Calcium Transients in the Garter Snake Vomeronasal Organ

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INTRODUCTION

The vomeronasal (VN) system of garter snakes is particularly well developed and is known to be critical for several species-specific behaviors including prey detection (Burghardt and Pruitt 1975; Graves and Duvall 1985; Halpern 1982, 1992; Halpern and Frumin 1979; Halpern and Kubie 1994; Kahmann 1932; Kubie and Halpern 1979; Naulleau 1965; Wilde 1938). The initial step in prey detection involves the interaction between a prey-derived chemoattractive ligand and specific receptors on the dendritic surfaces of bipolar neurons of the VN sensory epithelium. We have isolated and purified prey extracts and used them as selective chemoattractive stimuli. Among the compounds isolated are EW20 (Wang et al. 1988), a 20-kDa thiol-containing protein, EW3 (Wang et al. 1992, 1993), a low-molecular-weight chemoattractant [both derived from earthworm wash (EW)], and ES20 (Jiang et al. 1990), a 20-kDa glycoprotein derived from electric shock-induced earthworm secretion (ESS). ES20 specifically binds to the VN sensory epithelium in a saturable and reversible manner with a $K_d$ of $\sim0.3 \mu M$ (Jiang et al. 1990) and does not bind specifically to other organs, including brain and main olfactory epithelium. G proteins (G$_{o}$, G$_i$, and G$_s$) have been immunologically detected in the VN sensory epithelium of garter snakes and ES20 receptors probably are coupled to these G proteins (Luo et al. 1994).

Functional studies have demonstrated that ESS and ES20 evoke depolarizing currents in VN neurons and increase unit activity in the accessory olfactory bulb (AOB) mitral cells, the targets of the axons of receptor neurons of the VN epithelium (Jiang et al. 1990; Luo et al. 1994; Taniguchi et al. 1998, 2000). Binding of ES20 to VN receptors also results in increased levels of inositol 1,4,5-trisphosphate (IP$_3$), suggesting that this signal cascade pathway may be involved in chemosensory transduction. In contrast, ES20 significantly reduces basal levels of cAMP as well as GTP$_\gamma$S- or forskolin-induced high levels of cAMP (Luo et al. 1994). Nevertheless, an adenylylcyclase, AC$_{VN}$, has been cloned from a garter snake VN epithelial library, which shows high homology to AC type VI (Liu et al. 1998) and is sensitive to Ca$^{2+}$ regulation (Wang et al. 1997). Thus within the VN sensory epithelium components of two second-messenger systems, AC$_{VN}$ and phospholipase C (PLC), exist.

In VN neurons, the reversal potential induced by dialysis of IP$_3$ or its analogue 3-deoxy-3-fluoro IP$_3$ mimics the reversal currents generated by the chemoattractive stimuli (Taniguchi et al. 2000). IP$_3$, which is known to mobilize intracellularly sequestered Ca$^{2+}$ via the IP$_3$ receptor (IP$_3$R), causes elevations of cytosolic Ca$^{2+}$, which plays multiple functional roles in neurons including signal transduction (Berridge 1993; Clapham 1995). However, these events in VN neurons remain to be elucidated.

Taking advantage in the garter snake of the presence of specific prey extracts as selective chemoattractant stimuli (ESS), in the present study, we disclose mechanisms triggering stimulus-induced cytosolic Ca$^{2+}$ transients associated with chemosensory transduction. Optical recordings were performed in slices from VN neurons selectively loaded with the fluorescent Ca$^{2+}$ indicator Calcium Green-1 from their axonal terminals in the AOB by retrograde transport. We report here that chemoattractants produce initially a transient cytosolic Ca$^{2+}$ elevation even in the absence of extracellular Ca$^{2+}$, suggesting that in snake VN neurons, Ca$^{2+}$ release from intracellular stores is independent of a preceding Ca$^{2+}$ influx and both components are activated in parallel during early stages of chemosensory transduction. Once the response develops in apical dendritic segments, other mechanisms can also contribute to the amplification and modulation of these chemoattractant-mediated cytosolic Ca$^{2+}$ transients. In regions close to the cell bodies of the VN neurons, the activation of voltage-sensitive Ca$^{2+}$ channels and a Ca$^{2+}$-induced Ca$^{2+}$ release from intracellular ryanodine-sensitive stores secondarily boost initial cytosolic Ca$^{2+}$ elevations increasing their magnitude and durations. Return of intracellular Ca$^{2+}$ to prestimulation levels appears to involve a Ca$^{2+}$ extrusion mediated by a Na$^+$/Ca$^{2+}$ exchanger mechanism that probably plays an important role in limiting the magnitude and duration of the stimulation-induced Ca$^{2+}$ transients.

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cumulation of [Ca\(^{2+}\)], in dendritic regions via two pathways: a Ca\(^{2+}\) release from IP\(_3\)-sensitive intracellular stores and, to a lesser extent, a Ca\(^{2+}\) influx through the plasma membrane in dendritic regions. Although other mechanisms can secondarily participate in the modulation of these chemoattractant-induced cytosolic Ca\(^{2+}\) transients, cAMP seems not to have an important role in the generation these transient Ca\(^{2+}\) responses.

METHODS

Animals

Adult garter snakes, Thamnophis sirtalis, of both sexes were obtained from commercial suppliers. They had all resided in the laboratory for ≥2 months prior to use in the experiments described here.

Loading VN bipolar neurons with Calcium Green 1 using retrograde axonal transport from the accessory olfactory bulb

VN bipolar neurons were loaded with Calcium Green 1 by means of retrograde axonal transport of dye placed in the accessory olfactory bulb (AOB). Snakes were anesthetized with a subcutaneous injection of methohexital sodium (0.5 mg/g body wt; Eli Lilly, Indianapolis, IN). A hole above the AOB was drilled through the skull to expose the AOB bilaterally, and Calcium Green 1 was applied either as crystals or a 5-μl injection of Calcium Green solution injected deep into the glomerular layer. The snakes were allowed to recover for 3–4 days prior to the evaluation of Ca\(^{2+}\)-related fluorescence signals.

Preparation of VN sensory epithelial tissue slices

The methods are essentially similar to those described previously (Tanguchi et al. 1995, 1996, 2000). In brief, snakes were lightly anesthetized with methohexital sodium prior to decapitation. The vomeronasal neuroepithelium was dissected from the head after carefully removing the bony capsule and mushroom body, mounted onto a carrot block, and cut into slices, ~240 μm thick, with a vibrating slicer (Vibratome 3000, Technical Products International, St. Louis, MO) in snake Ringer solution. This solution consisted of (in mM) 119 NaCl, 4.1 KCl, 2.5 CaCl\(_2\), 1.5 MgCl\(_2\), 15 glucose, 5 Na-pyruvate, and 10 HEPES (pH 7.4). The tissue slice was then mounted onto a small plastic petri dish.

Preparation of ESS

The methods are the same as described earlier by Jiang et al. (1990). Secretions were obtained by passing an electric current from a 9-V battery (20 6-s bursts with an intershock interval of 30 s) through the worms. A yellowish mucus-like secretion was collected and centrifuged, and the supernatant was dialyzed against distilled water.

Preparation of VN sensory epithelial homogenate

Essentially we followed a standard method as previously reported (Luo et al. 1994). Dissected VN epithelia were homogenized in cold buffer (20 mM Tris/HCl, pH 8, 1 mM PMSF, 80 μg/ml DTT, 0.5 μg/ml antipain, 1 μg/ml leupeptin, 0.6 μg/ml chymostatin, and 0.6 μg/ml pepstatin) and centrifuged at 500 g for 5 min. The supernatant was recovered and referred to as homogenate.

Monitoring intracellular Ca\(^{2+}\) changes

OPTICAL SETUP. Video imaging of fluorescent changes was used to monitor calcium responses. The general plan of the optical setup is based on standard methods (Cinelli and Salzberg 1991, 1992; Cinelli et al. 1995). Essentially the system consists of an upright epi-illumination microscope (Nikon Epiphot) with a video camera (MV-1070; Marshall Electronics) in the photographic port. Light from a 150-W xenon lamp (Optic Quip 1600) is collimated and rendered quasi-monochromatic by one of several interference filters, focused by a quartz-UV-grade condenser (Nikon), and reflected to the preparation by a dichroic mirror. The wavelength for the excitation and emission filters and the dichroic mirror were selected according to the excitation and emission spectra of Calcium Green 1. To improve collection efficiency, fluorescent light from the cells was collected by high numerical aperture (n.a.) water-immersion objectives (×20 or ×40; Fluo; Nikon), which formed a real image on the CCD sensor of the video camera located in the image plane of the microscope. To further improve the sensitivity of this analog camera, image exposures were extended to increase light integration in the CCD sensor wells (Cinelli 1998). When fast acquisition rates were needed, an image intensifier was used to improve the sensitivity of the camera. Fluorescence emission usually remained constant during the experiments. To assure stability of the recordings and to avoid photobleaching effects, the acquisition light levels were reduced by neutral density filters until the fluorescence intensity remained constant for 200 s of illumination. No significant levels of autofluorescence were observed in VN neurons, and the drugs, at the concentrations used, did not affect or quench fluorescence levels.

CALCIUM IMAGING TECHNIQUES. Fluorescence measurements of Ca\(^{2+}\) levels were performed following standard protocols. Data are reported as fractional changes over background fluorescence levels (F/F\(_o\)). Standard procedures for background subtraction and calibration were used for calibration with solutions of known dye concentration (Tsien et al. 1985). After the experiments, in situ calibrations were performed. Cells were permeabilized with Ca\(^{2+}\)-ionophores (ionomycin) or membrane solvents (digitonin or saponin). F\(_{max}\) and F\(_{min}\) were determined in Ringer solution (1 mM Ca\(^{2+}\)\(^{+}\) to saturate the Ca\(^{2+}\) indicator and then by subsequently bathing the cells in low-Ca\(^{2+}\) Ringer solution supplemented with 5 mM EGTA. Calcium Green yielded increases in fluorescence signals proportional to Ca\(^{2+}\) bound; these levels were directly related to levels of [Ca\(^{2+}\)]\(^{i}\). Other terms of the equation were assessed by in situ calibration (see following text).

IMAGE PROCESSING. Images were digitized and stored in real time using a frame grabber board in a Pentium IBM-compatible computer system. Final images were analyzed by applying various digital filters or convolution algorithms (Cinelli 1998, 2000; Cinelli and Salzberg 1991, 1992; Cinelli et al. 1995). High spatial resolution during image acquisition was necessary to preserve the image details in fine dendrites even when low band-pass spatial filters were used. Background experiments indicated that low band-pass spatial filters were often required to suppress pixel noise from the detector or the image intensifier. High spatial resolutions were also necessary for the application of deconvolution techniques after low band-pass spatial filters. Deconvolution techniques were used for improving focus resolution and obtaining better resolution for visualizing particular cellular compartments such as the dendritic terminals of vomeronasal neurons. Temporal plots of Ca\(^{2+}\) transients were obtained from averaged values over 8 × 8 pixel kernels. In the figures, changes over time are illustrated by pseudocolors resulting from subtracting basal levels of [Ca\(^{2+}\)]\(^{i}\), from those obtained after experimental manipulation.

RESULTS

Retrogradely labeled VN neurons

Using slices of snake VN sensory epithelium, changes in cytosolic Ca\(^{2+}\) associated with chemosensory transduction were studied in VN neurons loaded with the Ca\(^{2+}\) indicator, Ca\(^{2+}\) Green 1. VN neurons were labeled by retrograde transport of this
dye from their axonal terminals in the AOB. This method allowed
the selective staining solely of mature VN neurons, which are the
only cell elements in the vomeronasal organ (VNO) that send their
axons to the AOB. Transport of the dye from the injection sites to
VN neurons was usually obtained within 72–84 h. Using this
approach, we observed that not all VN neurons in a slice prepa-
ration were found to be labeled with the fluorescence indicator (Fig. 1A), a condition that probably arose from the rather localized
application of the dye in the AOB. An important advantage of this
system for obtaining selective retrograde staining is that the VNO
and the AOB are in different and separated tissue compartments.
This condition prevents the undesirable diffusion of the dye to the
VNO and the nonspecific staining of other cell types. We consist-
ently observed in the slices that only mature VN neurons were
labeled with the fluorescence indicator (Fig. 1, A and B); no other
cellular elements, such as sustentacular cells or immature VN
neurons, were labeled. Therefore all of the Ca\(^{2+}\) responses eval-
uated in this study arose exclusively from fluorescence changes in
VN neurons sending their axons to the AOB.

In most of our recordings, the use of nonconfocal optics in

**ESS elicited Ca\(^{2+}\) Signals in VN Neurons**

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**Fig. 1.** Ca\(^{2+}\) transients in retrogradely labeled snake vomeronasal (VN) neurons. **A** shows a video image illustrating the
selective staining of VN neurons with Ca\(^{2+}\) Green after retrograde transport of this dye from their axonal terminals in the accessory
olfactory bulb (AOB). Observe the labeling in the cell bodies of mature VN neurons (red arrows) located in the intermediate region
of the sensory epithelium. No other cellular elements, such as sustentacular cells or immature VN neurons were labeled. L, lumen;
D, dendritic region; S, somata region; BL, basal lamina. Horizontal bar = 20 µm. **B**: a schematic diagram showing the laminar
organization of cellular elements in the snake VNO. As shown in **A**, the cell bodies of mature VN neurons (R) are located
exclusively in the middle epithelial laminae with dendrites projecting toward the epithelial lumen. Adjacent to the basal lamina are
basal cells (B); immature VN neurons (N) are located apical to the basal cells, and sustentacular cells (S) are located close to the
luminal surface, intermixed with the dendrites of VN neurons. **C**: the increase in [Ca\(^{2+}\)]\(_{i}\) recorded in the 1st, 3rd, 5th, and 7th frames
after electric shock-induced earthworm secretion (ESS, 2.0 mg protein/ml) application. Records taken from a 16 video sequence
(1 frame/s; 120-ms image exposure) showing the spatial distribution of Ca\(^{2+}\) responses. Abbreviations as in **A**. Optical signals were
obtained from single runs, and are coded as follow: green: 5–10%; yellow: 11–15%; orange: 16–20%; red: >20%. In situ
calibration performed after membrane permeabilization indicates that a 20% optical signal roughly corresponds to changes in
cytosolic Ca\(^{2+}\) levels on the order of 500 nM. **D**: the time course of the increase in [Ca\(^{2+}\)]\(_{i}\), recorded in the 1st, 3rd, 5th, and 7th frames
after electric shock-induced earthworm secretion (ESS, 2.0 mg protein/ml) application. Records taken from a 16 video sequence
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a rather thick slice preparation (240–300 μm) prevented us from obtaining, with certainty, Ca\(^{2+}\) signals at single-cell resolution. In fact, most of our optical recordings represented population responses from clusters of VN neurons. Despite this limitation, it was possible to study the spatial distribution of Ca\(^{2+}\) signals in different segments of VN neurons because the garter snake VNO exhibits a distinctive laminar pattern of cellular elements (Fig. 1B). In contrast to the less clear laminar organization of cellular elements found in other species (e.g., Halpern et al. 1995), the cell bodies of mature VN neurons are located exclusively in the middle epithelial laminae. Just above the basal lamina there are unstained cell clusters corresponding to basal cells and immature VN neurons. In the present study, the latter were not stained because their axons had not reached the AOB. Sustentacular cells and the dendrites of VN neurons are located in the apical epithelial regions close to the luminal surface. As shown in Fig. 1B, these sustentacular cells were also unstained in our slices because first they lack projections to the AOB and second no diffusion of the dye occurred from the AOB to the VNO. Thus despite the lack of single-cell resolution in our recordings, the lamination pattern of the snake VNO and the specificity of ESS ligands in eliciting Ca\(^{2+}\) responses among different epithelial sectors was rather constant. We interpret this finding as indicating that this heterogeneous pattern represents cumulative responses from similar VN neurons responding in different degrees to the ESS ligands.

**Resting Ca\(^{2+}\) levels**

Changes in cytosolic Ca\(^{2+}\) levels were determined with standardized optical-imaging techniques (Cinelli and Salzberg 1991, 1992; Cinelli et al. 1995). Using Calcium Green 1, single wavelength emission measurements of changes in fluorescence intensity represented estimates of [Ca\(^{2+}\)]\(_i\), variations. According to “in situ” calibrations performed at the end of the experiments (see METHODS), baseline [Ca\(^{2+}\)]\(_i\), was equivalent to 60.2 ± 15.4 (SD) nM (n = 12), and cytosolic Ca\(^{2+}\) response peaks were in the range of 104–740 nM elevations above resting levels, proportional to the intensity of stimulation. Experiments usually lasted 6–8 h. During this period, [Ca\(^{2+}\)]\(_i\), baseline levels remained steady (usually <70 nM), and no deterioration of the preparations was observed as judged by the similarity of the Ca\(^{2+}\) responses obtained throughout the experimental sessions.

**Characteristics of chemoattractant-induced Ca\(^{2+}\) responses**

To determine whether VN stimulants evoke cytosolic Ca\(^{2+}\) changes associated with excitatory responses, VN epithelial slices (240–300 μm) were exposed to prey chemoattractant ESS while [Ca\(^{2+}\)]\(_i\), was measured. As illustrated in Fig. 1C, Ca\(^{2+}\) transients in VN neurons elicited by the bath application of ESS ligands consisted of sharp elevations in cytosolic Ca\(^{2+}\) levels that usually reached a peak amplitude within 1 s. Response peaks were followed by a brief plateau and then a more prolonged decay phase in which cytosolic Ca\(^{2+}\) levels gradually declined to baseline levels within 16–32 s.

Ca\(^{2+}\) transients evoked by ESS ligands exhibited a rather widespread distribution. Figure 1, C and D, shows the spatial distribution and time course of typical Ca\(^{2+}\) transients evoked by bath application of ESS ligands (2.2 mg/ml). In general, patterns of activity displayed a scattered appearance with a heterogeneous organization in which it was possible to find nonuniform foci of activity distributed in multiple epithelial regions separated by silent sectors. Within each lamina there were important variations in the amplitude and time course of ESS responses, suggesting a different degree of activity among stimulated VN neurons. Usually Ca\(^{2+}\) signals from sectors showing the largest amplitudes exhibited the longest durations and lowest thresholds. Following successive stimuli at any given concentration of ESS, the overall organization of these Ca\(^{2+}\) responses among different epithelial sectors was rather constant. We interpret this finding as indicating that this heterogeneous pattern represents cumulative responses from similar VN neurons responding in different degrees to the ESS ligands.

ESS stimuli evoked clear dose-dependent Ca\(^{2+}\) transients in the concentration range of 0.5–4.5 mg/ml protein. Figure 2, A–C, shows typical changes in kinetics and spatial distributions of Ca\(^{2+}\) responses following ESS stimuli applied at different concentrations. The concentration threshold for eliciting detectable Ca\(^{2+}\) increases with ESS in our slice preparation was between 0.8 and 1.2 mg/ml protein. These Ca\(^{2+}\)-dependent fluorescence signals usually exhibited half-saturating peak responses at concentrations on the order of 7 mg/ml protein. In this study, most records were obtained with stimuli in the range of 2.2–4.4 mg/ml protein, and these concentrations are similar to those that evoke clear electrophysiological responses in the VN epithelium (Taniguchi et al. 1998). As a control for the specificity of ESS ligands in eliciting Ca\(^{2+}\) responses associated to chemosensory transduction mechanisms, we evaluated the effect of actin which is a behaviorally inactive compound (unpublished observations). In contrast to the clear responses evoked by ESS, the application of actin to the bath solution evoked no detectable Ca\(^{2+}\) changes (Fig. 2D) even at relatively high concentrations (4–6 mg/ml).

Once the stimulus threshold was reached, Ca\(^{2+}\) transients elicited by ESS exhibited relatively sharp response onsets with latency rise times in the range of 500–750 ms according to temporal plots obtained from video image sequences acquired at 250 ms/image (data not shown in the figures). Sharp response onsets were also observed even when ESS stimuli were applied at the lowest concentration (0.75 mg/ml, Fig. 2A, plot). Higher ESS concentrations evoked Ca\(^{2+}\) signals that exhibited similar response profiles but larger amplitudes and longer durations. As shown in Fig. 2, A–C, the applications of increasing ESS concentrations evoked a proportional increase in the amplitude and duration of Ca\(^{2+}\) transients in all epithelial regions. There were also important changes in the spatial distribution of these responses as ESS concentration increased. At low concentrations (e.g., 1 mg/ml), there was a limited number of activated sites that were located almost entirely in dendritic regions. As ESS stimulus concentration increased,
Three major effects were observed in the spatial distribution of these responses. First, Ca\(^{2+}\) transients spread from dendritic locations to the cell body region of VN neurons where magnitudes and duration comparable to those found in dendritic regions were attained. Second, we observed a relative enlargement of individual foci of activity, involving adjacent epithelial sectors. Finally, new discontinuous foci of activity appeared in previously silent epithelial regions (Fig. 2, B and C). These new sites of activity did not overlap with other active sectors, suggesting the recruitment of a different set of active VN neurons. These results suggest that the newly active VN neurons were probably responding to distinct chemical cues present in ESS ligands that exhibited different response thresholds.

**Characteristics of different cytosolic Ca\(^{2+}\) transients in VN neurons**

To elucidate possible mechanisms involved in the generation of ESS responses, we first established the general characteristics of different types of Ca\(^{2+}\) transients in VN neurons. Figure 3 compares the spatial distribution and kinetics of different types of Ca\(^{2+}\) signals evoked in snake VN neurons. Both ESS and caffeine (2–5 mM) applications gave rise to prolonged Ca\(^{2+}\) signals, which lasted 20–50 s depending on the stimulus concentrations. These two responses, however, differed in their spatial distribution and onset characteristics. In contrast with ESS responses, caffeine signals were found adjacent to the cell body region, and the pattern of activation was more uniform across different regions. Caffeine signals also exhibited a slower rise time with a more progressive build-up, a characteristic that was evident especially at low concentrations (<2.5 mM; Fig. 3C). High [K\(^+\)]\(_{o}\) elicited Ca\(^{2+}\) transients that were relatively rapid in onset and brief in duration compared with other types of Ca\(^{2+}\) signals found in snake VN neurons (Fig. 3B). They were found predominantly in the cell body region and, as with caffeine-induced responses, were practically absent in apical dendritic segments.

**Role of voltage-sensitive Ca\(^{2+}\) channels.** To evaluate the role of voltage-sensitive Ca\(^{2+}\) channels (VSCC) in the generation of cytosolic Ca\(^{2+}\) changes, we determined the properties of Ca\(^{2+}\) responses elicited by high KC1 (100 mM; Fig. 3B). The short duration and monotonic decay of these signals occurred even when high [K\(^-\)]\(_{o}\) was still present in the bath. This finding suggests a relatively rapid inactivation of VSCC in snake VN neurons. The total duration of these cytosolic Ca\(^{2+}\) changes was in the range of 3.5–5.5 s, lasting an average of 4.52 ± 1.87 (SD) s (n = 7). Thus the time course of these transients were considerably shorter than those elicited by ESS. Response latencies were also shorter, usually shorter than the fastest time resolution tested in this study (250 ms/image). Response peaks attained fluorescent fractional changes equivalent to 25–32%, values that corresponded to [Ca\(^{2+}\)]\(_{i}\) elevation on the order of 175–290 nM. These Ca\(^{2+}\) signals were reversibly suppressed when extracellular Ca\(^{2+}\) was removed from the medium (Fig. 4A). They were also completely blocked after the application of common VSCC blockers such as cadmium (Cd\(^{2+}\); Fig. 4B) or cobalt (Co\(^{2+}\); 50–100 μM; data not shown), confirming that they resulted primarily from a Ca\(^{2+}\) influx through VSCC.

To determine whether a Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) could be activated following an initial Ca\(^{2+}\) influx mediated through VSCC, we evaluated the characteristics of Ca\(^{2+}\) transients elicited by high-[K\(^+\)]\(_{o}\) depolarization following the depletion of internal Ca\(^{2+}\) stores. Depletion of intracellular stores was obtained either using thapsigargin or ryanodine. Thapsigargin depletes intracellular Ca\(^{2+}\) store because it is a potent inhibitor of the intracellular Ca\(^{2+}\) pump and prevents Ca\(^{2+}\) reuptake into these pools following spontaneous or stimulus-induced depletions. As a consequence of this action, thapsigargin was used to determine the effect obtained after the depletion of all intracellular Ca\(^{2+}\) pools. Under our experimental conditions, we found that the action of thapsigargin (1 μM for 10–15 min) in depleting internal Ca\(^{2+}\) stores was predominantly stimulus dependent because the spontaneous Ca\(^{2+}\) depletion from these stores was rather low. As a consequence, the effect of thapsigargin was assessed following repetitive stimulation (see following text). Under these conditions, we observed that thapsigargin could shorten the time course and reduce the magnitude of K\(^+\)-elicited Ca\(^{2+}\) signals, but this reduction was observed exclusively during the late phase of the response (n = 3; data not shown). Thus this result indicates that Ca\(^{2+}\) release enhanced the decay phase of VSCC-mediated signals.

To further determine the involvement of ryanodine-sensitive pools in this effect, we used ryanodine instead of thapsigargin to selectively deplete these stores. Ryanodine acts as a selective antagonist of one of the two types of Ca\(^{2+}\) stores by forcing their release channels into a permanently opened state. For this purpose, we applied ryanodine (10 μM) to the bath solution in the presence of high concentrations of caffeine (25 mM) for 10–15 min. In all cases (n = 18), this protocol completely depleted ryanodine-sensitive Ca\(^{2+}\) stores as judged by the suppression of caffeine-elicited responses (as in other cell systems, we also found that in snake VN neurons Ca\(^{2+}\) transients evoked by caffeine depend exclusively on a Ca\(^{2+}\) release from ryanodine-sensitive stores; see following text).

As illustrated in Fig. 4C, middle, following ryanodine treatment, K\(^+\)-elicited Ca\(^{2+}\) signals exhibited a shortened time course and a reduction in magnitude during the late phases of the response. These changes were observed in both dendritic and somatic regions but were more obvious in regions adjacent to the cell bodies of VN neurons. At this level, Ca\(^{2+}\) transients elicited by K\(^+\) depolarizations exhibited a reduction during the decay phase equivalent to 20–35% of control responses (n = 5). In no case was the initial onset or the peak amplitude of these responses affected. Thus K\(^+\) depolarization appears to elicit an initial Ca\(^{2+}\) influx through the activation of VSCC that in turn triggers a secondary Ca\(^{2+}\) release from ryanodine-sensitive internal stores, potentiating and prolonging these responses. Therefore both thapsigargin and ryanodine actions indicate the presence of a CICR mechanism enhancing the late phases of the Ca\(^{2+}\) signals mediated through the activation of VSCC.

**Characteristics of cytosolic Ca\(^{2+}\) elevations elicited by release from intracellular stores.** To determine the characteristics of the Ca\(^{2+}\) transients in snake VN neurons that depend on Ca\(^{2+}\) release from internal stores, we evaluated the action of caffeine. By readily crossing the plasma membrane in different cell systems, caffeine evokes strong Ca\(^{2+}\) release, which depends exclusively on its reversible binding to intra-
ESS Dose-Response Characteristics

A. ESS (1 mg/ml)

B. ESS (2 mg/ml)

C. ESS (3.3 mg/ml)

D. Actin (Control)
cellular ryanodine receptors (Usachev and Thayer 1999; Usachev et al. 1993). We found that relatively low doses of caffeine (2.5 mM) could trigger strong Ca$^{2+}$ transients in snake VN neurons (Fig. 3C). Similar to ESS responses, the amplitude and duration of these signals were dose dependent. Ca$^{2+}$ transients evoked by 2.5 mM caffeine had peak amplitudes similar to responses evoked by 2.2 mg/ml ESS (Fig. 3, A and C). Under these conditions, optical signals exhibited fluorescence increases of $28.5 \pm 4.7\%$ ($\Delta F/F_0$, mean $\pm$ SD; $n = 8$) that corresponded to [Ca$^{2+}$], elevations in the range of 190–200 mM. But in contrast to the ESS responses, Ca$^{2+}$ transients elicited by caffeine attained their largest amplitudes in the cell body region of VN neurons. As we mentioned before, this distribution was similar to the responses elicited by high-[K$^+$]o applications.

Caffeine responses exhibited moderate rise times, followed by a plateau, and then a prolonged declining phase in which [Ca$^{2+}$] slowly returned to baseline (Figs. 3A and C). In contrast to ESS transients, the rise times of caffeine signals were highly dependent on stimulus concentration. At low concentrations, they exhibited a slow onset, which progressively became sharper at higher concentrations.

Ca$^{2+}$ signals elicited by caffeine appear to be generated entirely by Ca$^{2+}$ release from internal pools. In all cases...
studied \((n = 7)\), the absence of \(\text{Ca}^{2+}\) from the medium \((0 \text{ [Ca}^{2+}]_o\), supplemented with 5 mM EGTA) did not affect the characteristics of caffeine responses evaluated immediately after the \(\text{Ca}^{2+}\) removal. Under these conditions, \(\text{Ca}^{2+}\) responses exhibited profiles, time courses \((14.8 \pm 2.3 \text{ s})\), and peak magnitudes \((29.5 \pm 3.7 \Delta F/F_o)\) similar to controls (Fig. 4).
FIG. 5. Properties of \( Ca^{2+} \) signals evoked by caffeine in snake VN neurons. A: the characteristics of caffeine responses obtained following the removal of \( Ca^{2+} \) from the medium. Left: corresponds to control responses obtained from a 4 × 4 pixel area in the layer of VN neuron somata. Middle: plots of \( Ca^{2+} \) signals elicited by caffeine (10 mM) following the removal of \( Ca^{2+} \) from the medium (0 [Ca\(^{2+}\)]\(_o\) with 5 mM EGTA). Although no significant change was observed in the initial response (—), successive caffeine applications evoked a use-dependent gradual reduction in consecutive \( Ca^{2+} \) signals, as shown in responses obtained after 4 and 8 caffeine applications (— — — and — — — —, respectively). Right: the recovery of this \( Ca^{2+} \) transient after restitution of normal [Ca\(^{2+}\)]\(_o\) (2–3 min). B: the effect of depleting internal \( Ca^{2+} \) stores with thapsigargin on caffeine-elicited \( Ca^{2+} \) signals. Left: a control response; middle: traces correspond to the progressive reduction in these \( Ca^{2+} \) responses following 2, 4, and 8 caffeine stimulations after incubation of the slice with thapsigargin (1 μM, 15 min). Observe that on the 8th trial, no detectable signals were obtained, and this suppression was not reversed following repetitive rinses with normal Ringer solution (right). C: \( Ca^{2+} \) signals evoked by caffeine following the depletion of ryanodine-sensitive internal stores. Left: corresponds to a control signal obtained before the application of ryanodine (20 μM, 15 min). Middle: the absence of caffeine-induced \( Ca^{2+} \) transients after cytosolic \( Ca^{2+} \) levels returned to basal levels following ryanodine treatment (see further details in text). Similar to thapsigargin effects, the ryanodine suppression of optical signals did not recover after rinsing the preparation in normal Ringer solution. Tissue slices in B and C were tested in normal snake Ringer solution. Plots correspond to \( Ca^{2+} \) signals from the cell body region of VN neurons taken from a single 16 image sequence (1 frame/s; 120-ms image exposure). The 1st point in the plots corresponds to basal cytosolic values and the 2nd point to the 1st response obtained 1 s after stimulus application.
5A, middle, 1st trace). However, we consistently found that successive applications of caffeine in Ca²⁺-free medium evoked Ca²⁺ transients that exhibited a progressive reduction, first in their amplitude and then in their duration \((n = 7; \text{Fig. 5A, middle, - - - and · · · · ·})\). The most likely explanation for this result is that these reductions are a consequence of the inability of intracellular Ca²⁺ stores to be fully refilled in Ca²⁺-free medium (Usachev and Thayer 1999). This interpretation is corroborated by the finding that there was a complete recovery of caffeine responses when normal \([\text{Ca}^{2+}]_o\) was restored to the medium (Fig. 5A, right).

Further confirmation about the role of intracellular Ca²⁺ release in the generation of caffeine responses was obtained by evaluating these signals after thapsigargin or ryanodine treatment. Both thapsigargin (1 μM; Fig. 5B) and ryanodine (20 μM; Fig. 5C) suppressed caffeine-induced Ca²⁺ transients but with some differences in their actions. VN slices preincubated with thapsigargin (10–15 min) evoked no major changes in basal cytosolic Ca²⁺ levels, and initial caffeine applications elicited Ca²⁺ signals that exhibited only a slight reduction in their amplitude and duration. Under these conditions, however, successive caffeine applications evoked a progressive reduction in magnitude and duration of Ca²⁺ transients that led, after 8–15 trials, to a complete response suppression (Fig. 5A, series of traces in the middle plot). No major change was observed using different intervals between trials \((n = 5)\). In contrast, the magnitude of these reductions was use-dependent because it was related to the magnitude and duration of the previous caffeine responses. Altogether this evidence suggests that the reduction and eventual suppression of caffeine responses were caused by the gradual and irreversible depletion of internal Ca²⁺ stores.

Ryanodine depletion also suppressed caffeine-elicited Ca²⁺ signals (Fig. 5C). But in contrast to the gradual effects observed with thapsigargin, the protocol used here to deplete ryanodine-sensitive stores (see preceding text) evoked a complete suppression of all caffeine responses from the first trial (Fig. 5C, middle and right, respectively). This irreversible suppression of caffeine responses following ryanodine treatment not only confirms that these Ca²⁺ transients arise entirely from internal Ca²⁺ release but further indicates that this release depends primarily on ryanodine-sensitive pools.

Role of the IP₃ signaling cascade in the generation of Ca²⁺ transients

Previous studies have suggested that phosphoinositide turnover leading to IP₃ formation may constitute the second-messenger system mediating chemosensory transduction in the VN system (Holy et al. 2000; Kroner et al. 1996; Luo et al. 1994; Sasaki et al. 1999; Wekesa and Anholt 1997). Thus we were interested in determining whether the activation of this pathway could give rise to cytosolic Ca²⁺ changes similar to those elicited by ESS ligands. In different cell systems, bradykinin (BK) stimulates phosphoinositide turnover, which, in turn, among other actions, can increase production of IP₃ (Kirischuk et al. 1995; Seymour-Laurent and Barish 1995; Verkhartsky and Kettenmann 1996). Thus it has been used rather extensively to evaluate the role of IP₃ in intracellular Ca²⁺ signaling (see Berridge 1993, 1998). To determine the effect of BK in snake VNO and compare it to ESS ligands, we evaluated the actions of BK and ESS stimuli on IP₃ levels in homogenates of the snake VN sensory epithelium. IP₃ production was measured following protocols previously reported (Luo et al. 1994). VN homogenates (50 μg protein) were incubated either with ESS (13 μg) or BK (300 nM) in a reaction solution (500 μl) of 25 mM Tris-acetate, pH 7.6, 5 mM MgAc₂, 0.5 mM ATP, 1 mM DTT, 0.01 mM GTP, 0.1 mM CaCl₂, and 0.1 mg/ml BSA. As a control, distilled water was used instead of ESS or BK. In all cases the incubation time was 1 min. Under these experimental conditions, ESS evoked IP₃ levels equivalent to 210 ± 10 (SD) pmol/mg proteins \((n = 4)\) while BK evoked IP₃ levels equivalent to 255 ± 5.0 pmol/mg proteins \((n = 4)\). These IP₃ increases differed significantly from those obtained under control conditions \([75 ± 15 (SD) \text{ pmol/mg proteins}; n = 4]\), suggesting that both ESS and BK induce similar phosphinositide turnover leading to IP₃ formation. To further determine whether these increases elicited by BK correspond to IP₃ changes arising from VN neurons, IP₃ levels were determined also in homogenates from VN preparations previously deafferented from the AOB. These VN preparations lack mature VN neurons because the sectioning of their axons induce their degeneration. In contrast with the intact VNO, in deafferented homogenates, BK and ESS incubations exhibited IP₃ levels that did not differ significantly from those found under control conditions. Thus these data indicate that both ESS and BK increase IP₃, and these increases take place predominantly in mature snake VN neurons.

Ca²⁺ signals evoked by BK applications also exhibited strong similarities with the Ca²⁺ transients elicited by ESS ligands. As shown in Fig. 6, BK stimuli evoked Ca²⁺ transients that consisted of an initial sharp \([\text{Ca}^{2+}]_i\) rise followed by a brief plateau and then a prolonged declining phase in which cytosolic Ca²⁺ levels slowly returned to basal values, with a halftime in the range of 9–14 s, depending on the stimulus concentration. BK responses were also dose dependent in the concentration range tested in this study (50–300 nM) with amplitudes and durations directly proportional to stimulus intensity. Application of 200 nM of BK evoked peak fluorescent signals attaining fractional changes \((\Delta F/F_0)\) equivalent to 25–40%. These values roughly corresponded to elevation in \([\text{Ca}^{2+}]_i\) on the order of 150–390 nM. Under these conditions, Ca²⁺ signals exhibited a time course in the range of 12–16 s. Like ESS responses, BK signals exhibited relatively sharp rise times even at low concentrations (Fig. 6B), characteristics that differed from the Ca²⁺ responses elicited by caffeine (Fig. 3C). Ca²⁺ transients elicited by ESS and BK also shared a similar spatial distribution with responses distributed in somatic as well as in dendritic sectors (Fig. 6, A and C). This presence of Ca²⁺ signals in dendritic regions, even those adjacent to the luminal surface, differed from the localization of caffeine responses found largely in the cell body region of VN neurons.

Despite their similarities, we found some differences between the overall distribution of BK and ESS elicited Ca²⁺ signals. Unlike ESS responses, BK patterns of activity were more uniformly distributed across different sectors of the epithelium, and lacked the multifocal appearance characteristic of ESS responses. As illustrated in Fig. 6, even at relatively low concentrations, BK-evoked responses were homogeneously distributed over relatively large epithelial regions. This more uniform distribution probably reflects a lack of selectivity by
BK in the activation of VN neurons that have different chemosensory specificities.

Source of Ca$^{2+}$ signals related to IP$_3$ production

To further characterize the sources of the Ca$^{2+}$ responses evoked by ESS and BK stimuli, we determined whether these responses depend on a Ca$^{2+}$ release from intracellular stores or a Ca$^{2+}$ influx through the plasma membrane. According to our present results (see preceding text), both BK and ESS stimuli appeared to trigger an IP$_3$ increase in VN neurons, and this molecule can elicit important cytosolic Ca$^{2+}$ elevations that, in different cell systems, depend entirely on a Ca$^{2+}$ release from internal pools (for review, see Berridge 1998). On the other hand, IP$_3$ can also evoke cytosolic Ca$^{2+}$ increases through a plasma membrane Ca$^{2+}$ influx via an IP$_3$-activated cation conductance as has been demonstrated in some invertebrate chemosensory systems (Munger et al. 2000; see also Schild and Restrepo 1998 for review).

BK RESPONSES IN CA$^{2+}$-FREE MEDIUM. To determine whether BK responses in snake VN neurons depend on Ca$^{2+}$ entry...
through the plasma membrane or a release from internal pools, we first evaluated these responses in \([\text{Ca}^{2+}]_o\)-free medium. In general, the absence of extracellular \(\text{Ca}^{2+}\) did not abolish BK responses, suggesting that these signals depend largely on a \(\text{Ca}^{2+}\) release from internal stores. In all the slices evaluated (\(n = 6\)), applications of BK (100–200 mM) in 0 \([\text{Ca}^{2+}]_o\) medium (supplemented with 2 mM EGTA) evoked \(\text{Ca}^{2+}\) signals that exhibited similar characteristics and kinetics to control responses. Under these conditions, most \(\text{Ca}^{2+}\) transients preserved their relatively sharp onsets, magnitudes, and durations.

Although in most epithelial regions there was no modification, \(\text{Ca}^{2+}\)-free conditions affected BK signals in apical dendritic regions close to the luminal surface of the epithelium. In this sector, we consistently observed that immediately after the removal of \(\text{Ca}^{2+}\) from the bath there was a sudden but constant reduction in the magnitude of BK responses equivalent to a decrease of 10–18%. These decreases were observed in the amplitude at the peak of the responses as well as during their early decay phase. These changes are illustrated in Fig. 7A, which shows BK signals recorded in apical epithelial regions before (—) and after (– – –) the removal of \(\text{Ca}^{2+}\) from the medium. Full response recovery was always obtained without delay after the restitution of normal \([\text{Ca}^{2+}]_o\) in the bath. This evidence indicates that \(\text{Ca}^{2+}\) signals elicited by BK stimuli in these apical dendritic segments of VN neurons has a component generated by a \(\text{Ca}^{2+}\) influx through the plasma membrane. In this region, however, there is still a response remaining that is unaffected by removal of \(\text{Ca}^{2+}\) from the medium. This component, present in \(\text{Ca}^{2+}\)-free medium, appears to be largely dependent on \(\text{Ca}^{2+}\) release from intracellular stores as is the case for BK-evoked signals obtained in more basal regions.

In contrast with the initial, sudden reduction found in apical dendritic regions, a different type of response decrease was found in \(\text{Ca}^{2+}\)-free medium following repetitive BK stimulations (trials 8–20). These changes were observed in all epithelial regions, including the apical dendritic locations, and consisted of a progressive and cumulative decrease in response magnitude and duration following subsequent applications of BK (6–20; Fig. 7B). The degree of reduction in these \(\text{Ca}^{2+}\) signals was dependent on the number and the concentration of previous BK applications. As was the case for \(\text{Ca}^{2+}\) transients elicited by repetitive applications of caffeine when the VN slices were in \(\text{Ca}^{2+}\)-free medium, these reductions probably arise from the progressive inability of intracellular \(\text{Ca}^{2+}\) stores to be replenished in 0 \([\text{Ca}^{2+}]_o\).

**BK RESPONSES AFTER DEPLETING \(\text{Ca}^{2+}\) STORES.** To further characterize the component of BK responses that was independent of \(\text{Ca}^{2+}\) release, we evaluated these signals after the depletion intracellular \(\text{Ca}^{2+}\) stores. As with \(\text{Ca}^{2+}\) transients evoked by caffeine, the preincubation of VN slices with thapsigargin (1 \(\mu\)M for 10–15 min) did not affect initial BK responses to a large extent (Fig. 8B). Subsequent BK applications, however, evoked a progressive decrease affecting both the amplitude and duration of these \(\text{Ca}^{2+}\) signals. These reductions were observed equally in all epithelial regions and depended on the stimulus concentration and the number of previous stimulations. There was, however, some difference in the evolution of these changes as repetitive BK stimuli were delivered. In regions corresponding to the cell bodies of VN neurons, cumulative reduction progressed until all \(\text{Ca}^{2+}\) responses elicited by BK were abolished. Depending on the stimulus concentration, this occurred usually after 8–20 consecutive applications of BK. In apical dendritic regions, however, responses failed to be completely suppressed. Instead, the reduction progressed until it revealed a small component that remained insensitive to

**FIG. 7.** Characteristics of \(\text{Ca}^{2+}\) signals evoked in apical dendritic regions of VN neurons by BK in the absence of \([\text{Ca}^{2+}]_o\). A: responses to BK (300 nM) obtained in normal Ringer solution (control) and immediately following removal of \(\text{Ca}^{2+}\) from the bath \([\text{Ca}^{2+}]_o\), with 2 mM EGTA (1st trial). Observe the rapid reduction in the peak amplitude of the \(\text{Ca}^{2+}\) signal that occurred without delay after removing extracellular \(\text{Ca}^{2+}\). B: the effect of repetitive BK applications in \(\text{Ca}^{2+}\)-free medium. After their initial reduction, \(\text{Ca}^{2+}\) responses in dendritic regions maintained rather constant characteristics for a few runs (2–5) and progressively decreased following subsequent BK applications. This cumulative decrease gradually affected both the amplitude and duration of \(\text{Ca}^{2+}\) responses until all cytosolic \(\text{Ca}^{2+}\) changes become undetectable (usually after 20 runs using this stimulus concentration; bottom trace). C: full recovery shown of BK-elicited \(\text{Ca}^{2+}\) signals after the VN slices were returned to normal Ringer solution for 5 min. All \(\text{Ca}^{2+}\) signals were taken from single runs of 16 image sequences (1 frame/s; 120-ms image exposure). The 1st point in the plots corresponds to basal cytosolic values and the 2nd point to the 1st response obtained 1 s after stimulus application.
thapsigargin (n = 7) even after multiple BK applications (12–25 trials). This component attained its largest amplitude in locations adjacent to the epithelial luminal surface with a magnitude equivalent to 15–24% of the control responses and a duration of ∼4–6 s. The real amplitude and duration of these Ca$^{2+}$ transients, however, could be underestimated in our recordings because it appears to be generated in a highly localized dendritic region that cannot be independently assessed due to the population nature of our optical signals.

Figure 8A illustrates the characteristics of BK responses recorded in apical dendritic regions after the depletion of internal Ca$^{2+}$ stores by thapsigargin. As seen in the middle plots, BK applications in thapsigargin-treated slices evoked a reduction of Ca$^{2+}$ signals, and after the sixth trial, it is possible to distinguish the emergence of a thapsigargin-resistant component, which maintained a rather constant magnitude and duration during subsequent stimulus applications (12–20 trials; middle and right). Under this condition, caffeine applications failed to evoke any detectable [Ca$^{2+}$]$_i$ changes in responses recorded in the soma region (data not shown), indicating that this dendritic component does not depend on a partial depletion of intracellular Ca$^{2+}$ stores. In contrast, the removal of Ca$^{2+}$ from the medium reversibly abolished this thapsigargin-resistant component. Thus altogether this evidence indicates that this component is not generated by an internal Ca$^{2+}$ release but instead is dependent on a Ca$^{2+}$ influx through the plasma membrane. Confirming this notion, the magnitude and duration of this component matches the initial reductions in control BK responses recorded in the same region when Ca$^{2+}$ was removed from the medium (Fig. 7A).

In contrast with thapsigargin, ryanodine-induced Ca$^{2+}$ depletion did not greatly affect the characteristics of Ca$^{2+}$ tran-

**FIG. 8.** Ca$^{2+}$ signals recorded in apical dendritic regions of VN neurons evoked by BK following depletion of internal Ca$^{2+}$ stores. A: the effects of depleting internal Ca$^{2+}$ stores on responses following the preincubation of a VN slice with thapsigargin (1 μM). Left: Ca$^{2+}$ signals under control conditions. Middle: traces illustrate Ca$^{2+}$ responses evoked by multiple BK stimuli tested 15 min after the application of thapsigargin; note the reduction, but not total suppression, of these Ca$^{2+}$ signals. Right: the thapsigargin-resistant portion of the original BK response remained constant after 20 trials. B: Ca$^{2+}$ signals in dendritic and somatic regions evoked by BK after the depletion of ryanodine-sensitive stores. Left: BK responses under control conditions. Middle: Ca$^{2+}$ signals obtained after the depletion of ryanodine stores (see protocol in the text). Observe that the Ca$^{2+}$ signal from dendritic locations shows no detectable changes, while that from the somata region exhibits a significant reduction during its decaying phase. Right: after ryanodine treatment there was no detectable Ca$^{2+}$ signal evoked by caffeine stimulation, corroborating the full depletion of ryanodine-sensitive stores under this condition. Ca$^{2+}$ signals obtained from single runs of 16 image sequences (1 frame/s; 120-ms image exposure). The 1st point in the plots corresponds to basal cytosolic values and the 2nd point to the 1st response obtained 1 s after stimulus application.
These changes were also fully reversible. After Ca\(^{2+}\) depletion from ryanodine stores (application of 10 \(\mu M\) ryanodine with high concentrations of caffeine, 25 mM), Ca\(^{2+}\) transients elicited by BK applications were affected significantly only in the cell body region of the epithelium (Fig. 8B, middle, •••). But even changes in this region were limited because they consisted of only slight reductions in peak amplitudes (equivalent to 12–18%) and a minor shortening in duration (in the range of 30%) of these responses. Figure 8B illustrates typical Ca\(^{2+}\) signals evoked in dendritic (–) and somatic regions (•••) before (left) and after (middle) the depletion of ryanodine-sensitive stores. Practically no change is observed in the signals evoked by BK in dendritic regions (a), and the Ca\(^{2+}\) signals from the somatic regions (b) exhibited only a slight reduction in magnitude, which occurred predominantly during its decay phase. Under these conditions, however, caffeine applications (2–5 mM) evoked no detectable Ca\(^{2+}\) signals (Fig. 8B, right), indicating that the lack of major changes in BK responses was not caused by an incomplete depletion of ryanodine stores. Therefore these data suggest that BK responses (especially in dendritic regions) depend largely on a Ca\(^{2+}\) release from nonryanodine-sensitive pools, probably IP\(_3\)-sensitive stores.

Furthermore, the relative lack of action of ryanodine on these BK-induced Ca\(^{2+}\) signals indicates that at least in dendritic regions a functional separation between ryanodine- and nonryanodine-sensitive Ca\(^{2+}\) pools probably exists. Assuming that this interpretation is correct, the reduction observed in somatic regions after the depletion of ryanodine-sensitive stores might be secondary and reflect an impairment in CICR mechanisms, which perhaps participate in amplifying BK responses. This interpretation is consistent with the observation that the effect is predominantly in the decaying portion of these responses. This hypothesis is also consistent with the reduction in the Ca\(^{2+}\) signals evoked by K\(^+\) depolarization observed following the depletion of ryanodine-sensitive stores (see preceding text; Fig. 4C).

**ESS RESPONSES IN Ca\(^{2+}\)-FREE MEDIUM.** The mechanisms involved in the generation of Ca\(^{2+}\) signals elicited by ESS ligands were analyzed using protocols similar to those used with BK stimulation. First, we evaluated whether Ca\(^{2+}\) transients evoked by ESS ligands depend on Ca\(^{2+}\) influx or reflect a release from internal stores. As with BK signals we found that the absence of Ca\(^{2+}\) in the medium (0 [Ca\(^{2+}\)], supplemented with 5 mM EGTA) did not suppress ESS responses. Immediately after the removal of Ca\(^{2+}\) from the medium, Ca\(^{2+}\) signals in most epithelial locations exhibited kinetics and characteristics similar to control responses. Ca\(^{2+}\) signals obtained in apical dendritic regions adjacent to the epithelial lumen, however, exhibited a slight reduction in their peak amplitudes of ~12–20% (Fig. 9B). As observed with reductions in BK responses, the late phase of these responses was less affected. These changes were also fully reversible.

In contrast with these local and immediate effects, repetitive ESS stimuli under Ca\(^{2+}\)-free conditions evoked a different type of response reduction. As with the effects found in BK signals, numerous ESS applications (8–20 trials) in Ca\(^{2+}\)-free medium evoked a progressive decrease in Ca\(^{2+}\) signals in all epithelial regions. These reductions were use dependent because they were related to the number and concentration of preceding stimulations and affected both the size and duration of the Ca\(^{2+}\) signals until they disappeared. Figure 9B illustrates the progressive decay of ESS responses following repeated stimulation in Ca\(^{2+}\)-free medium. This type of change usually appeared after the fourth ESS application, and following the eighth stimulus, Ca\(^{2+}\) signals started to exhibit major reductions in peak amplitude (70–85%) and duration. At this stage, Ca\(^{2+}\) transients also exhibited slower rise times and longer

![Figure 9](http://jn.physiology.org/)
peak latencies. A complete recovery of ESS responses was observed after 5–10 min incubation in normal [Ca\(^{2+}\)_i] (Fig. 9C). As with similar use-dependent reductions found in caffeine and BK responses, this progressive response decrease following repetitive ESS stimulation in Ca\(^{2+}\)/H11001-free medium probably reflects the inability of intracellular Ca\(^{2+}\)_i stores to be replenished.

ESS RESPONSES AFTER THE DEPLETION OF Ca\(^{2+}\)_i STORES. To confirm the characteristics of the components dependent and independent of internal Ca\(^{2+}\)_i release, ESS responses were evaluated after the depletion of these intracellular stores. As with BK signals, following the application of ESS ligands, VN slices preincubated with thapsigargin (1 \mu M for 10–15 min) evoked Ca\(^{2+}\)_i signals that exhibited a gradual reduction in amplitude and duration, consistent with a progressive depletion of internal Ca\(^{2+}\)_i stores (Fig. 10A). In the cell body region, ESS responses were completely suppressed by multiple ESS applications (8–10 trials), indicating that at this level these responses depend entirely on Ca\(^{2+}\)_i release from internal stores.

In apical dendritic regions, however, the depletion induced by thapsigargin failed to completely suppress all Ca\(^{2+}\)_i signals, even following numerous applications of ESS ligands (>20 trials). As with BK responses, there was a thapsigargin-resistant component that was revealed after the disappearance of the overlapping ESS response dependent on intracellular Ca\(^{2+}\)_i release (Fig. 10A). Fig. 10A, right, shows that this ESS-evoked
component persisted after numerous stimulus applications (20 trials). This component was, however, reversibly suppressed in Ca\(^{2+}\)-free medium, supporting the notion that it arises directly from Ca\(^{2+}\) influx through the plasma membrane. This remaining ESS response exhibited its largest amplitudes in locations just adjacent to the luminal surface of the epithelium (Fig. 10A, right), similar to the BK-induced response. In this location, the response attained a magnitude equivalent to 15–20% of the full response obtained before the thapsigargin-induced Ca\(^{2+}\) depletion. However, as we previously mentioned for the similar component in BK signals, the actual size of this Ca\(^{2+}\) influx could be underestimated in our population optical recordings due to the difficulty of accurately determining cytosolic Ca\(^{2+}\) levels from localized regions.

These data indicate that Ca\(^{2+}\) transients associated with chemosensory transduction in VN neurons depend on two different mechanisms: a widespread component generated by Ca\(^{2+}\) release from intracellular stores and a more restricted component that depends on Ca\(^{2+}\) influx in apical dendritic regions. In addition, the strong similarities between the kinetics and properties of BK and ESS responses suggest that both Ca\(^{2+}\) transients are closely interrelated, partially sharing similar mechanisms linked to PLC activation and IP\(_3\) production.

Because we found previously a functional separation between ryanodine- and nonryanodine-sensitive stores, we determined next whether cytosolic Ca\(^{2+}\) elevation evoked by ESS stimuli depends on Ca\(^{2+}\) release from ryanodine (IP\(_3\)-sensitive) stores. For this purpose, we evaluated the presence and characteristics of ESS responses after the depletion of ryanodine-sensitive stores. As with BK responses, no major changes in the Ca\(^{2+}\) signals elicited by ESS ligands were observed following the depletion of ryanodine-sensitive stores. The depletion of ryanodine stores only affected ESS signals in the cell body region of VN neurons (Fig. 10B, a). Consistent with similar effects observed in Ca\(^{2+}\) transients elicited by K\(^+\) depolarization (Fig. 4C) and BK stimuli (Fig. 8B), it is likely that these effects depend on an impairment in CICR mechanisms that we have interpreted previously to be mediated predominantly by Ca\(^{2+}\) release from ryanodine-sensitive pools.

Possible role of cAMP in responses to ESS

In the main olfactory system, odor transduction and associated Ca\(^{2+}\) transients rely on the activation of a cAMP pathway. The binding of odorants to olfactory receptors causes a G-protein activation of adenylate cyclase, which, via cAMP, activates a cyclic nucleotide-gated (CNG) channel. The opening of this channel generates an inward transduction current carried by Na\(^+\) and Ca\(^{2+}\), and this influx is largely responsible for the Ca\(^{2+}\) transients elicited during odor stimulation. To determine whether similar CNG channel activity participates in a Ca\(^{2+}\) influx in snake VN neurons, we evaluated ESS responses after the application of the specific CNG channel blockers LY 83583 and t-cis-dilitiazem (LCD), which are known to abolish Ca\(^{2+}\)-related odor responses in olfactory receptor neurons (ORNs) (Kolesnikov et al. 1990; Leinders-Zufall et al. 1997, 1998). As illustrated in Fig. 11, preincubation of VN slices with either LY 83583 (80 \(\mu M\); B) or LCD (40 \(\mu M\); C) for 10–15 min did not affect the general spatial distribution (Fig. 11, B and C) or the kinetics (Fig. 11E) of Ca\(^{2+}\) transient elicited by ESS ligands. The amplitudes, latencies, and time courses of ESS responses remained practically unchanged after the application of either of these CNG channel blockers (Fig. 11E). A similar absence of effect was observed in all the slices evaluated (n = 5). Furthermore, no changes were observed even in the dendritic regions adjacent to the luminal surface of the epithelium where we found a component arising from Ca\(^{2+}\) influx.

To eliminate the possibility that in VN neurons ESS responses are mediated by CNG channels that are insensitive to the blockers tested or by the participation of an unknown cAMP-related mechanisms, we evaluated the effects of forskolin on cytosolic Ca\(^{2+}\) changes in these cells. As in other cell systems, forskolin appears to act as an adenylate cyclase (AC) activator directly inducing an increase in cAMP levels in VN neurons (Luo et al. 1994; Wang et al. 1997). Despite this action, we found that the addition of forskolin (10 \(\mu M\)) to the bath evoked no detectable [Ca\(^{2+}\)] changes, at least in the time frame in which Ca\(^{2+}\) responses were analyzed in this study (n = 5; Fig. 10F). Thus altogether these results indicate that cAMP mechanisms do not directly participate in mediating the Ca\(^{2+}\) transients that occur during the early phases of chemosensory transduction in snake VN neurons.

Role of Na\(^+/Ca^{2+}\) exchanger in ESS responses

We also investigated whether the Ca\(^{2+}\) influx observed during ESS response was dependent on a reverse mode of operation of the Na\(^+/Ca^{2+}\) exchanger. In different cell systems, the operation of the Na\(^+/Ca^{2+}\) exchanger is fully reversible and can mediate either a Ca\(^{2+}\) influx or efflux depending on the ionic gradients present across the plasma membrane (DiPolo and Beaute 1987, 1999). Thus it is conceivable that during VN chemosensory transduction an increase in cytosolic Na\(^+\) could activate the Na\(^+/Ca^{2+}\) exchanger in its reverse mode forcing Ca\(^{2+}\) into the cell as has been reported recently in squid (Danaceau and Lucero 2000). To characterize the functional role of the Na\(^+/Ca^{2+}\) exchanger in VN neurons, we evaluated ESS responses under conditions that inhibit its activity. Because monovalent cations cannot substitute for Na\(^+\) on the Na\(^+/Ca^{2+}\) exchanger (Dipolo and Beaute 1987, 1999; Fiero et al. 1998), we blocked the exchanger by replacing external Na\(^+\) with Li\(^+\) or choline. After the substitution of Na\(^+\) by equimolar concentrations of Li\(^+\) or choline, we observed no permanent changes in basal [Ca\(^{2+}\)] in some cases (n = 3), however, immediately after the substitution of Na\(^+\) we observed increases in cytosolic Ca\(^{2+}\) that returned to normal values within 1–2 min. We interpret these Ca\(^{2+}\) elevations as reflecting a brief Ca\(^{2+}\) influx through the exchanger operating in reverse mode following a sharp depletion of [Na\(^+\)].

During ESS responses, however, we found that the Na\(^+/Ca^{2+}\) exchanger operates in the forward mode, mediating instead a Ca\(^{2+}\) efflux. In all the cases studied (n = 6), we found that the blockade of exchanger activity following the replacement of Na\(^+\) by Li\(^+\) enhanced ESS responses. Under this condition, Ca\(^{2+}\) signals exhibited slight increases in their peak amplitude, and major increases in their duration and magnitude. As seen in Fig. 12B, the changes in amplitudes were more prominent during the late phases of ESS responses. These Ca\(^{2+}\) signals exhibited longer plateaus and slower decay rates, characteristics that increased the total duration of these transients by 35–50%. Because Li\(^+\) may
interfere with IP₃ mechanisms (Worley et al. 1988) and ESS responses seem to rely on this pathway, we also tested the effect of blocking the exchanger by choline substitution (n/H11005). Consistent with our previous results, the replacement of Na/H11001 by choline yielded similar effects to those obtained using Li/H11001. As illustrated in Fig. 12C, under this condition, ESS responses also exhibited larger amplitudes and longer durations, and these changes resulted mainly from the slower decay rate of the cytosolic Ca²⁺ elevations elicited by ESS ligands. Thus both Li/H11001 and choline substitution confirm that during VN transduction the Na⁺/Ca²⁺ exchanger participates in the clearance of Ca²⁺ overloads.

**DISCUSSION**

**General characteristics of optical signals in snake VN neurons**

In this study we have shown the presence of major cytosolic Ca²⁺ transients associated with chemosensory transduction in the VNO of the garter snake. The combined use of optical techniques with the selective labeling of VN neurons by retrograde transport of the Ca²⁺ indicator made it possible for us to assess the characteristics and sources of cytosolic Ca²⁺ elevations elicited by a well-defined chemoattractant exclusively in VN neuron. In our slice preparation, the relatively low resolution of the images in relation to the image blur caused by the lack of confocal optics (Cinelli 2000) prevented single-cell resolution of fluorescence signals in most cases. Nevertheless, two important experimental conditions have facilitated the analysis of Ca²⁺ responses in snake VN neurons. First, fluorescence signals in this study arose exclusively from VN neurons because only these cells were retrogradely labeled with the Ca²⁺ indicator from the AOB. Therefore no optical signals could arise from cell types other than mature VN neurons whose axons project to the AOB. Another advantage in the snake VNO is its laminar organization. As illustrated in Fig. 1B, the cell bodies of mature VN neurons are located in the
intermediate region of the sensory epithelium with dendrites projecting apically, toward the epithelial lumen. Thus optical signals recorded at intermediate epithelial levels represented Ca\(^{2+}\) changes in the cell body regions, while signals toward the luminal epithelial surface corresponded to Ca\(^{2+}\) responses from VN dendrites.

In general we found that the average basal \([\text{Ca}^{2+}]_i\) values in snake VN neurons were in reasonable agreement with those found in other neurons, including ORNs. The cytosolic Ca\(^{2+}\) elevations elicited by the chemoattractant ESS also exhibited characteristics and kinetics similar to the Ca\(^{2+}\) responses evoked in rat VN neurons (Leinders-Zufall et al. 2000) and ORNs (Leinders-Zufall et al. 1997, 1998; Restrepo and Boyle 1991; Restrepo et al. 1990, 1993; Sato et al. 1991; Tareilus et al. 1995) during chemosensory transduction.

Spatial distribution of Ca\(^{2+}\) transient elicited by ESS ligands

An interesting finding was that Ca\(^{2+}\) signals elicited by ESS ligands in snake VN neurons exhibited a rather broad distribution with optical signals arising from an apparently large population of VN neurons. These Ca\(^{2+}\) transients, however, were not uniformly distributed and exhibited dissimilar thresholds, characteristics that suggest the presence of heterogeneous responses in different VN neurons. We also observed a recruitment of new VN neurons that became active as ESS concentration increased. But even at the highest concentration tested not all VN neurons responded to ESS stimuli, suggesting a certain degree of selectivity in the response characteristics of these neurons. Corroborating this notion, the heterogeneous response characteristics among different VN neurons con-
trasted with the more uniform signals evoked by other stimuli such as caffeine, high-[K+]o, depolarization and BK.

The broad and heterogeneous responses elicited by ESS stimuli in snake VN neurons contrast with the more restricted patterns found in mouse VN neurons following stimulation with single chemicals (Leinders-Zufall et al. 2000). In this case, Ca2+ transients were found only in limited VN neurons, the number of which remained constant at higher stimulus concentrations. It is likely that the differences observed in the distribution of Ca2+ signals in these two preparations may arise as a consequence of the different types of stimuli employed. Natural compounds such as ESS ligands have a heterogeneous molecular structure that probably comprises multiple chemical cues. Thus it is likely that each of these cues could activate different sets of VN neurons having distinct molecular receptive ranges. In contrast, pure single chemicals such as those tested in the mouse VNO probably activate a more selective and uniform set of VN neurons. Supporting this hypothesis, recent electrophysiological studies in rat VNO have shown that complex pheromonal stimuli found in urine elicit broad activation of large numbers of VN neurons (Holy et al. 2000). Moreover, similar to our findings in snake VN neurons, higher concentrations of this natural stimulus also seem to increase the number of active VN neurons. In general, in both systems it is conceivable that the simultaneous detection of multiple cues by a large number of VN neurons may reflect a distributed coding strategy that is useful for the detection of complex chemotactants. In the garter snake, such a strategy would be useful in identifying a large number of chemical cues associated with different types of prey. A similar combinatorial code strategy has been proposed as the basis for odor recognition in the main olfactory system (Cinelli et al. 1995; Friedrich and Korsching 1997; Rubin and Katz 1999) and certain complex pheromonal compounds in invertebrates (for review, see Sorensen et al. 1998).

Mechanisms involved in Ca2+ transients in VN neurons

Our results indicate that in snake VN neurons, Ca2+ responses elicited by ESS ligands depend on two different mechanisms: a Ca2+ influx through the plasma membrane and a Ca2+ release from intracellular stores. We can assume that these two components correspond to cytosolic Ca2+ elevations exclusively from VN neurons because only these cellular elements were stained and contribute to the optical response evaluated here (see preceding text). The component generated by a Ca2+ influx was found after depleting intracellular stores with thapsigargin and exclusively observed in the apical dendritic region of VN neurons. This distribution is interesting because it suggests that this component occurs at locations in close proximity to the sites where VN transduction takes place and could be related to the activation of a primary transduction current. The component dependent on Ca2+ release from internal stores displayed a more widespread distribution with responses spreading to different cellular segments. Nevertheless, it seems likely that this component also starts in apical dendritic regions because there it exhibited its lowest threshold. If this is the case, it is interesting that both Ca2+ influx and Ca2+ release seem to coexist in similar dendritic domains.

Within the limitation imposed by the time resolution of our recordings, both components seem to appear almost simultaneously because we were unable to detect any difference either in their latencies or their rise times. It is unlikely that this characteristic results from the “in vitro” conditions used here because ESS ligands should only stimulate VN neurons through the activation of VN receptors. Thus even in our slice preparation, only responses associated with VN transduction should give rise to these Ca2+ signals.

We also found these two components to be relatively independent because the suppression of one of them did not abolish the other. In fact, the depletion of internal stores by thapsigargin abolishes most Ca2+ transients elicited by ESS ligands except those arising from a Ca2+ influx. On the other hand, this Ca2+ influx was reversibly abolished when Ca2+ was removed from the medium, but this condition did not suppress the Ca2+ signals elicited from Ca2+ release. Therefore we cannot consider either of these two components to be secondary to the other. Instead it is likely that both components constitute primary responses that appear to be triggered in parallel by a common mechanism associated with the initial stages of VN signal transduction.

BK applications evoked Ca2+ transients that exhibited kinetics and properties similar to the Ca2+ signals evoked by ESS ligands. The main difference observed was in relation to their spatial distribution. In contrast to the heterogeneous appearance of ESS responses, Ca2+ signals elicited by BK stimuli were distributed more uniformly among different VN neurons. This characteristic probably reflects the lack of selectivity of the BK stimulus in activating different VN neurons. But similar to ESS responses, Ca2+ signals elicited by BK also exhibited two components, one arising from Ca2+ influx and the other from Ca2+ release. The component arising from Ca2+ influx was also found exclusively in apical dendritic regions close to the epithelial lumen. This is an interesting finding because in our “in vitro” slice preparation, BK stimuli directly applied to the bath probably activate BK receptors from all cellular segments because it is believed that in general these receptors are broadly distributed in neurons (Koizumi et al. 1999). Despite this putative global effect, however, we observed in VN neurons only a Ca2+ influx in apical dendritic segments, indicating that this component probably exists only at this cellular level. This evidence provides further support for the notion that it might represent the opening of a conductance that is normally activated by VN transduction. In addition, as with ESS signals, these two components also appear to be relatively independent because the suppression of one did not abolish the presence of the other. Finally, Ca2+ release in response to both ESS and BK was largely unaffected by the selective depletion of ryanodine-sensitive stores (see following text). This finding indicates that this Ca2+ release depends predominantly on nonryanodine-sensitive stores, probably those stores activated by IP3 (Berridge 1998). Only the Ca2+ responses from the cell body seem to be affected by the depletion of ryanodine stores but with reductions that appear to be secondary because only the late phases of these Ca2+ transients are affected.

Taken together with the evidence obtained in the biochemical assay indicating that both ESS and BK stimuli evoked similar IP3 elevations, these results lead to the interpretation that the Ca2+ transients elicited by ESS ligands depend primarily on mechanisms linked to IP3 production. Consistent with this interpretation, previous functional studies have sug-
gests that IP$_3$ is a good candidate as a second messenger in VN transduction. In the garter snake VN epithelium, exposure of prey-derived chemosensory pheromones to female porcine VNO incubated with boar seminal fluid or urine (Wekesa and Anholt 1997). Aphrodisin, which is a pheromone evoked by female hamsters, also induces IP$_3$ accumulation in the VN epithelium of male hamsters (Kroner et al. 1996). Finally, urinary pheromones evoke IP$_3$ increases in rat VN epithelium (Sasaki et al. 1999), and the VN neuronal discharges evoked by these urinary pheromones are selectively blocked by the PLC inhibitor U-73122 (Holy et al. 2000).

Besides the putative IP$_3$-mediated Ca$^{2+}$ responses, we also found that other mechanisms contribute to the generation of cytosolic Ca$^{2+}$ elevations elicited by chemosensory pheromones. Figure 13 is a summary diagram of the mechanisms that might participate in the generation and regulation of Ca$^{2+}$ transients in VN neurons during chemosensory transduction. Basically, our results seem to reveal two levels of organization in the generation of ligand-induced Ca$^{2+}$ signals. First, the binding of specific ligands to receptors triggers the activation of a secondary signaling mechanism in which IP$_3$ production appears to be involved. This signaling molecule, in turn, appears to mediate both an increase in cytosolic Ca$^{2+}$ levels through a release from internal stores and a Ca$^{2+}$ influx through the plasma membrane. These initial Ca$^{2+}$ elevations are then amplified through secondary mechanisms localized predominantly in the cell body region. These mechanisms probably include a Ca$^{2+}$ influx through VSCC activated by membrane depolarization associated with transduction mechanisms and a further Ca$^{2+}$ release from ryanodine-sensitive internal pools through a CICR mechanism. Thus the first stage of these Ca$^{2+}$ transients occurs predominantly in dendritic regions, whereas secondary events occur predominantly in the somata region. Finally, the Na$^+$/Ca$^{2+}$ exchanger participates in maintaining basal cytosolic Ca$^{2+}$ by extruding the Ca$^{2+}$ excess resulting from activation of chemosensory transduction mechanisms. In accordance with our results, this model does not include the participation of a cAMP activation of CNG channels in the generation of the Ca$^{2+}$ transients associated with VN transduction.

Both high [K$^+$]$_o$ and caffeine evoked Ca$^{2+}$ transients near the cell body region of VN neuron, but there were important differences between these Ca$^{2+}$ signals. High [K$^+$]$_o$ elicited relatively brief Ca$^{2+}$ signals that depended on the activation of VSCC because they were suppressed by the removal of Ca$^{2+}$ from the medium and by common VSCC blockers. Caffeine also evoked major cytosolic Ca$^{2+}$ elevations in the somata region, but these signals exhibited a more prolonged time course and depended exclusively on Ca$^{2+}$ release from internal stores. These caffeine responses also differed from ESS signals at least in three aspects. First, Ca$^{2+}$ transients evoked by caffeine exhibited a more gradual rise-time with a progressive buildup. Second, caffeine signals were absent in apical dendritic regions, and the removal of extracellular Ca$^{2+}$ did not reduce the magnitude of these responses in any region. Third, Ca$^{2+}$ signals elicited by caffeine were completely abolished by depletion of ryanodine-sensitive stores, indicating that these responses arise entirely from Ca$^{2+}$ release from these pools.

An interesting finding of this study was that depletion of ryanodine stores did not affect the overall characteristics of ESS responses. These data indicate that a Ca$^{2+}$ release from ryanodine-sensitive stores does not constitute the primary source of the Ca$^{2+}$ elevations associated with chemosensory transduction. In addition, it suggests that in snake VN neurons a functional separation exists between IP$_3$ and ryanodine-sensitive Ca$^{2+}$ pools, which appear to be unevenly distributed among different cellular segments. In this regard, we observed that caffeine responses were found predominantly in the cell body region, while ESS and BK responses were more broadly distributed toward dendritic regions. Our data also suggest that ryanodine-sensitive pools mediate an amplification of Ca$^{2+}$ responses through a CICR mechanism because depletion of these stores affected the decay phase of the Ca$^{2+}$ transients evoked either by ESS or high-[K$^+$]$_o$, depolarization. In rat ORNs, a similar CICR mechanism also seems to amplify Ca$^{2+}$ signals elicited by odor stimuli (Zufall et al. 2000).
Component of ESS response dependent on a Ca\(^{2+}\) release

An unexpected finding of this study was the presence of major Ca\(^{2+}\) transients elicited by ESS ligands in the absence of extracellular Ca\(^{2+}\). This result differs from otherwise similar Ca\(^{2+}\) signals associated with chemosensory transduction in mouse VN neurons (Leinders-Zufall et al. 2000) and in ORNs (Leinders-Zufall et al. 1997; Restrepo et al. 1990, 1993; Sato et al. 1991; Schild et al. 1995; Tareilus et al. 1995), which are completely suppressed in 0 [Ca\(^{2+}\)]_o. It is interesting to note that Ca\(^{2+}\) release from internal Ca\(^{2+}\) pools also occurs in ORNs, but this mechanism appears to be secondary to an initial Ca\(^{2+}\) influx that is mediated through the opening CNG channels (Leinders-Zufall et al. 1998; Zufall et al. 2000).

In snake VN neurons, we found that Ca\(^{2+}\) release can be directly triggered by VN transduction mechanisms, a condition that may depend on two factors. First, in this system transduction seems to be mediated by phosphoinositide turnover, and it is known that IP\(_3\) elevations in multiple cell systems mediate major Ca\(^{2+}\) mobilization from intracellular pools (for review, see Berridge 1993, 1998). Second, in snake VN neurons, it appears that a tight coupling exists between the IP\(_3\) elevation associated with VN signal transduction and the activation of Ca\(^{2+}\) release from intracellular stores. Previous ultrastructural studies of these cells demonstrated well developed cisternae of endoplasmic reticulum (ER) extending close to microvillar regions extending to microvillar processes (Wang and Halpern 1980a,b). Perhaps, the proximity of these potential Ca\(^{2+}\) stores to the microvilli permits efficient coupling for the activation of Ca\(^{2+}\) release during VN signal transduction.

Component of ESS response dependent on Ca\(^{2+}\) influx

Besides the contribution of Ca\(^{2+}\) release, we found, in the region adjacent to the luminal surface of the VN epithelium, a component of ESS responses that depends on Ca\(^{2+}\) influx. In our recordings, this component only represents 12–20% of the total cytosolic Ca\(^{2+}\) transients elicited by ESS ligands, but the magnitude could be underestimated, since our optical signals represent population responses from several VN neurons together. Under these conditions, stronger neighboring signals arising from Ca\(^{2+}\) release might minimize the real magnitude of this Ca\(^{2+}\) influx, which probably occurs in confined regions of the microvilli.

It is interesting that this Ca\(^{2+}\) influx does not appear to be a cAMP-mediated mechanism because specific blockers of CNG channels do not affect, to any measurable degree, the characteristics of this component, nor does the direct application of forskolin evoke detectable [Ca\(^{2+}\)]_i changes. This interpretation contrasts with the primary mechanism generating Ca\(^{2+}\) responses in ORNs (Leinders-Zufall et al. 1997, 1998) and leads to the suggestion that in snake VN neurons a cAMP signaling pathway does not play a fundamental role during the initial stages of VN signal transduction. This Ca\(^{2+}\) influx does not depend either on the reverse operation of the Na\(^+\)/Ca\(^{2+}\) exchanger (DiPolo and Beauge 1987) because we found that its blockage resulted in an increase in the amplitude and duration of the Ca\(^{2+}\) transients. Thus this exchanger probably participates in restoring basal [Ca\(^{2+}\)]_i, as has been reported for other neurons (Fierro et al. 1998), including ORNs (Jung et al. 1994; Noe et al. 1997; Reisert and Matthews 1998; Zufall et al. 2000). Because we found that both ESS and BK responses seem to depend on IP\(_3\) production, it appears likely that this Ca\(^{2+}\) influx depends on activation of an IP\(_3\) conductance in VN dendritic membranes. The existence of IP\(_3\)-gated cation channels has been proposed in different chemosensory systems, but unfortunately their role is still unclear (for review, see Schuld and Restrepo 1998). The best available evidence for the existence of these types of channels in chemosensory transduction is in lobster ORNs, where it has been possible to characterize an IP\(_3\)-gated channel coupled to a G protein (Munger et al. 2000). In vertebrates, however, the presence of an IP\(_3\) transduction pathway in olfaction is still controversial. Transgenic mice deficient in either CNG channels or G-protein-coupled adenylate cyclase type III (Golf) fail to respond to odors (Belluscio et al. 1999; Brunet et al. 1996), suggesting that IP\(_3\) cannot be considered an alternate transduction pathway in this system.

Among the possible candidates involved in mediating the Ca\(^{2+}\) influx found here are members of the transient receptor potential (TRP) channels. Some TRP proteins are nonspecific cation-permeable, store-operated channels (SOC) activated by the depletion of intracellular Ca\(^{2+}\) stores (Boulay et al. 1999; Minke and Selinger 1996a,b; Vannier et al. 1999; Zhu et al. 1996). However, it has been reported that some members in this family seem to be activated also by phosphoinositide turnover but not after store depletion (Okada et al. 1998; Schaefer et al. 2000). This may involve both functional and physical interactions between IP\(_3\) receptors and TRP channels (Birnbaumer et al. 2000; Boulay et al. 1999; Kiselyov et al. 1998). In situ hybridization and immunohistochemical studies (Limon et al. 1999) in rats indicate that the mRNA of a member of this channel type (rTRP2) is expressed exclusively in the receptor cells of the vomeronasal sensory epithelium and that the protein is localized to the microvilli of these neurons. Interestingly, this location corresponds to the Ca\(^{2+}\) influx here. If similar TRP channels are expressed in snake VN neurons in this region, it is unlikely that they would be solely SOC because our results indicate that depletion of intracellular Ca\(^{2+}\) stores alone failed to elicit the Ca\(^{2+}\) influx observed in this study.

Still to be resolved are the relationship between these two components found in responses to ESS and BK as well as the exact role of these cytosolic Ca\(^{2+}\) elevations during VN transduction. It is likely, however, that these cytosolic Ca\(^{2+}\) elevations play a significant role in the regulation of VN responses. In the main olfactory system, cytosolic Ca\(^{2+}\) elevations with similar kinetics play an important role, among other functions, in odor adaptation. In ORNs, the main mechanism of odor adaptation depends on an increase in cytosolic Ca\(^{2+}\) that activates a calmodulin (CAM)-dependent protein kinase II (CAM-kinase II) that, in turn, phosphorylates the adenyl cyclase and lowers CNG channel activity (Chen and Yau 1994; Kurahashi and Menini 1997). A similar mechanism is unlikely to occur in snake VN neurons because we found that Ca\(^{2+}\) transients associated with VN transduction do not depend on the activation of CNG channels. Other mechanisms, however, can still be triggered by cytosolic Ca\(^{2+}\) and act on either the primary transduction currents or downstream. Interestingly, in Caenorhabditis elegans (Colbert et al. 1997) and Drosophila (Störrkühl et al. 1999) mutants defective in some TRP members exhibit impairment in olfactory adaptation. In addition,
cytosolic Ca\(^{2+}\) elevations could also regulate VN responses by modulating membrane excitability as has been demonstrated in a variety of neurons (Congar et al. 1997; Llano et al. 1991; Partridge and Valenzuela 1999; Partridge et al. 1994). IP\(_3\) dialyzed into snake vomeronasal receptor cells produces a depolarizing current, which has been attributed to chloride (Taniguchi et al. 2000). According to our results, it is possible that IP\(_3\), either as a depolarizing current or localized to the plasma membrane of olfactory receptor cell axons (Kleene 1993, 1997; Kleene and Gesteland 1991; Kurashashi and Yau 1993). In addition cytosolic Ca\(^{2+}\) levels could also modulate the activity of other conductances such as K\(^{+}\) channels and VSCC as occurs in ORNs (for review, see Schmid and Restrepo 1998).

On the other hand, if TRP channels constitute the primary transduction currents in VN neurons, Ca\(^{2+}\) release occurring in or adjacent to the microvilli could regulate the activity of these channels. Although there are some members of the TRP channel family that are not store operated, still the depletion of intracellular stores could enhance their activity (Strubing et al. 2001). Under such conditions, the depletion of intracellular Ca\(^{2+}\) stores could potentiate the cation influx through these channels, a condition that could eventually enhance transduction currents. Thus the presence of cytosolic Ca\(^{2+}\) transients arising from two sources simultaneously in VN neurons is of special interest in light of the accumulating body of evidence indicating that cytosolic Ca\(^{2+}\) plays an important role in modulating sensory transduction at different levels.

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CHEMOATTRACTANT-INDUCED CALCIUM RELEASE


