Calcium Transients in the Garter Snake Vomeronasal Organ

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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; Naillau 1965; Wilde 1938). The initial step in prey detection involves the interaction between a prey-derived chemosensory 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 $\sim 0.3$ μM (Jiang et al. 1990) and does not bind specifically to other organs, including brain and main olfactory epithelium. G proteins ($G_\alpha$, $G_i$, 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 adenylyl cyclase, 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 transient IP$_3$-related ac-

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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 (Taniguchi 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 on a piece of filter paper (Valapar, Canada) 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 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, 0.5 μg/ml aprotinin, 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; Marshal 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 means of 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; Fluor; 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 excitation 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 ($F/F_0$). Standard procedures for background subtraction and calibration were used for calibration with solutions of known dye concentrations (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$^{2+}$]. 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 filtering 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$^{2+}$], 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 preparation 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 consistently observed in the slices that only mature VN neurons were labeled with the fluorescence indicator (Fig. 1A and B); no other cellular elements, such as sustentacular cells or immature VN neurons, were labeled. Therefore all of the Ca^{2+} responses evaluated 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 Ca²⁺ Signals in VN Neurons**

![Fig. 1](http://jn.physiology.org/)

**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+}] 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+}], over baseline levels following ESS application from the entire sequence (16 frames) as determined from 3 different sites (8 × 8 pixels) shown in the first image in C (a–c). The 1st point in the plots corresponds to basal cytosolic values and the 2nd point to the first response obtained 1 s after stimulus application. Arrows indicate the times corresponding to the 4 images illustrated in C.
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 elements 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 specific staining of only VN neurons allowed us to define the source of the optical signals at least in three cellular compartments: the cell body region, the dendritic shaft region, and the apical dendritic region of VN neurons adjacent to the luminal surface. Consequently, as seen in all figure labels, Ca$^{2+}$ responses recorded toward the base and in the middle of the epithelium corresponded to transients predominantly generated in or close to the cell bodies, optical signals from the upper VN epithelial sectors related primarily to Ca$^{2+}$ transients from dendritic shafts, and fluorescence changes adjacent to the VN lumen corresponded to Ca$^{2+}$ responses arising from the most apical segments of VN dendrites.

**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$^{2+}$], variations. According to “in situ” calibrations performed at the end of the experiments (see METHODS), baseline [Ca$^{2+}$], 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$^{2+}$], 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 chemotaxtractant ESS while [Ca$^{2+}$], 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 dissimilar 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\textsuperscript{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\textsuperscript{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\textsuperscript{2+} transients in VN neurons. Figure 3 compares the spatial distribution and kinetics of different types of Ca\textsuperscript{2+} signals evoked in snake VN neurons. Both ESS and caffeine (2–5 mM) applications gave rise to prolonged Ca\textsuperscript{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\textsuperscript{+}]\textsubscript{o} elicited Ca\textsuperscript{2+} transients that were relatively rapid in onset and brief in duration compared with other types of Ca\textsuperscript{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\textsuperscript{2+} CHANNELS.** To evaluate the role of voltage-sensitive Ca\textsuperscript{2+} channels (VSCC) in the generation of cytosolic Ca\textsuperscript{2+} changes, we determined the properties of Ca\textsuperscript{2+} responses elicited by high KCl (100 mM; Fig. 3B). The short duration and monotonic decay of these signals occurred even when high [K\textsuperscript{+}]\textsubscript{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\textsuperscript{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\textsuperscript{2+}], elevation on the order of 175–290 nM. These Ca\textsuperscript{2+} signals were reversibly suppressed when extracellular Ca\textsuperscript{2+} was removed from the medium (Fig. 4A). They were also completely blocked after the application of common VSCC blockers such as cadmium (Cd\textsuperscript{2+}; Fig. 4B) or cobalt (Co\textsuperscript{2+}; 50–100 μM; data not shown), confirming that they resulted primarily from a Ca\textsuperscript{2+} influx through VSCC.

To determine whether a Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) could be activated following an initial Ca\textsuperscript{2+} influx mediated through VSCC, we evaluated the characteristics of Ca\textsuperscript{2+} transients elicited by high-[K\textsuperscript{+}]\textsubscript{o} depolarization following the depletion of internal Ca\textsuperscript{2+} stores. Depletion of intracellular stores were obtained either using thapsigargin or ryanodine. Thapsigargin depletes intracellular Ca\textsuperscript{2+} store because it is a potent inhibitor of the intracellular Ca\textsuperscript{2+} pump and prevents Ca\textsuperscript{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\textsuperscript{2+} pools. Under our experimental conditions, we found that the action of thapsigargin (1 μM over 10–15 min) in depleting internal Ca\textsuperscript{2+} stores was predominantly stimulus dependent because the spontaneous Ca\textsuperscript{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\textsuperscript{+}-elicited Ca\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{2+} transients evoked by caffeine depend exclusively on a Ca\textsuperscript{2+} release from ryanodine-sensitive stores; see following text).

As illustrated in Fig. 4C, middle, following ryanodine treatment, K\textsuperscript{+}-elicited Ca\textsuperscript{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\textsuperscript{2+} transients elicited by K\textsuperscript{+} 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\textsuperscript{+} depolarization appears to elicit an initial Ca\textsuperscript{2+} influx through the activation of VSCC that in turn triggers a secondary Ca\textsuperscript{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\textsuperscript{2+} signals mediated through the activation of VSCC.

**CHARACTERISTICS OF CYTOSOLIC Ca\textsuperscript{2+} ELEVATIONS ELICITED BY RELEASE FROM INTRACELLULAR STORES.** To determine the characteristics of the Ca\textsuperscript{2+} transients in snake VN neurons that depend on Ca\textsuperscript{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\textsuperscript{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 ± 4.7% (\(\Delta F/\Delta F_0\); mean ± SD; \(n = 8\)) that corresponded to [Ca\(^{2+}\)] \(_e\) elevations in the range of 190–200 nM. 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\(^{2+}\)] \(_e\) slowly returned to baseline (Figs. 3C and 5A). In contrast to ESS transients, the rise times of caffeine signals were highly dependent on stimulus concentration. At low concentrations, they exhibited a slower onset, especially in small amplitude responses (see trace a). Optical signals were obtained from single runs (1 frame/s; 120-ms image exposure) and superimposed on a fluorescent image of the VN slice. 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. Cytosolic Ca\(^{2+}\) levels were coded as described in Fig. 1C. Abbreviations in Fig. 1A. Horizontal bar in C = 25 μm.

**FIG. 2.** General characteristics of Ca\(^{2+}\) transients evoked by ESS ligands in garter snake VN neurons. A–C: patterns of activity show 8 consecutive records from 16 frame sequences illustrating [Ca\(^{2+}\)] \(_e\) over baseline levels, just before (1st frame) and following (remaining 7 frames) the application of ESS stimuli at 3 different concentrations (l, 2, and 3.3 mg protein/ml). Note that the luminal surface of the epithelium (L) is to the right, the basolateral space (BL) to the left. D: a similar sequence but with records obtained following the application of actin (3 mg/ml), which was used as a control and produced no detectable Ca\(^{2+}\) changes. Observe that in B and C, the increases in odorant concentrations evoked larger and more widespread responses over different regions of the vomeronasal sensory epithelium. Plots on the right illustrate the corresponding time course of Ca\(^{2+}\) signals from the whole sequence of images (16) at the 3 sites shown in A. 1st image (a–c) are indicated by —, —, and ··, respectively. In all cases, [Ca\(^{2+}\)] \(_e\) returns to baseline levels after 12–16 s, but higher concentrations produce longer response durations. Optical signals were obtained from single runs (1 frame/s; 120-ms image exposure). The 1st 2 points in the plots correspond to basal cytosolic values and the 3rd point to the 1st response obtained 1 s after stimulus application. Cytosolic Ca\(^{2+}\) levels were coded in pseudocolors as in Fig. 1C and overlapped on a fluorescent image of labeled VN neurons. — in D = 75 μm.
studied ($n = 7$), the absence of Ca$^{2+}$ from the medium (0 [Ca$^{2+}$]$_o$, supplemented with 5 mM EGTA) did not affect the characteristics of caffeine responses evaluated immediately after the Ca$^{2+}$ removal. Under these conditions, Ca$^{2+}$ responses exhibited profiles, time courses (14.8 ± 2.3 s), and peak magnitudes (29.5 ± 3.7 Δ$F/F_o$) similar to controls (Fig. 4).
FIG. 5. Properties of Ca\textsuperscript{2+} signals evoked by caffeine in snake VN neurons. \textbf{A}: the characteristics of caffeine responses obtained following the removal of Ca\textsuperscript{2+} from the medium. \textit{Left}: corresponds to control responses obtained from a 4 × 4 pixel area in the layer of VN neuron somata. \textit{Middle}: plots of Ca\textsuperscript{2+} signals elicited by caffeine (10 mM) following the removal of Ca\textsuperscript{2+} from the medium (0 [Ca\textsuperscript{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\textsuperscript{2+} signals, as shown in responses obtained after 4 and 8 caffeine applications (— — and — — —, respectively). \textit{Right}: the recovery of this Ca\textsuperscript{2+} transient after restitution of normal [Ca\textsuperscript{2+}]o (2–3 min).

\textbf{B}: the effect of depleting internal Ca\textsuperscript{2+} stores with thapsigargin on caffeine-elicited Ca\textsuperscript{2+} signals. \textit{Left}: a control response; \textit{middle}: traces correspond to the progressive reduction in these Ca\textsuperscript{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).

\textbf{C}: Ca\textsuperscript{2+} signals evoked by caffeine following the depletion of ryanodine-sensitive internal stores. \textit{Left}: corresponds to a control signal obtained before the application of ryanodine (20 μM, 15 min). \textit{Middle}: the absence of caffeine-induced Ca\textsuperscript{2+} transients after cytosolic Ca\textsuperscript{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 \textbf{B} and \textbf{C} were tested in normal snake Ringer solution. Plots correspond to Ca\textsuperscript{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 Ca^{2+}-free medium evoked Ca^{2+} transients that exhibited a progressive reduction, first in their amplitude and then in their duration \((n = 7; \text{Fig. 5A}, \text{middle}, \cdots)\). The most likely explanation for this result is that these reductions are a consequence of the inability of intracellular Ca^{2+} stores to be fully refilled in Ca^{2+}-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 Ca^{2+} 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 Ca^{2+} transients but with some differences in their actions. VN slices preincubated with thapsigargin (10–15 min) evoked no major changes in basal cytosolic Ca^{2+} levels, and initial caffeine applications elicited Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} stores.

Ryanodine depletion also suppressed caffeine-elicited Ca^{2+} 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 Ca^{2+} transients arise entirely from internal Ca^{2+} release but further indicates that this release depends primarily on ryanodine-sensitive pools.

### Role of the IP\(_3\) signaling cascade in the generation of Ca\(^{2+}\) transients

Previous studies have suggested that phosphoinositide turnover leading to IP\(_3\) 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 Ca\(^{2+}\) 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 IP\(_3\) (Kirischuk et al. 1995; Seymour-Laurent and Barish 1995; Verkhovsky and Kettenmann 1996). Thus it has been used rather extensively to evaluate the role of IP\(_3\) in intracellular Ca\(^{2+}\) 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 IP\(_3\) levels in homogenates of the snake VN sensory epithelium. IP\(_3\) 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 nM Tris-acetate, pH 7.6, 5 mM MgAc\(_2\), 0.5 mM ATP, 1 mM DTT, 0.01 mM GTP, 0.1 mM CaCl\(_2\), 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 IP\(_3\) levels equivalent to 210 ± 10 (SD) pmol/mg proteins \((n = 4)\) while BK evoked IP\(_3\) levels equivalent to 255 ± 5.0 pmol/mg proteins \((n = 4)\). These IP\(_3\) differences significantly from those obtained under control conditions \((75 ± 15 \text{ (SD) pmol/mg proteins}; n = 4)\), suggesting that both ESS and BK induce similar phosphoinositide turnover leading to IP\(_3\) formation. To further determine whether these increases elicited by BK correspond to IP\(_3\) changes arising from VN neurons, IP\(_3\) 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 IP\(_3\) levels that did not differ significantly from those found under control conditions. Thus these data indicate that both ESS and BK increase IP\(_3\), and these increases take place predominantly in mature snake VN neurons.

Ca\(^{2+}\) signals evoked by BK applications also exhibited strong similarities with the Ca\(^{2+}\) transients elicited by ESS ligands. As shown in Fig. 6, BK stimuli evoked Ca\(^{2+}\) 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 Ca\(^{2+}\) 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, Ca\(^{2+}\) 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 Ca\(^{2+}\) responses elicited by caffeine (Fig. 3C). Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) 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
BK Responses in 0 [Ca\(^{2+}\)]_o

**A**

- Control
- 1st Trial

**B**

- Control
- 1st Trial
- 2nd
- 4th
- 8th
- 20th

**C**

After Rinse

**FIG. 7.** Characteristics of Ca\(^{2+}\) signals evoked in apical dendritic regions of VN neurons by BK in the absence of [Ca\(^{2+}\)]_o. A: responses to BK (300 nM) obtained in normal Ringer solution (control) and immediately following removal of Ca\(^{2+}\) from the bath [0 [Ca\(^{2+}\)]_o]. B: the effect of repetitive BK applications in Ca\(^{2+}\)-free medium. After their initial reduction, 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 Ca\(^{2+}\) responses until all cytosolic Ca\(^{2+}\) changes become undetectable (usually after 20 runs using this stimulus concentration; bottom trace). C: full recovery shown of BK-elicited Ca\(^{2+}\) signals after the VN slices were returned to normal Ringer solution for 5 min. All 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.

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

Although in most epithelial regions there was no modification, 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 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% of maximal responses. 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 Ca\(^{2+}\) from the medium. Full response recovery was always obtained without delay after the restitution of normal [Ca\(^{2+}\)]_o in the bath. This evidence indicates that Ca\(^{2+}\) signals elicited by BK stimuli in these apical dendritic segments of VN neurons has a component generated by a Ca\(^{2+}\) influx through the plasma membrane. In this region, however, there is still a response remaining that is unaffected by removal of Ca\(^{2+}\) from the medium. This component, present in Ca\(^{2+}\)-free medium, appears to be largely dependent on 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 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 Ca\(^{2+}\) signals was dependent on the number and the concentration of previous BK applications. As was the case for Ca\(^{2+}\) transients elicited by repetitive applications of caffeine when the VN slices were in Ca\(^{2+}\)-free medium, these reductions probably arise from the progressive inability of intracellular Ca\(^{2+}\) stores to be replenished in 0 [Ca\(^{2+}\)]_o.

**BK RESPONSES AFTER DEPLETING Ca\(^{2+}\) STORES.** To further characterize the component of BK responses that was independent of Ca\(^{2+}\) release, we evaluated these signals after the depletion intracellular Ca\(^{2+}\) stores. As with Ca\(^{2+}\) transients evoked by caffeine, the preincubation of VN slices with thapsigargin (1 μM 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 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 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
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 Ca²⁺ 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 Ca²⁺ stores by thapsigargin. As seen in the middle plots, BK applications in thapsigargin-treated slices evoked a reduction of Ca²⁺ 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 [Ca²⁺]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 Ca²⁺ stores. In contrast, the removal of Ca²⁺ from the medium reversibly abolished this thapsigargin-resistant component. Thus altogether this evidence indicates that this component is not generated by an internal Ca²⁺ release but instead is dependent on a Ca²⁺ influx through the plasma membrane. Confirming this notion, the magnitude and duration of this component matches the initial reductions in control BK responses observed in the same region when Ca²⁺ was removed from the medium (Fig. 7A).

In contrast with thapsigargin, ryanodine-induced Ca²⁺ depletion did not greatly affect the characteristics of Ca²⁺ tran-
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\(^{2+}\)]\(_{\text{in}}\)) 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 used 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
peak latencies. A complete recovery of ESS responses was observed after 5–10 min incubation in normal \([\text{Ca}^{2+}]_0\) (Fig. 9C). As with similar use-dependent reductions found in caffeine and BK responses, this progressive response decrease following repetitive ESS stimulation in \(\text{Ca}^{2+}\)-free medium probably reflects the inability of intracellular \(\text{Ca}^{2+}\)-stores to be replenished.  

ESS RESPONSES AFTER THE DEPLETION OF \(\text{Ca}^{2+}\) STORES. To confirm the characteristics of the components dependent and independent of internal \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\) signals that exhibited a gradual reduction in amplitude and duration, consistent with a progressive depletion of internal \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\) release from internal stores. In apical dendritic regions, however, the depletion induced by thapsigargin failed to completely suppress all \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\) release (Fig. 10A). Fig. 10A, right, shows that this ESS-evoked

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**ESS -Responses after \(\text{Ca}^{2+}\) Store Depletion**

**A** Thapsigargin

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<th>After Thapsigargin</th>
<th>Trial 10</th>
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**B** Ryanodine

<table>
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<th>After Ryanodine</th>
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![Image](image6.png)
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. 10, 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, probably 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 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, probably sharing similar mechanisms linked to PLC activation and IP\(_3\) production.

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 Beauge 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 Beauge 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

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 \(\lambda\)-cis-diltiazem (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\(^{2+}\)]i changes, at least in the time frame in which Ca\(^{2+}\) responses were analyzed in this study \((n = 5; \text{Fig. 10F})\). 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 chemo- sensory transduction in snake VN neurons.
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\textsuperscript{2+} changes in the cell body regions, while signals toward the luminal epithelial surface corresponded to Ca\textsuperscript{2+} responses from VN dendrites.

In general we found that the average basal [Ca\textsuperscript{2+}]\textsubscript{i} values in snake VN neurons were in reasonable agreement with those found in other neurons, including ORNs. The cytosolic Ca\textsuperscript{2+} elevations elicited by the chemoattractant ESS also exhibited characteristics and kinetics similar to the Ca\textsuperscript{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\textsuperscript{2+} transient elicited by ESS ligands

An interesting finding was that Ca\textsuperscript{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\textsuperscript{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⁺], 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 stimulants found in urinal 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 chemoattractants. 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 IP₃ is a good candidate as a second messenger in VN transduction. In the garter snake VN epithelium, exposure of prey-derived chemoattractants induced dose-dependent IP₃ accumulation (Luo et al. 1994), and similar IP₃ elevations have been found in microvillar membranes from prepubertal female porcine VNO incubated with boar seminal fluid or urine (Wekesa and Anbolt 1997). Aphrodisin, which is a pheromone excreted by female hamsters, also induces IP₃ accumulation in the VN epithelium of male hamsters (Kroner et al. 1996).

Finally, urinary pheromones evoke IP₃ 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 IP₃-mediated Ca²⁺ responses, we also found that other mechanisms contribute to the generation of cytosolic Ca²⁺ elevations elicited by chemoattractants. Figure 13 is a summary diagram of the mechanisms that might participate in the generation and regulation of Ca²⁺ transients in VN neurons during chemosensory transduction. Basically, our results seem to reveal two levels of organization in the generation of ligand-induced Ca²⁺ signals. First, the binding of specific ligands to receptors triggers the activation of a secondary signaling mechanism in which IP₃ production appears to be involved. This signaling molecule, in turn, appears to mediate both an increase in cytosolic Ca²⁺ levels through a release from internal stores and a Ca²⁺ influx through the plasma membrane. These initial Ca²⁺ elevations are then amplified through secondary mechanisms localized predominantly in the cell body region. These mechanisms probably include a Ca²⁺ influx through VSCC activated by membrane depolarization associated with transduction mechanisms and a further Ca²⁺ release from ryanodine-sensitive internal pools through a CICR mechanism. Thus the first stage of these Ca²⁺ transients occurs predominantly in dendritic regions, whereas secondary events occur predominantly in the somata region.

Finally, the Na⁺/Ca²⁺ exchanger participates in maintaining basal cytosolic Ca²⁺ by extruding the Ca²⁺ 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 Ca²⁺ transients associated with VN transduction.

Both high [K⁺]₀ and caffeine evoked Ca²⁺ transients near the cell body region of VN neuron, but there were important differences between these Ca²⁺ signals. High [K⁺]₀ elicited relatively brief Ca²⁺ signals that depended on the activation of VSCC because they were suppressed by the removal of Ca²⁺ from the medium and by common VSCC blockers. Caffeine also evoked major cytosolic Ca²⁺ elevations in the somata region, but these signals exhibited a more prolonged time course and depended exclusively on Ca²⁺ release from internal stores. These caffeine responses also differed from ESS signals at least in three aspects. First, Ca²⁺ 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 Ca²⁺ did not reduce the magnitude of these responses in any region. Third, Ca²⁺ signals elicited by caffeine were completely abolished by depletion of ryanodine-sensitive stores, indicating that these responses arise entirely from Ca²⁺ 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 Ca²⁺ release from ryanodine-sensitive stores does not constitute the primary source of the Ca²⁺ elevations associated with chemosensory transduction. In addition, it suggests that in snake VN neurons a functional separation exists between IP₃ and ryanodine-sensitive Ca²⁺ 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 Ca²⁺ responses through a CICR mechanism because depletion of these stores affected the decay phase of the Ca²⁺ transients evoked either by ESS or high-[K⁺]₀, depolarization. In rat ORNs, a similar CICR mechanism also seems to amplify Ca²⁺ 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; Schilt 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 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+}], 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+}], 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 Schilt 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 adenylyl 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 (Liman 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 (Störtkuhl 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\(_3\) diazylated 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\(_3\) dialysis induces a cytotoxic Ca\(^{2+}\) increase that in turn could activate a Cl\(^{-}\) conductance. In this regard, Ca\(^{2+}\)-dependent Cl\(^{-}\) conductance is well characterized in ORNs and constitutes an important mechanism for boosting the initial membrane depolarization caused by the opening of CNG channels (Kleene 1993, 1997; Kleene and Gesteland 1991; Kura-hashi 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 Schild 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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