induced by subthreshold depolarization, and reduced sustained increases in $[Ca^{2+}]_i$ when applied after several minutes of depolarization ($[K^+]_o$ = 20 mM). *B*, differential actions of low and high concentrations of Ni^{2+} on increases in $[Ca^{2+}]_i$ induced by 20 and 50 mM-K^+. Ni^{2+} at 200 μM reduced $[Ca^{2+}]_i$ responses, and at 1 mM virtually eliminated depolarization-induced increases in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ responses returned after removal of high concentrations of Ni^{2+}.

Shown in panel *A* are the results of an experiment in which 200 μM-Ni^{2+} was applied during periods of depolarization to approximately −40 mV ($[K^+]_o$ = 20 mM). When Ni^{2+} was present before and during the initiation of depolarization, the depolarization-induced elevation of $[Ca^{2+}]_i$ was slowed (rightmost record). Application

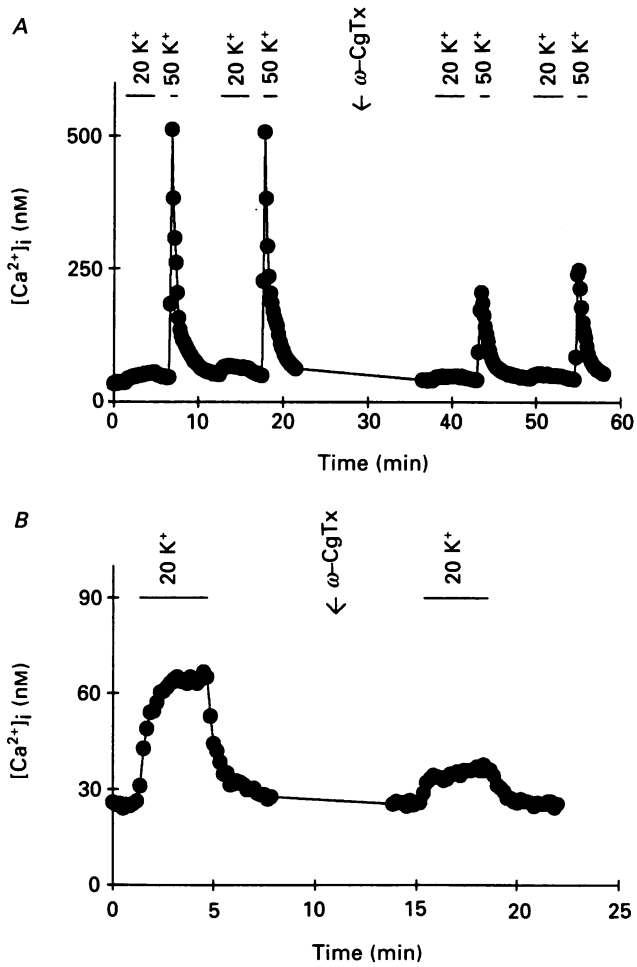

Fig. 8. Reduction in increases in $[Ca^{2+}]_i$ during both sub- and suprathreshold depolarizations by ω-CgTx. *A*, in this experiment, after two pairs of test depolarizations in 20 and 50 mM-K^+-containing solutions, data acquisition was halted and a puffer pipette containing 5·5 μM-ω-CgTx in normal solution was placed in the bath and used to apply toxin to the cell. Data acquisition resumed after the puffer pipette was removed from the bath. *B*, effect on the waveform of responses to 20 mM-K^+. A protocol similar to that described in *A* was followed.

of Ni^{2+} for 4 min during a 20 mM-K^+-induced depolarization reversibly reduced $[Ca^{2+}]_i$, but did not abolish the sustained component of the response (centre record).

Higher concentrations of Ni^{2+} effectively and reversibly blocked depolarization-induced increases in $[Ca^{2+}]_i$. As shown in Fig. 7*B*, 200 μM-Ni^{2+} reduced the increases in $[Ca^{2+}]_i$ associated with applications of 20 and 50 mM-K^+-containing solutions, and

1 mM-Ni^{2+} almost but not completely abolished them. A single exposure to 1 mM-Ni^{2+} did not substantially reduce a subsequent $[Ca^{2+}]_i$ response to 50 mM-K^+, and after the second Ni^{2+} application the response was still robust.

ω-Conotoxin

ω-Conotoxin (ω-CgTx, 5·5 μM) reduced $[Ca^{2+}]_i$ responses at sub- and suprathreshold voltages, as shown in Fig. 8. Panel *A* shows $[Ca^{2+}]_i$ responses from a cell during

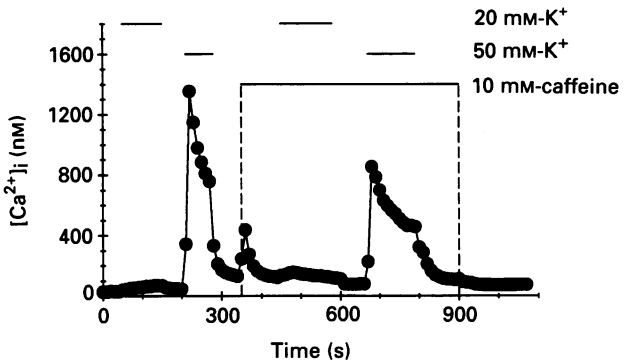

Fig. 9. Effect of caffeine on resting $[Ca^{2+}]_i$ and increases in $[Ca^{2+}]_i$ gated by 20 and 50 mM-K^+. Caffeine (10 mM) caused a transient release of Ca^{2+}, and changed the waveform of the increase in $[Ca^{2+}]_i$ gated by 20 mM-K^+ from a steady increase to a more transient event. The amplitude of the response to 50 mM-K^+ was also reduced.

exposure to 20 and 50 mM-K^+-containing solutions, and panel *B* from a different cell during exposure to 20 mM-K^+-containing solution only. In both cases, after acquisition of control responses, ω-CgTx was applied from a puffer pipette that was positioned, pressurized, and removed from the bath during the gaps in the $[Ca^{2+}]_i$ record. As can be seen in panel *A*, application of ω-CgTx reduced responses to both 20 and 50 mM-K^+; in three experiments, ω-CgTx reduced responses to 20 mM-K^+ by $30 \pm 8\%$, and those to 50 mM-K^+ by $66 \pm 10\%$ (mean ± S.D.). ω-Conotoxin reduced the amplitudes of responses to 20 mM-K^+, but did not markedly change their waveforms (panel *B*). Despite the preference of the ω-CgTx for HVA over T-type Ca^{2+} currents (Barish, 1991), ω-CgTx affected the rising and sustained phases of the $[Ca^{2+}]_i$ responses to 20 mM-K^+ in approximately equal proportion.

Inhibition of intracellular Ca^{2+} release

Application of caffeine to a variety of peripheral neurones results in a transient increase in $[Ca^{2+}]_i$ and reduction of the $[Ca^{2+}]_i$ response to K_o^+-induced depolarizations (see Neering & McBurney, 1984; Lipscombe, Madison, Poenie, Reuter, Tsien & Tsien, 1988). The effects of caffeine on *Xenopus* spinal neurones are illustrated in Fig. 9. After measurement of control $[Ca^{2+}]_i$ responses to 20 and 50 mM-K^+, application of 10 mM-caffeine resulted in a transient increase in $[Ca^{2+}]_i$. These caffeine-induced $[Ca^{2+}]_i$ surges were similar to but smaller than those observed by Lipscombe *et al.* (1988); $\Delta[Ca^{2+}]_i$ for *Xenopus* neurones was 457 ± 221 nM ($n = 6$). Caffeine also increased the resting $[Ca^{2+}]_i$ to a variable extent, the increase in this cell was relatively large. In the presence of caffeine, $[Ca^{2+}]_i$ responses to 20 mM-K^+ rose to a peak and then decayed to a lower plateau level, and responses to 50 mM-K^+ were

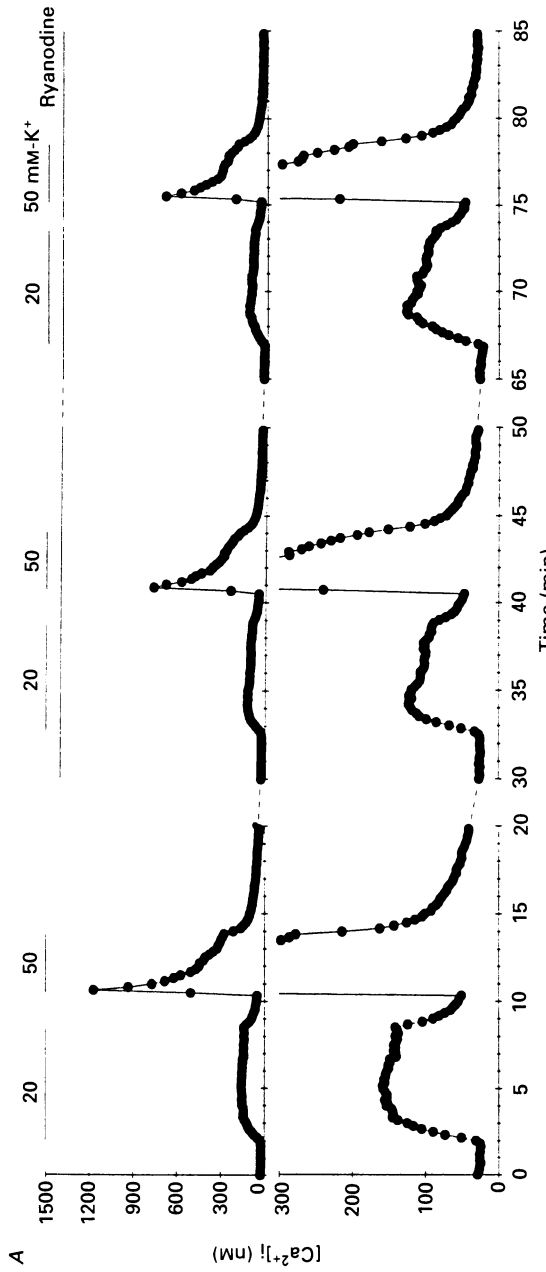

Fig. 10. Effects of ryanodine on depolarization-induced $[Ca^{2+}]_i$ transients. *A*, paired application of 20 and 50 mM-K^+-containing solutions before and in the presence of 10 μM-ryanodine. The data are also plotted below on an expanded vertical scale. Ryanodine (10 μM) reduced $[Ca^{2+}]_i$ responses to 20 and 50 mM-K^+, particularly the sustained phase of the 20 mM-K^+ response. *B*, higher concentrations of ryanodine (100 μM) completely blocked the sustained phase of the $[Ca^{2+}]_i$ response to 20 mM-K^+ while sparing the initial rising phase.

reduced in amplitude. In the aggregate ($n = 4$) caffeine reduced the increase in $[Ca^{2+}]_i$ elicited by 50 mM-K^+ by $56 \pm 12\%$ (mean $\pm$ S.D.) and the response to 20 mM-K^+ (measured at the peak in the presence of caffeine) by $37 \pm 4\%$.

Ryanodine is a plant alkaloid that in skeletal and cardiac muscle reduces Ca^{2+} release from sarcoplasmic reticulum (Jenden & Fairhurst, 1969; Sutko, Ito & Kenyon, 1985). In neurones, it inhibits several caffeine-sensitive mechanisms thought to be due to intracellular Ca^{2+} release, including spike after-hyperpolarization and spontaneous hyperpolarization in rat sympathetic (superior cervical ganglion) neurones (Kawai & Watanabe, 1989), and to a lesser extent and in conjunction with caffeine, increases in $[Ca^{2+}]_i$ resulting from voltage clamp depolarizations and activation of Ca^{2+} currents (Thayer, Hirning & Miller, 1988). The effects of 10 μM-ryanodine on depolarization-induced increases in $[Ca^{2+}]_i$ are shown in Fig. 10*A*; the lower series of panels show the data plotted on an expanded vertical scale. In this cell release of Ca^{2+} was not seen at the time of application of ryanodine, and such release was only seen in one of five cells examined. During application of 20 mM-K^+, ryanodine only slightly reduced the maximum initial $[Ca^{2+}]_i$ response (to $85 \pm 16\%$ of control; mean $\pm$ S.D.) but inhibited the sustained phase during minutes-long depolarizations. Ryanodine also affected the peak amplitude of the response to 50 mM-K^+ (mean amplitude $58 \pm 7\%$ of control). These effects of ryanodine remained stable during successive applications of 20 and 50 mM-K^+-containing solutions, as illustrated in panel *A*.

A higher dose of ryanodine, 100 μM, could almost completely eliminate the sustained phase of the $[Ca^{2+}]_i$ response to 20 mM-K^+ while minimally affecting the initial rising phase of the response (Fig. 10*B*).

DISCUSSION

This report has described elevations of intracellular Ca^{2+} concentration induced by depolarization of embryonic *Xenopus* spinal neurones to sub- and suprathreshold voltages. The waveforms of $[Ca^{2+}]_i$ increases could be divided into transient and sustained phases, with the proportion of $[Ca^{2+}]_i$ responses occupied by these two phases varying such that responses at more positive voltage were both larger and more transient. The pharmacological sensitivities of these elevations of $[Ca^{2+}]_i$ were found in many but not all cases to agree with predictions based on the patterns of voltage-gated Ca^{2+} currents described in the preceding paper (Barish, 1991). Ca^{2+} influx through voltage-gated Ca^{2+} channels appeared to trigger a secondary release of Ca^{2+} from intracellular stores, as the amplitudes and waveforms of depolarization-induced increases in $[Ca^{2+}]_i$ were sensitive to puturbation of Ca^{2+} release.

Distribution of changes in Ca^{2+} ion concentrations

Elevated Ca^{2+} concentrations in nuclei as observed here (which are independent of the depressions in reported $[Ca^{2+}]_i$ induced by yolk granules) have been seen in several other investigations of excitable tissues, including those of cultured *Xenopus* neurones similar to those studied here (Holliday & Spitzer, 1990), smooth muscle (Williams, Fogarty, Tsien & Fay, 1985) and bull-frog sympathetic neurones (Hernández-Cruz, Sala & Adams, 1990). The sources of nuclear–cytoplasmic

gradients in $[Ca^{2+}]$ are not well understood, but they do not appear to be artifactual, as gradients are abolished by Ca^{2+} ionophores (Williams *et al.* 1985), and not observed after neurone culture in Ca^{2+}-free medium (Holliday & Spitzer, 1990). Under some circumstances Fura-2 signals in cells loaded with the acetoxymethylester form of the dye may originate from non-cytoplasmic intracellular compartments (Almers & Neher, 1985; Connor & Tseng, 1988; Silver, Lamb & Bolsover, 1989), and one mechanism that may establish such gradients is ATP-dependent uptake of Ca^{2+} into the nuclear compartment, as has been described for isolated rat liver nuclei (Nicotera, McConkey, Jones & Orrenius, 1989). However, elevation of nuclear Ca^{2+} ion concentrations is not characteristic of all neurones; in cultured rat diencephalon and mouse hippocampal neurones, the Ca^{2+} ion concentration of the nucleus is reported to be lower than that of the surrounding cytoplasm (Connor, 1986; M. E. Barish, unpublished observation).

Relationship of changes in $[Ca^{2+}]_i$ *to voltage-gated* Ca^{2+} *currents*

In these embryonic *Xenopus* neurones the major Ca^{2+} current activated at subthreshold voltages is T-type current (Barish, 1991). Within a window centred on -40 mV activation and inactivation curves for T-type Ca^{2+} current overlap such that Ca^{2+} current will not completely inactivate, and sustained Ca^{2+} influx at subthreshold voltages may occur. Several observations on $[Ca^{2+}]_i$ made during subthreshold depolarizations are consistent with a role of sustained T-type Ca^{2+} current activation in mediating sustained increases in $[Ca^{2+}]_i$ – increases in $[Ca^{2+}]_i$ were reduced, but not eliminated, by exposure to blocking doses of Ni^{2+}, removal of extracellular Ca^{2+} eliminated $[Ca^{2+}]_i$ increases, and Mn^{2+} quenched Fura-2 fluorescence. Similar voltage overlap of activation and steady-state inactivation curves has been reported for T-type current in other cells, but the physiological significance of this property has remained for the most part obscure. However, Cohen, McCarthy, Barrett & Rasmussen (1988) concluded that in adrenal glomerulosa cells a similar overlap of activation and inactivation curves for T-type current permitted a steady Ca^{2+} influx responsible for the sensitivity of aldosterone secretion to small changes in extracellular K^+.

$[Ca^{2+}]_i$ increases during subthreshold depolarizations were also sensitive to ω-CgTx and verapamil, and these blockers of HVA Ca^{2+} current affected $[Ca^{2+}]_i$ waveforms differently. In measurements of $[Ca^{2+}]_i$ responses to 20 mM-K^+, ω-CgTx reduced both the rising and sustained phases of responses such that overall waveforms remained similar. Results of electrophysiological experiments have indicated that ω-CgTx irreversibly blocks the major portion of both HVA–relaxing and HVA–sustained currents (McCleskey, Fox, Feldman, Cruz, Olivera, Tsien & Yoshikami, 1987; Plummer, Logothetis & Hess, 1989; Barish, 1991) but only reversibly affects T-type Ca^{2+} currents (McCleskey *et al.* 1987). Thus, an effect on subthreshold $[Ca^{2+}]_i$ responses was not expected, as the toxin-filled puffer pipette was present in the bath only during the period of drug application. This paradox suggests either that an additional very small ω-CgTx-sensitive component of HVA Ca^{2+} current may be active at subthreshold voltages, or that ω-CgTx may affect an electrically silent Ca^{2+} entry pathway possibly related to Ca^{2+} loading of intracellular stores (see, for example, Putney, 1986; Penner, Matthews & Neher, 1988), or both. Verapamil, in

contrast, selectively reduced the sustained portion of the subthreshold $[Ca^{2+}]_i$ response, while sparing the rising phase. In electrophysiological experiments verapamil preferentially affected the non-inactivating component of HVA current (Barish, 1991). Thus this pattern of verapamil sensitivity could reflect block of a small HVA-sustained current activated at subthreshold voltages, or verapamil might affect Ca^{2+} release, possibly by a mechanism similar to that by which verapamil analogues block initiation of contraction in skeletal muscle (Hui, Milton & Eisenberg, 1984).

Release of intracellular Ca^{2+}

In addition to Ca^{2+} entry, release of intracellular Ca^{2+} made a contribution to increase in $[Ca^{2+}]_i$ consequent to depolarization. Application of caffeine to resting cells elicited a transient $[Ca^{2+}]_i$ increase, suggesting that significant Ca^{2+} was retained in intracellular stores. In addition, increases in $[Ca^{2+}]_i$ in response to depolarizations to approximately -15 mV (attained with 50 mM-K^+) were attenuated by pre-exposure to caffeine or ryanodine. Responses to 20 mM-K^+ showed a complex pattern of sensitivities to blockers of Ca^{2+} release. In these cases, while inhibition of Ca^{2+} influx slowed the rising phase of $[Ca^{2+}]_i$ responses, caffeine or ryanodine selectively reduced the sustained phases of the responses. Ryanodine was able to give a more complete reduction of the sustained phase than caffeine; differences in the behaviours of ryanodine and caffeine have also been reported for Ca^{2+} release in vascular smooth muscle cells (Erne & Hermsmeyer, 1988). These data indicate the activity of a Ca^{2+}-induced Ca^{2+}-release mechanism similar to that described for cardiac and skeletal muscle (Sutko *et al.* 1985).

Increases in $[Ca^{2+}]_i$ during K_o^+-induced depolarizations showing waveforms similar to those seen here, transient large increase followed by a reduced sustained component, have been reported by a number of investigators, including Lipscombe *et al.* (1988) for bull-frog sympathetic neurones, and Thayer *et al.* (1988) for rat superior cervical ganglion (SCG) neurones. Comparison of these results with those presented here indicates that the contribution of intracellular Ca^{2+} release to depolarization-induced $[Ca^{2+}]_i$ transients can vary widely between cell types and even between similar cells in different species. In bull-frog sympathetic ganglion cells, prior exposure to caffeine reduced the amplitude of increases in $[Ca^{2+}]_i$ during application of 30 mM-K^+ by more than 50%, and also caused significant reduction of responses to 60 mM-K^+ (Lipscombe *et al.* 1988). In contrast, in rat SCG neurones, the combined application of caffeine and ryanodine resulted in only a small decrease in the $[Ca^{2+}]_i$ increase induced by voltage clamp depolarizations. The results obtained in the present study on *Xenopus* spinal neurones indicate a contribution of Ca^{2+}-induced Ca^{2+} release that is smaller than that in bull-frog sympathetic neurones, but nevertheless one that substantially amplifies the effects of Ca^{2+} entry through voltage-gated Ca^{2+} channels.

Persistent increases in $[Ca^{2+}]_i$ at subthreshold voltages

The data suggest a model for regulation of $[Ca^{2+}]_i$ at subthreshold voltages that involves Ca^{2+} influx via T-type Ca^{2+} channel activation (an influx that may be long lasting due to a window of overlap between inactivation and activation curves), an

additional ω-CgTx-sensitive Ca^{2+} entry pathway, and discharge of caffeine- and ryanodine-sensitive intracellular stores by a mechanism of Ca^{2+}-induced Ca^{2+} release. Basal levels of $[Ca^{2+}]_i$ at subthreshold voltages will thus depend on degree of membrane polarization, Ca^{2+} channel density (T-type and possibly others) and the charging/discharging properties of intracellular stores.

Regulation of $[Ca^{2+}]_i$ by subthreshold voltage excursions may occur *in vivo*. Inhibitory and excitatory neurotransmitter receptors appear early in *Xenopus* development (Bixby & Spitzer, 1982, 1984; Sands & Barish, 1989; S. B. Sands & M. E. Barish, unpublished observations) and growing neurones release neurotransmitter before synaptic connections are formed (Hume, Role & Fischbach, 1983; Young & Poo, 1983). Thus the resting potential of early cells may not be a well-defined value, but rather may vary depending on exposure to synaptically or extrasynaptically released neurotransmitters and other humoral influences.

Long-lasting increases in $[Ca^{2+}]_i$ could have multiple consequences for neural differentiation. Numerous developmental events have been shown to be dependent on depolarization and Ca^{2+} entry (see, for reviews, Harris, 1981; Brenneman & Nelson, 1985), and in embryonic *Xenopus* neurones, process of neurite outgrowth and voltage-gated K^+ current maturation appear to be modulated by $[Ca^{2+}]_i$ (Holliday & Spitzer, 1990; Desarmenien & Spitzer, 1990). The dependence of $[Ca^{2+}]_i$ on subthreshold membrane potential described here may serve to couple early electrical activity to neuronal development before development of mature synaptic connections.

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