

Hypocretin/Orexin Peptide Signaling in the Ascending Arousal System: Elevation of Intracellular Calcium in the Mouse Dorsal Raphe and Laterodorsal Tegmentum

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Submitted 23 January 2004; accepted in final form 27 February 2004

Kohlmeier, Kristi A., Takafumi Inoue, and Christopher S. Leonard. Hypocretin/orexin peptide signaling in the ascending arousal system: elevation of intracellular calcium in the mouse dorsal raphe and laterodorsal tegmentum. *J Neurophysiol* 92: 221–235, 2004. First published March 3, 2004; 10.1152/jn.00076.2004. Dysfunction of the hypocretin/orexin (Hcrt/Orx) peptide system is closely linked to the sleep disorder narcolepsy, suggesting that it is also central to the normal regulation of sleep and wakefulness. Indeed, Hcrt/Orx peptides produce long-lasting excitation of arousal-related neurons, including those in the laterodorsal tegmentum (LDT) and the dorsal raphe (DR), although the mechanisms underlying these actions are not understood. Since Hcrt/Orx mobilizes intracellular calcium ($[Ca^{2+}]_i$) in cells transfected with orexin receptors and since receptor-mediated Ca^{2+} transients are ubiquitous signaling mechanisms, we investigated whether Hcrt/Orx regulates $[Ca^{2+}]_i$ in the LDT and DR. Changes in $[Ca^{2+}]_i$ were monitored by fluorescence changes of fura-2 AM loaded cells in young mouse brain slices. We found Hcrt/Orx (Orexin-A, 30–1,000 nM) evoked long-lasting increases in $[Ca^{2+}]_i$ with differing temporal profiles ranging from spiking to smooth plateaus. A fragment of Hcrt/Orx (16–33) failed to evoke changes in $[Ca^{2+}]_i$ and changes were not blocked by TTX or ionotropic glutamate receptor antagonists, suggesting they resulted from specific activation of postsynaptic orexin receptors. Unlike orexin receptor-transfected cells, Hcrt/Orx-responses were not attenuated by depletion of Ca^{2+} stores with cyclopiazonic acid (CPA; 3–30 μ M), thapsigargin (3 μ M), or ryanodine (20 μ M), although store-depletion by either CPA or ryanodine blocked Ca^{2+} mobilization by the metabotropic glutamate receptor agonist (\pm)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (*trans*-ACPD; 30 μ M). In contrast, Hcrt/Orx responses were strongly attenuated by lowering extracellular Ca^{2+} (\sim 20 μ M) but were not inhibited by concentrations of KB-R7943 (10 μ M) selective for blockade of sodium/calcium exchange. Nifedipine (10 μ M), inhibited Hcrt/Orx responses but was more effective at abolishing spiking than plateau responses. Bay K 8644 (5–10 μ M), an L-type calcium channel agonist, potentiated responses. Finally, responses were attenuated by inhibitors of protein kinase C (PKC) but not by inhibitors of adenylyl cyclase. Collectively, our findings indicate that Hcrt/Orx signaling in the reticular activating system involves elevation of $[Ca^{2+}]_i$ by a PKC-involved influx of Ca^{2+} across the plasma membrane, in part, via L-type calcium channels. Thus the physiological release of Hcrt/Orx may help regulate Ca^{2+} -dependent processes such as gene expression and NO production in the LDT and DR in relation with behavioral state. Accordingly, the loss of Hcrt/Orx signaling in narcolepsy would be expected to disrupt calcium-dependent processes in these and other target structures.

INTRODUCTION

The recently discovered hypothalamic peptides known both as the hypocretins and orexins (Hcrt/Orx) (de Lecea et al. 1998; Kilduff and Peyron 2000; Peyron et al. 1998; Sakurai et al. 1998) were originally shown to stimulate feeding (Sakurai et al. 1998). A remarkable collection of evidence has now linked impaired signaling by this peptide system to narcolepsy (Chemelli et al. 1999; Lin et al. 1999; Nishino et al. 2000; Peyron et al. 2000; Thannickal et al. 2000), a disabling sleep disorder in which features of REM sleep intrude into wakefulness. Those findings and others suggest that the Hcrt/Orx system regulates feeding and autonomic function and is crucial for the normal regulation of wakefulness and sleep (for review, see Kilduff and Peyron 2000; Taheri et al. 2002). Since expression of REM and wakefulness is controlled in part by the reticular activating system, which includes neurons of the laterodorsal tegmentum (LDT) and dorsal raphe (DR), it is significant that these structures receive Hcrt/Orx input (Peyron et al. 1998) and express Hcrt/Orx receptors (Marcus et al. 2001). Moreover, brain slice recordings indicate that Hcrt/Orx evokes prolonged excitation of neurons in these (Brown et al. 2001; Burlet et al. 2002) and other arousal related structures such as locus ceruleus (LC) (Horvath et al. 1999; Ivanov and Aston-Jones 2000; van den Pol et al. 2002), tuberomammillary nucleus (TM) (Eriksson et al. 2001), basal forebrain (BF) (Eggermann et al. 2001), and nonspecific thalamic nuclei (Bayer et al. 2002).

Hcrt/Orx might also regulate $[Ca^{2+}]_i$ in these structures since Hcrt/Orx elevates $[Ca^{2+}]_i$ in cells transfected with orexin receptors (Lund et al. 2000; Sakurai et al. 1998; Smart et al. 1999), cultured hypothalamic and spinal cord neurons (van den Pol 1999; van den Pol et al. 1998), and dissociated ventral tegmental neurons (Uramura et al. 2001). Indeed, we have recently shown that Hcrt/Orx induces rises in $[Ca^{2+}]_i$ in TM neurons (Willie et al. 2003). This effect may be an important general action of Hcrt/Orx since elevation of free $[Ca^{2+}]_i$ regulates key neuronal functions including gene expression, neurotransmitter release, plasticity, neuronal excitability, and enzyme activity (Berridge 1998). In LDT neurons, regulation of $[Ca^{2+}]_i$ appears of particular importance since the spike-evoked Ca^{2+} influx is regulated by neurotransmitters (Kohlmeier and Leonard 2002; Leonard et al. 2000), and these neurons express high levels of the Ca^{2+} -dependent enzyme NO

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synthase (Vincent and Kimura 1992). In fact, NO is produced locally by these neurons in a Ca^{2+} -influx dependent manner (Leonard et al. 2001) and at their axonal terminals in thalamus (Williams et al. 1997) where production varies with the sleep-wakefulness state. Because of the important role Ca^{2+} may play in nuclei involved in arousal systems and the demonstrated role Hcrt/Orex plays in arousal related dysfunctions, we have examined whether Hcrt/Orex stimulates changes in $[\text{Ca}^{2+}]_i$ in neurons within the LDT and DR.

Our data show that Hcrt/Orex elevates $[\text{Ca}^{2+}]_i$ in neurons of the LDT and DR and that these increases result from Ca^{2+} influx, in part, via L-type calcium channels rather than from store-release as suggested by studies with transfected cells and involve PKC rather than PKA-related mechanisms. Accordingly, these Ca^{2+} signals may function to couple the physiological release of Hcrt/Orex to vital regulatory processes within these key nuclei controlling behavioral state.

METHODS

Brain slices

Frontal brain slices were prepared from normal 6- to 17-day-old C57/Bl6 mice (Charles River Laboratories) as previously described (Burlet et al. 2002). All procedures complied with National Institutes of Health and institutional guidelines for ethical use of animals and were approved by NYMC. Briefly, animals were deeply anesthetized by inhalation of isoflurane and decapitated into ice-cold artificial cerebral spinal fluid (ACSF). The brain was rapidly removed, blocked, and sectioned at 250 μm with a vibratome (VT1000S, Leica). Slices containing the DR and LDT were incubated in ACSF at 36°C for 15 min and were stored in oxygenated ACSF at room temperature following this incubation. For recordings, slices were submerged in a chamber (volume: 1.4 ml) perfused at 1-1.7 ml/min with warmed ACSF (24-28°C), which was set on a fixed stage of an Olympus upright BX50WI microscope. This slow perfusion system had a larger volume and slower flow-rate than one previously used (Burlet et al. 2002). Hence, the time course of Hcrt/Orex actions measured here are slower and not precisely comparable with that previously measured.

Solutions

The standard ACSF solution for recording was gassed with 95% O_2 -5% CO_2 and contained (in mM) 121 NaCl, 5 KCl, 1.2 Na_2PO_4 , 2.7 CaCl_2 , 1.2 MgSO_4 , 26 NaHCO_3 , 20 dextrose, and 4.2 lactic acid. In some experiments, extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) was reduced to $\sim 20 \mu\text{M}$ (calculated with Patcher's Power Tools for Igor Pro) by adding 2.7 mM EGTA to the ACSF and elevating MgSO_4 to 3.9 mM. Tissue was incubated in low $[\text{Ca}^{2+}]_o$ solution for ≥ 20 min before test drug application.

Drugs

All drug-containing solutions were freshly prepared for the experiments. Hcrt/Orex (Orexin-A, Sigma or American Peptides) was diluted to 100 μM in distilled water and frozen. On the day of the experiment, minutes before the peptide was to be applied, the aliquot was unfrozen and diluted in ACSF to a final concentration of 300 nM, unless noted otherwise. Preliminary experiments indicated that following a 40-min washout of Hcrt/Orex, Ca^{2+} responses were reproducible with a second application of peptide. Therefore ≥ 40 min was the recovery time utilized for the present experiments. An amide fragment of Hcrt/Orex (Orexin-A, peptide sequence 16-33; Phoenix

Pharmaceuticals) was dissolved in normal ACSF and superfused at 300 nM or 1 μM as a control for nonspecific peptide actions. TTX (Alomone Labs) was dissolved in ACSF to a final concentration of 500 nM and applied to the slice for ≥ 10 min prior to the peptide to ensure complete block of voltage-dependent sodium channels. Cyclopiazonic acid (CPA, Calbiochem) aliquots (30 mM) were prepared in dimethyl sulfoxide (DMSO) and applied at a final concentration of 3-30 μM in ACSF and 500 nM TTX. Tissue was incubated in CPA/TTX for 15-30 min prior to test drug application to ensure block of the smooth endoplasmic reticulum calcium ATPase (SERCA) pump, and washout of this reversible inhibitor was ≥ 45 min. Thapsigargin (Calbiochem) was diluted in DMSO at a stock concentration of 3 mM and delivered in ACSF containing 500 nM TTX at a final concentration of 3 μM . Slices were incubated in this irreversible blocker of the SERCA pump for 30 min prior to application of Hcrt/Orex. Aliquots of ryanodine (Sigma) were prepared in DMSO at a stock concentration of 20 mM and applied in ACSF at a final concentration of 20 μM . KB-R7943 mesylate (Tocris), an inhibitor of the sodium/calcium exchanger, was dissolved in water, and aliquots were added to ACSF at a final concentration of either 80 or 10 μM . Nifedipine (Sigma) and (\pm)-Bay K 8644 (Calbiochem) were prepared fresh on the day of the experiment in DMSO and light protected until delivered to the slice at a final concentration of 10 and 5-10 μM , respectively. Bisindolylmaleimide I (Calbiochem) was used at a final concentration of 1 μM . Aliquots of 10 mM phorbol 12, 13-dibutyrate (Sigma) dissolved in DMSO were added to ACSF and delivered at a final concentration of 10 μM . 2',5'-Dideoxyadenosine (DDA; Calbiochem) was initially dissolved in DMSO and applied at a final concentration of 50 μM in ACSF for ≥ 30 min before Orexin. (\pm)-1-Aminocyclopentane-*trans*-1,3-dicarboxylic acid (t-ACPD, Tocris) was delivered in the ACSF at a final concentration of 30 μM for a total application time of 2 min.

Ca^{2+} imaging

DR and LDT cells were loaded with the Ca^{2+} indicator, fura-2 by incubating slices in ACSF containing 15 μM fura-2 AM (Molecular Probes) prepared from a 3.3 mM stock of fura-2 AM in DMSO. Slices were incubated for 30 min at 36°C in a small volume equilibrated with carbogen (5% CO_2 -95% O_2). Slices were transferred to the recording chamber and rinsed for ≥ 30 min to ensure fura-2 AM de-esterification and temperature equilibration. Localization of the DR and LDT was determined with a $4\times$ objective by brightfield illumination. Individual cells were then imaged using video-enhanced DIC optics using a $40\times$ water immersion lens (Olympus; NA 0.8). In the majority of experiments, Ca^{2+} transients were monitored by measuring the emission at 515 nm resulting from excitation of fura-2 with 380 nm (F_{380}) using the 71000 Chroma fura 2 filter set and a shuttered 75-W Xenon light source (Osram, Berlin-München, Germany). While Hcrt/Orex-induced changes recovered, and in many cases, showed fast rise and decay times, to verify that changes in F_{380} reflected peptide-induced activity and not changes in dye concentration, or optical path length, we utilized ratiometric measures (F_{340}/F_{380}) by manually altering excitation between 340 and 380 nm. These measures corroborated the F_{380} data. Optical recordings were made using a frame-transfer cooled CCD camera system (EEV 57 chip, Micromax System, Roper Scientific). Initially a full frame image (512×512 pixels) of the entire field that encompassed many filled cells was acquired and compared with full frame images taken under DIC-bright field illumination to identify putative neurons. Following this identification, another image was taken but binned on chip at 4×4 pixels. Regions of interests (ROIs) were selected to encompass identified cells and analysis of fluorescence was conducted. Images had exposures of 600 ms and were collected every 1-4 s. The cooled CCD camera was controlled by, and

the data were collected with custom software written by, one of the authors (T. Inoue) and run on a Mac OS computer.

Relation of $[Ca^{2+}]_i$ levels to changes in fluorescence

Changes in fluorescence (F) were measured as dF/F , where F is the fluorescence at rest within a ROI following subtraction of background fluorescence that was defined as fluorescence measured from a region of the slice without labeled cells. dF/F is the change in fluorescence following subtraction of F measured before stimulation. dF/F was corrected for bleaching that occurred during the run. Using fura-2, rises in Ca^{2+} are reflected as a decrease in F_{380} and an increase in F_{340} . dF/F ratios in all figures are presented such that upgoing traces represent rises in $[Ca^{2+}]_i$. Ratio measurements were not converted to absolute values of $[Ca^{2+}]_i$ due to several well known uncertainties (as discussed in Connor and Cormier 2000). Differences between means were determined by utilization of a paired Student's t -test or a one-way ANOVA. Nonparametric comparisons were conducted using χ^2 analysis.

Whole cell patch-clamp recordings

To verify that dF/F signals arose from neurons, we established whole cell patch recordings from fura-2-loaded cells that had first been identified as responding to Hcrt/Orex. Patch pipettes were fabricated from borosilicate glass (A-M Systems, 8250) and were filled with a solution containing (in mM) 144 K-gluconate, 0.2 EGTA, 3 $MgCl_2$, 10 HEPES, 0.3 NaGTP, and 4 Na_2ATP . Gigaseals were obtained under visual control using an Axoclamp 2A (Axon Instruments) operated in continuous voltage-clamp mode to monitor seal resistance (3-KHz output filter). After establishing whole cell recordings, the amplifier was switched to current-clamp mode (10-KHz output filter), and constant-current pulses were delivered to determine if the cells fired action potentials and were hence neurons. Pipettes also contained 50 μM of Alexa-594 (Molecular Probes) to visualize the recorded cell by using a Chroma 41004 Texas Red filter set.

RESULTS

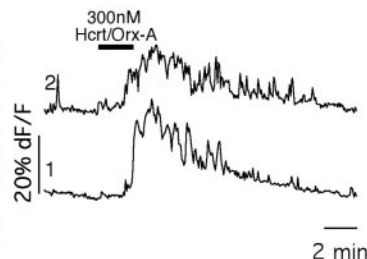
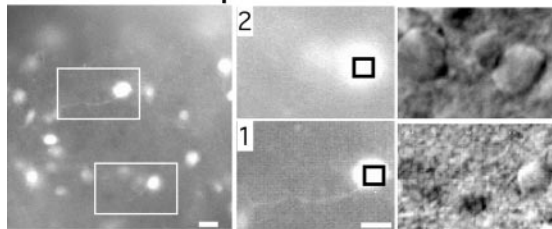
Hcrt/Orex elevated $[Ca^{2+}]_i$ in the DR and LDT

Changes in cellular fluorescence were monitored from regions determined to be in DR and LDT by initial visual inspection of the slices at low power ($4\times$ objective). Fura-2 loading of these slices resulted in a discrete pattern of cellular fluorescence readily observable using a $40\times$ objective (Fig. 1, A and B). Comparing DIC images with fluorescence images verified that the fluorescence arose from cells that appeared to be neurons based on their size and processes (~ 13 – $18\ \mu m$; Fig. 1, A and B). Application of Hcrt/Orex (Orexin-A, 300 nM) induced consistent, long-lasting but reversible changes in dF/F in many of these cells, suggesting that Hcrt/Orex evokes somatic elevations of $[Ca^{2+}]_i$ (Fig. 1, A and B). To control for possible alternative explanations of these fluorescence changes, we monitored fura-2 fluorescence ratiometrically (F_{340}/F_{380}) in a subset of cells before, during, and after this effect. Hcrt/Orex also reversibly increased the F_{340}/F_{380} ratios, strongly indicating that Hcrt/Orex stimulates rises in $[Ca^{2+}]_i$ in these cells (Fig. 1C).

Hcrt/Orex-induced rises in Ca^{2+} showed multiple temporal profiles

The transients induced by Hcrt/Orex varied from cell to cell but could be broadly categorized into three main profiles: 1) "Plateau" responders had relatively slow rise times (10–90% rise time, 90.0 ± 11.3 s, 2 DR slices, $n = 5$, 3 LDT slices, $n = 6$) that rose to a peak and returned to baseline over the course of several minutes; 2) "Spiker" responders had shorter transients with more rapid onsets (10–90% rise time, 11.2 ± 3.5 s, 2 DR slices $n = 4$, 2 LDT slices, $n = 4$), and shorter durations with faster recovery times (seconds) that exhibited a burst or

A Dorsal Raphe



B LDT

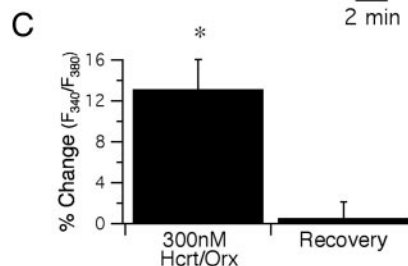
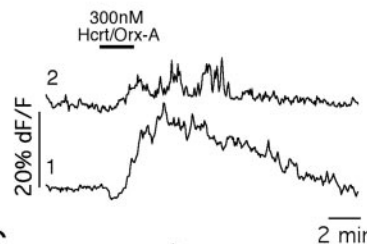
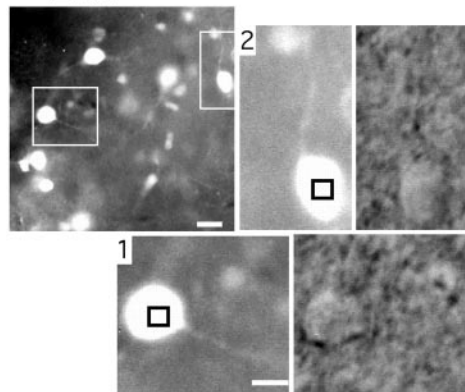


FIG. 1. Hypocretin/orexin (Hcrt/Orex) induced $[Ca^{2+}]_i$ rises in both the dorsal raphe (DR) and the lateral dorsal tegmental (LDT) nucleus. A and B: left: full frame image of fura-2-loaded cells (F_{380}) within the DR and LDT from which fluorescent changes were monitored. White boxes indicate the regions shown expanded to right. F_{380} of fura-2-loaded cells are shown in the middle panels, and the same cells are shown under bright field DIC conditions in right panels. Regions of interest (ROI) from which fluorescence signals were averaged are indicated by squares over the somata of these cells. Hcrt/Orex-induced $[Ca^{2+}]_i$ rises are reflected by changes in dF/F as indicated in the right panels. C: histogram of ratiometric measures (F_{340}/F_{380}) showing the percent response following Hcrt/Orex application normalized to the baseline level of $[Ca^{2+}]_i$. Data corroborate the single wavelength measures and indicate that Hcrt/Orex elevates $[Ca^{2+}]_i$. Scale bars are 20 μm on full frame images and 10 μm on expanded images.

oscillation behavior arising from a more stable baseline; and 3) "Plateau/Spiker" responders displayed a combination of the others in that briefer transients arose from a slower rising baseline. Figure 2, *A* and *B*, shows the heterogeneity of these Hcrt/Orx responses observed within single image fields in the DR and LDT. Response latency from the onset of Hcrt/Orx exposure was also variable (typical range: 2.7–5 min) but did not appear to be correlated with response profile. Different response types were often found to co-exist within the same field along with cells that failed to respond. Although we did not explicitly examine this issue due to differences in focal planes of each cell in relation to the chosen best imaged focal plane, our impression was that spiker responses appeared more prevalent among smaller cells. The peak amplitudes of transients exhibited a wide range; however, the average change in dF/F measured from DR cells displaying plateau responses was $13.5 \pm 1.1\%$ (32 DR slices, $n = 199$) and from the LDT was $11.6 \pm 1.0\%$ (24 LDT slices, $n = 155$; $P > 0.05$). We also found that the effects of Hcrt/Orx were repeatable after allowing a recovery time of 40–60 min between applications ($P >$

0.05, 2 DR slices, $n = 4$, 3 LDT slices, $n = 6$). In some cases, imaging from proximal dendrites was also possible, and there, Hcrt/Orx induced changes in dF/F as well with similar kinetics to those seen in the soma (Fig. 2*C*).

We determined the percentage of cells that responded to Hcrt/Orx in each nucleus and the type of response elicited by counting the number of cells identified as such with DIC optics in each nucleus at the imaged focal plane and determining the number of these cells that responded to Hcrt/Orx. This examination revealed that Hcrt/Orx induced rises in $[Ca^{2+}]_i$ in 68.7% of DIC-identified cells in the DR (8 slices, 33/48) and 65.7% of the DIC-identified cells (11 slices, 50/76) in the LDT. The frequency of encountering each response type in the DR and LDT is summarized in Fig. 2*C* and indicates that each response type occurred with a similar frequency in both the DR and LDT.

Hcrt/Orx actions were dose-dependent and specific

Previous experiments suggested that maximum Hcrt/Orx-induced excitation of LDT neurons occurred at 300 nM (Burlet

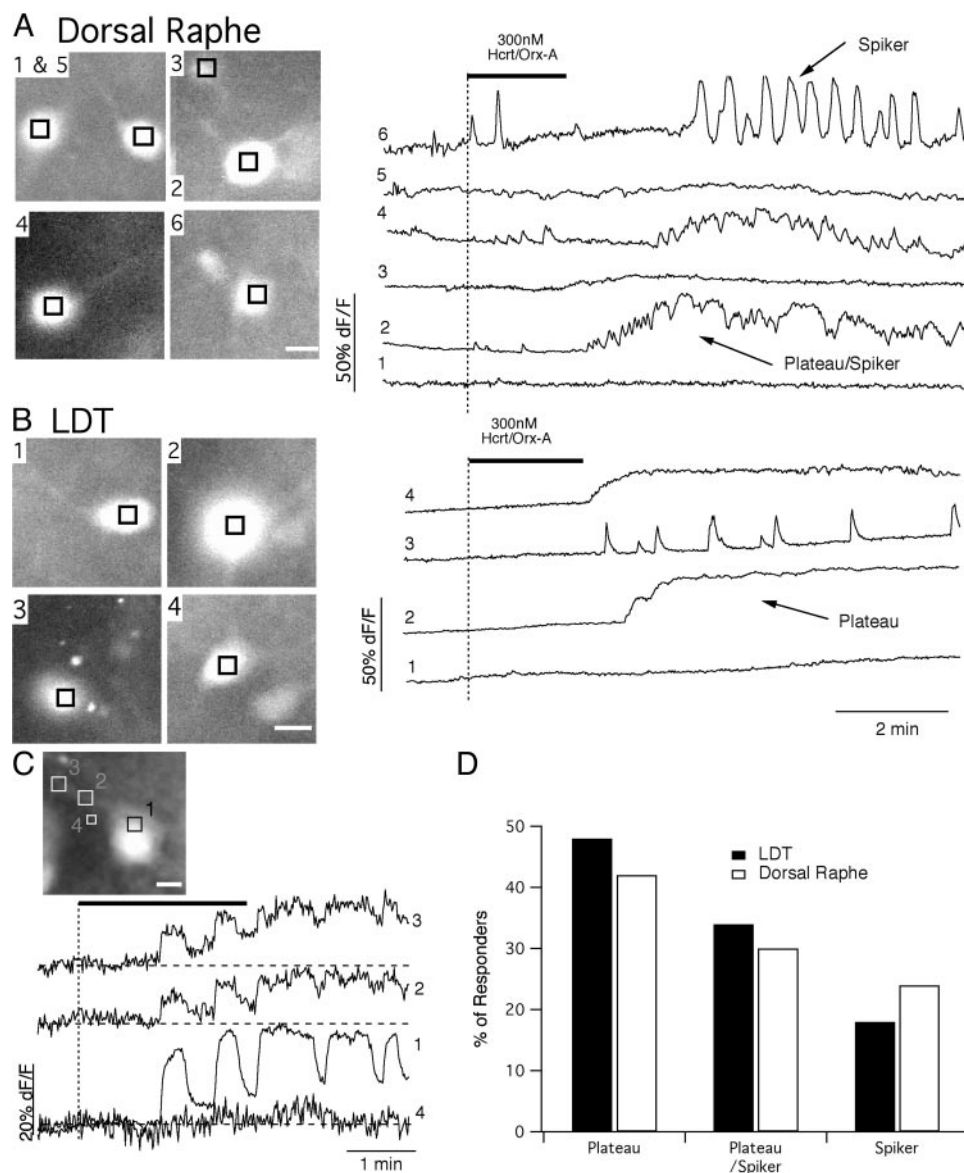


FIG. 2. Hcrt/Orx-induced $[Ca^{2+}]_i$ rises exhibited multiple temporal profiles in both the DR and LDT. *F*₃₈₀ images of cells within the (A) DR and (B) LDT from which changes in fluorescence were monitored. Numbers by fluorescence images correspond to each numbered dF/F trace on right, which reflects changes in fluorescence within the ROIs. Responses to Hcrt/Orx could be broadly categorized into "Plateau," "Spiker," or Plateau/Spiker" as indicated. *C*: dF/F traces showing that spiking responses also occurred in dendrites and that the transients recorded at dendrites exhibited similar kinetics to those recorded at the soma. Lack of response in ROI 4 that is adjacent to the soma and dendrite indicates the similarity of the dendritic signals is not due to scatter from the soma. *D*: histogram comparing response types between the DR and LDT reveal that the likelihood of Hcrt/Orx eliciting a particular response type was similar in each nucleus. Scale bar = 10 μ m and applies to each image group.

et al. 2002). We therefore examined concentrations of Hcrt/Orx ranging from 3–1,000 nM. We found that 30 nM produced just barely detectable fluorescence changes ($0.1 \pm 0.1\%$, 2 raphe slices, $n = 4$, 1 LDT slice, $n = 3$), while 1 μM produced the largest responses (Fig. 3). We also found that response profile was stable across a wide range of Hcrt/Orx concentrations. For example, we never found a case in which a plateau response turned into a transient response at higher doses over the range of 100 nM–1 μM (2 DR slices, $n = 5$, 3 LDT slices, $n = 5$). These data suggest that the different response types were not simply due to ligand concentration differences between cells.

To verify that the observed changes in $[\text{Ca}^{2+}]_i$ required the functional Hcrt/Orx peptide, we examined the changes in dF/F produced by the amide fragment (16–33) of Hcrt/Orx on eight DR and LDT cells (2 DR slices, $n = 4$, 2 LDT slices, $n = 4$). The fragment had no effect, but subsequent application of the full peptide produced robust responses ($19.2 \pm 3.7\%$, $n = 8$; Fig. 3D). We therefore conclude that increases of $[\text{Ca}^{2+}]_i$ following Hcrt/Orx application do not result from nonspecific peptide actions.

Hcrt/Orx-induced rises in Ca^{2+} persisted in TTX and ionotropic glutamate receptor antagonists

Since Hcrt/Orx application evokes prolonged repetitive firing in LDT (Burlet et al. 2002) and DR neurons (Brown et al. 2001), we tested whether the changes in dF/F produced by Hcrt/Orx were sensitive to action potential blockade by TTX (0.5 μM). In the first group of cells studied (9 DR slices $n = 33$, 5 LDT slices $n = 26$), we found that all three response profiles were encountered in the presence of TTX, suggesting that none of these profiles required sodium-dependent action potentials. Moreover, the likelihood of encountering each type of response was not different in TTX.

In a separate population of cells, we also compared the effect of Hcrt/Orx applied after action potentials were blocked by TTX and again following 1 h of wash out of TTX, which was sufficient for recovery of action potentials as determined in separate experiments. Using plateau responses which were most easily quantifiable, we found that the response increased from 10.3 ± 0.4 to $13.5 \pm 0.3\%$ following washout of TTX ($P < 0.05$, 3 DR slices, $n = 15$, 3 LDT slices, $n = 20$; Fig. 4). TTX also had minor effects on spiking and plateau/spiking responses indicating that action potential-evoked Ca^{2+} influx contributed little to the elevation of $[\text{Ca}^{2+}]_i$ we measured following Hcrt/Orx application.

Since Hcrt/Orx evokes glutamate release in the LDT (Burlet et al. 2002) and activation of ionotropic glutamate receptors elevates $[\text{Ca}^{2+}]_i$ in LDT neurons (Leonard et al. 2001), we examined whether activation of ionotropic glutamate receptors was involved with the Hcrt/Orx-induced Ca^{2+} transients. We found that in the presence of blockers of AMPA and *N*-methyl-D-aspartate (NMDA) receptors, the Hcrt/Orx responses were not different from those elicited under control conditions in the same cells. Control responses in TTX were $10.2 \pm 2.2\%$ dF/F and responses in the presence of APV/CNQX/TTX were $10.2 \pm 2.0\%$ dF/F ($P > 0.05$; 1 DR slice, $n = 3$, 2 LDT slices $n = 7$; data not shown). Additionally, all three response types were found to be elicited in the presence of APV/CNQX/TTX. Thus Hcrt/Orx-induced $[\text{Ca}^{2+}]_i$ rises were not mediated by the activation of ionotropic glutamate receptors. Collectively,

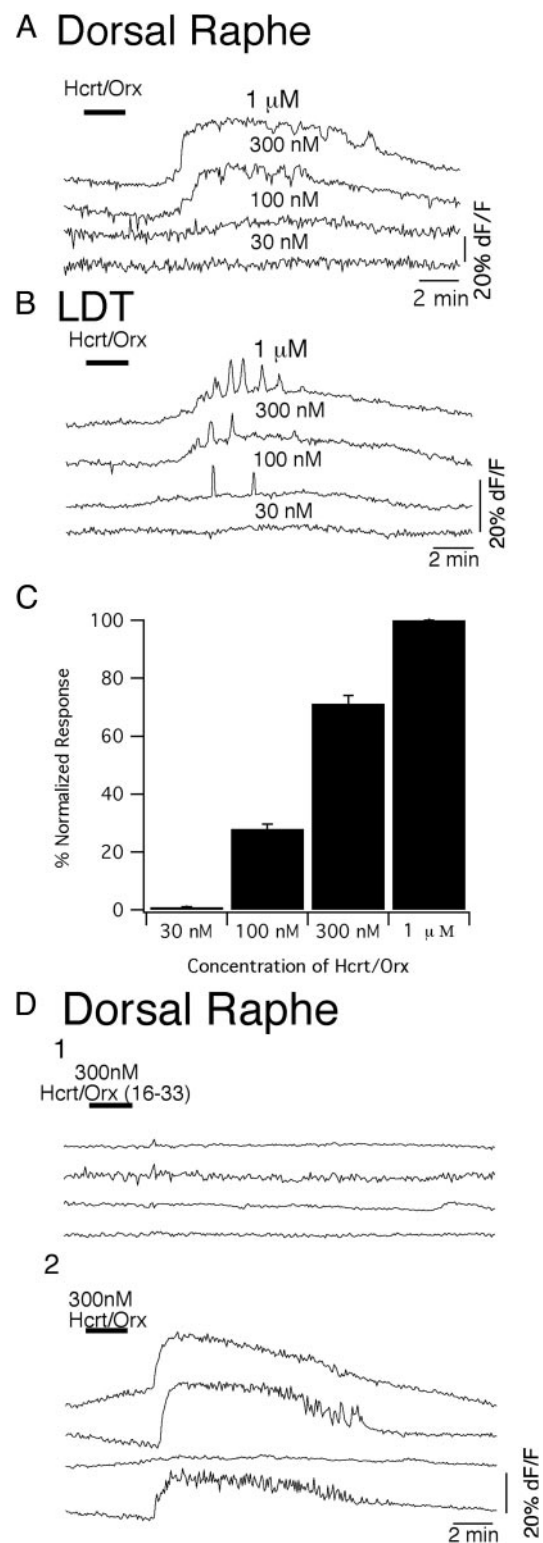


FIG. 3. Hcrt/Orx-induced responses were dose-dependent and were not elicited by an Hcrt/Orx amide fragment. *A* and *B*: responses of 2 different cells to 4 concentrations of Hcrt/Orx. *A* shows that the response category appeared independent of the concentration of Hcrt/Orx. For example, cells that exhibited a plateau response at 100 nM also exhibited plateau responses at 300 nM and 1 μM (*A*). (*C*) Histogram detailing the response of DR and LDT cells to 4 concentrations of Hcrt/Orx. Responses are normalized to the response elicited at 1 μM . (*D1*) An amide fragment of Hcrt/Orx (Hcrt/Orx (16–33)) did not elicit changes in dF/F in DR or LDT cells in which subsequent applications of Hcrt/Orx did elicit changes (*D2*).

LDT

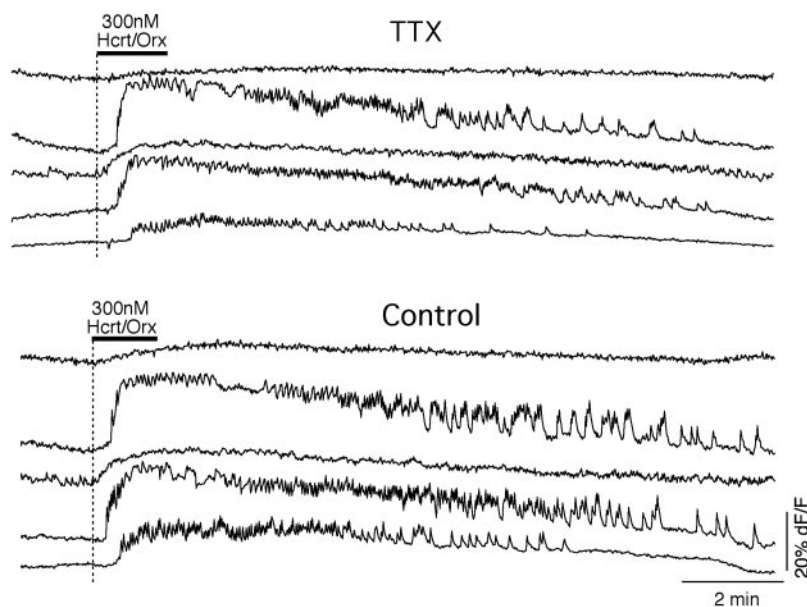


FIG. 4. All response categories were still evident following the blockade of voltage-gated sodium channels with TTX. Hcrt/Orx-evoked changes in dF/F were similar during spike blockade with TTX (top) and after washout of TTX. However, note that while 4 of the 5 cells that responded to Hcrt/Orx in control artificial cerebrospinal fluid (ACSF) also responded in TTX, the response was reduced in amplitude and spikes were reduced in amplitude and frequency during the blockade.

these data indicate Hcrt/Orx elevates $[Ca^{2+}]_i$ independently of TTX-sensitive action potentials and ionotropic glutamate receptors and suggest that these rises in $[Ca^{2+}]_i$ result from a direct action of Hcrt/Orx on the imaged cells.

Hcrt/Orx-induced rises in $[Ca^{2+}]_i$ were resistant to depletion of intracellular Ca^{2+} stores

To determine if these Hcrt/Orx-induced rises in $[Ca^{2+}]_i$ involved the release of Ca^{2+} from intracellular stores, as observed in heterologous expression systems (Sakurai et al. 1998; Smart et al. 1999), we depleted intracellular Ca^{2+} stores with the SERCA pump inhibitors, thapsigargin (3 μ M), or the reversible cyclopiazonic acid (CPA; 3–30 μ M). Continuous application of thapsigargin or CPA in the presence of TTX produced a transient rise in dF/F , suggesting depletion of Ca^{2+} from SERCA pump-dependent intracellular stores was successful (latency: 85 ± 4.2 s). Nevertheless, all three response profiles observed prior to depletion were observed in the presence of CPA or thapsigargin. Indeed, the average amplitude of the plateau response ($12.3 \pm 2.3\%$) was not different from that in control conditions ($12.5 \pm 2.6\%$; 4 DR slices, $n = 12$, 4 LDT slices, $n = 13$; $P > 0.05$; Fig. 5, A, C, and D).

To verify that CPA successfully depleted SERCA pump-dependent stores, we explored the possibility that metabotropic glutamate receptors (mGluR) trigger Ca^{2+} release from intracellular stores as described elsewhere (Conn and Pin 1997; Nicoletti et al. 1986; Sladeczek et al. 1985). Indeed, we found that activation of mGluRs with t-ACPD induced robust rises in $[Ca^{2+}]_i$ in the LDT (Fig. 5B). Moreover, in contrast to the persistence of Hcrt/Orx actions following treatment with CPA, the responses elicited by t-ACPD were completely abolished by CPA (Fig. 5B) and thapsigargin (6 LDT slices, $n = 36$). These data strongly indicate that CPA and thapsigargin effectively depleted SERCA-pump-dependent pools of $[Ca^{2+}]_i$.

While the data indicate that Hcrt/Orx-induced Ca^{2+} transients in the CNS are SERCA pump independent, it has been

reported in other cell types that calcium-induced calcium-release (CICR) channels (ryanodine receptors) can trigger Ca^{2+} release from stores that are resistant to depletion by thapsigargin or CPA (Golovina and Blaustein 1997; Murphy and Miller 1989; Thayer et al. 1988) suggesting that in some cell types, SERCA pump and ryanodine receptor-mediated stores are separate (Murphy and Miller 1989; Thayer et al. 1988). We therefore examined the possibility that Hcrt/Orx-induced Ca^{2+} rises were influenced by ryanodine receptors (calcium-induced calcium release channels) located on thapsigargin/CPA-insensitive stores. Responses to Hcrt/Orx were examined following preincubation of the slices in 20 μ M ryanodine that blocks the ryanodine receptor in the open configuration (Rousseau et al. 1987). Application of ryanodine alone did not cause detectable changes in fluorescence, as has been previously reported (McPherson et al. 1991). In the presence of ryanodine, each response type previously observed following Hcrt/Orx application were also observed, and there was no significant difference in the amplitude of the plateau responses observed in control ($18.2 \pm 4.5\%$) and ryanodine conditions ($23.1 \pm 7.6\%$; $P > 0.05$; 2 DR slices, $n = 10$, 2 LDT slices, $n = 14$; Fig. 6A), indicating that CICR is not necessary for Hcrt/Orx-induced Ca^{2+} transients.

Since ryanodine receptors might be located on SERCA-pump dependent stores and since mGluR receptor-mediated store release may rely on ryanodine receptors (Fagni et al. 2000), we also examined the effectiveness of ryanodine in preventing t-ACPD induced Ca^{2+} rises. Preincubation of slices in 20 μ M ryanodine strongly attenuated the t-ACPD induced Ca^{2+} rise by 89.1% (3 LDT slices, $n = 9$; Fig. 6B). Thus ryanodine receptor-mediated store depletion was successful in this preparation, indicating that Hcrt/Orx mediated rises did not depend on ryanodine receptors. Moreover, the failure of t-ACPD to mobilize $[Ca^{2+}]_i$ in the presence of CPA, thapsigargin and its significant attenuation in the presence of ryanodine suggest that IP_3 and ryanodine receptors are located on the same intracellular Ca^{2+} store(s) in LDT cells as has been

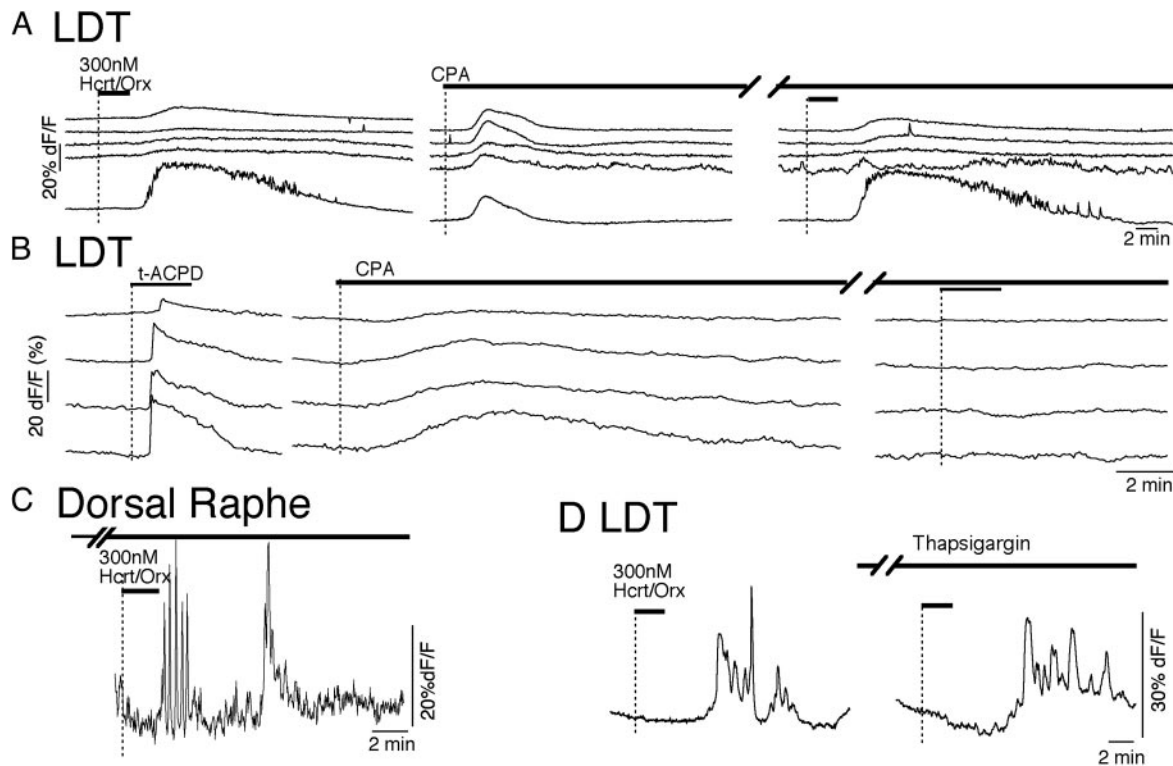


FIG. 5. Depletion of intracellular Ca^{2+} stores with CPA or thapsigargin did not attenuate Hcrt/Orx-induced rises in $[\text{Ca}^{2+}]_i$. A: dF/F values for 5 cells from the same field are displayed following Hcrt/Orx application, prior to (left) and following CPA application (right). Rises in dF/F were seen following CPA application (middle). B: CPA effectively depleted intracellular Ca^{2+} stores since t-ACPD-mediated mobilization of Ca^{2+} from these stores was abolished by CPA. C: baseline spiking was also present in CPA-store depleted conditions. D: responses to Hcrt/Orx were not attenuated following depletion of intracellular stores of Ca^{2+} by thapsigargin. Thus smooth endoplasmic reticulum calcium ATPase (SERCA) pump-mediated intracellular Ca^{2+} stores are not involved in Hcrt/Orx induced $[\text{Ca}^{2+}]_i$ rises. TTX was present throughout these experiments.

described in other neurons (Nakamura et al. 1999; Seymour-Laurent and Barish 1995).

Hcrt/Orx-induced rises in $[\text{Ca}^{2+}]_i$ were nearly abolished by lowering extracellular $[\text{Ca}^{2+}]_o$

To determine if Hcrt/Orx induced rises required external Ca^{2+} , we compared responses in control and low extracellular Ca^{2+} ($[\text{Ca}^{2+}]_o$) conditions. At a calculated $[\text{Ca}^{2+}]_o$ of $20 \mu\text{M}$, we found that all three profiles of Hcrt/Orx-induced responses were significantly attenuated from those elicited in the same DR and LDT cells under control conditions (Fig. 7, A and B). The average plateau response was reduced by $83.8 \pm 0.7\%$ from that seen in control conditions ($P < 0.05$, 9 DR slices, $n = 17$, 5 LDT slices, $n = 18$; Fig. 7C). Moreover, spiking responses observed in control conditions were abolished in low $[\text{Ca}^{2+}]_o$ conditions in the same cells. Thus lowering the $[\text{Ca}^{2+}]_o$ strongly attenuated the response which recovered to near 100% following reintroduction of $[\text{Ca}^{2+}]_o$ ($P > 0.05$, 1 DR slice, $n = 2$, 1 LDT slice, $n = 1$; Fig. 7A).

Hcrt/Orx-induced rises in Ca^{2+} appear independent of the sodium/calcium exchanger

A possible explanation for the dependence on $[\text{Ca}^{2+}]_o$ is that the Hcrt/Orx receptor(s) couple to the sodium/calcium exchanger (NCE). Indeed, it has recently been reported that a drug that interferes with the NCE attenuates Hcrt/Orx-induced

inward currents (Burdakov et al. 2003; Eriksson et al. 2001; Wu et al. 2002). While forward operation of the pump that would generate an inward current and extrude Ca^{2+} from the cell could not be responsible for Hcrt/Orx-induced responses observed here, it is possible that reverse operation of the pump could contribute to the observed responses. Therefore we examined the effect of the NCE inhibitor, KB-R7943, at two concentrations on responses to Hcrt/Orx. We found that at $10 \mu\text{M}$, a concentration reported to be at the high end of specificity of inhibition of the pump (Iwamoto et al. 1996), all three response types to Hcrt/Orx were similar to those seen in control conditions (plateau response in $10 \mu\text{M}$ KB-R7943, $98.9 \pm 1.0\%$ of control, $P > 0.05$, 1 DR slice, $n = 2$; 2 LDT slices, $n = 4$, Fig. 8). At $80 \mu\text{M}$, responses to Hcrt/Orx were significantly attenuated (plateau response, $49.8 \pm 0.5\%$ of control, $P < 0.05$, 4 DR slices, $n = 4$, 3 LDT slices, $n = 4$); however, at this concentration the drug is probably not acting specifically (Iwamoto et al. 1996). Hence, these data suggest that the Hcrt/Orx-induced calcium influx in DR and LDT cells does not depend on operation of the NCE.

Hcrt/Orx-induced rises in $[\text{Ca}^{2+}]_i$ involve L-type calcium channels

Antagonists of L-type calcium channels have been reported to reduce the Hcrt/Orx evoked increase in $[\text{Ca}^{2+}]_i$ in ventral tegmental neurons (Uramura et al. 2001). Since L-type channels are present on LDT neurons (Kohlmeier and Leonard

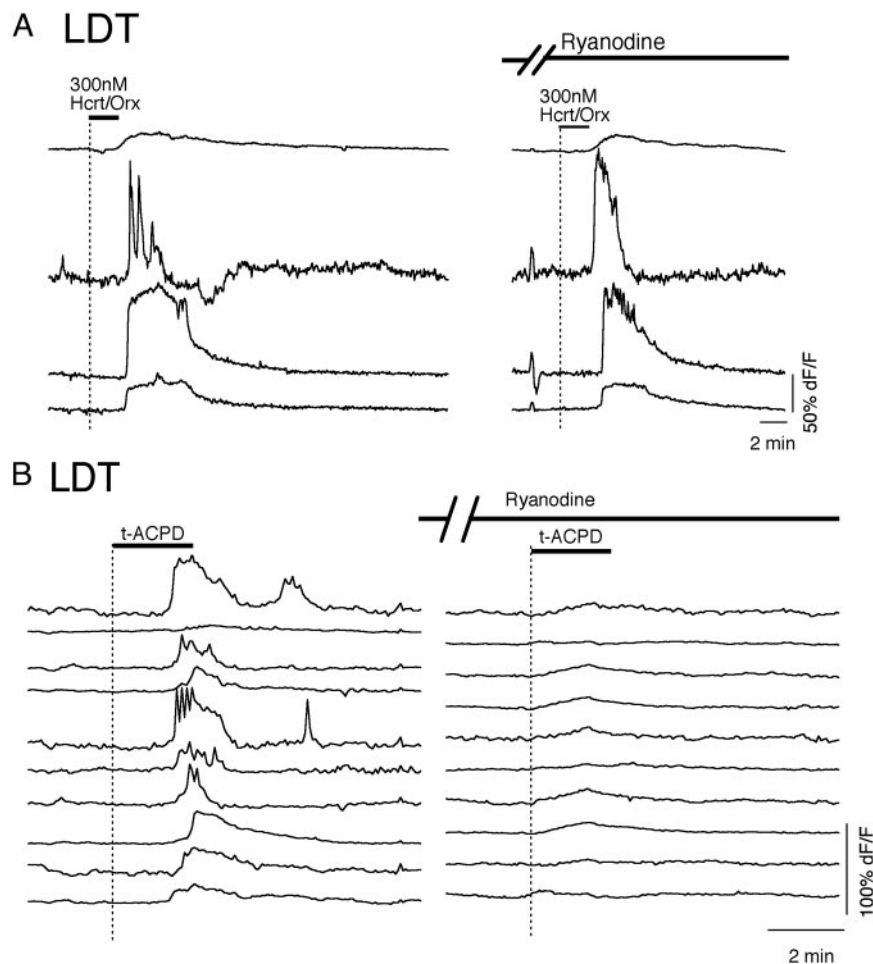


FIG. 6. Blockade of the ryanodine receptor did not inhibit Hcrt/Orx responses. *A*: control responses (*left*) were not attenuated after application of ryanodine (*right*). *B*: in contrast, control responses to t-ACPD (*left*) were nearly abolished (*right*) by application of ryanodine. Thus Hcrt/Orx-induced rises in $[Ca^{2+}]_i$ were not dependent on mobilization of Ca^{2+} from these intracellular stores. TTX was present throughout these experiments.

2002) and DR neurons (Penington et al. 1991), we examined the effects of nifedipine, an L-channel inhibitor on responses induced by Hcrt/Orx in DR and LDT neurons. Ten micromolar nifedipine attenuated Hcrt/Orx-induced plateau responses to $44.5 \pm 5.6\%$ of control ($P < 0.05$; 8 DR slices, $n = 16$; 6 LDT slices, $n = 12$; Fig. 9) and abolished ($n = 13/22$) or attenuated ($n = 5/22$) the majority of spiker responses (Fig. 9A). Since nifedipine was more effective at abolishing baseline spiking than plateau responses ($P < 0.05$; χ^2 test), it appears L-type calcium channels play a greater role in generating Hcrt/Orx-induced spiking than plateau responses. Nevertheless, nifedipine-insensitive spontaneous spiking was also observed in some cells, suggesting that nifedipine-sensitive channels are not necessary for spiking behavior in some cells.

Exposure of the slice to 5–10 μM Bay K 8644, an agonist of L-type calcium channels, increased Hcrt/Orx-induced Ca^{2+} transients significantly in a subpopulation of cells ($n = 15/21$). In these cells, the response was increased from control by $20.1 \pm 3.5\%$ (4 DR slices, $n = 8$; 3 LDT slices, $n = 7$; $P < 0.05$). In addition, Bay-K 8644 alone sometimes induced spiking that was independent of Hcrt/Orx application (Fig. 9C), and in some cases, this spiking obscured the possible effects of Hcrt/Orx (Fig. 9B). Taken together, these data indicate that L-type calcium channels participate in Hcrt/Orx-evoked plateau responses and play an even more important role in Hcrt/Orx-evoked spiking.

To investigate the possibility that Hcrt/Orx-mediated depolarization alone could account for the Ca^{2+} transients, we monitored the response of DR and LDT neurons to bath application of 1 μM AMPA, which produces greater inward somatic current in LDT neurons than does Hcrt/Orx (compare Burlet et al. 2002; Sanchez and Leonard 1996) and evokes repetitive firing in quiescent DR and LDT neurons (data not shown). AMPA produced an increase in somatic $[Ca^{2+}]_i$ that was completely abolished by TTX, indicating that the Ca^{2+} signal resulted from a spike-evoked Ca^{2+} influx rather than the AMPA-mediated depolarization ($n = 8$). In contrast, in the presence of TTX, Hcrt/Orx evoked both plateau and spiker responses in these same cells (Fig. 9C₃). These data suggest that depolarization alone is not the mechanism by which Hcrt/Orx induces somatic Ca^{2+} transients.

Hcrt/Orx-induced rises in $[Ca^{2+}]_i$ depend in part on PKC

Hcrt/Orx receptors have been shown to stimulate PLC (Lund et al. 2000). Because the $[Ca^{2+}]_i$ rises in the LDT and DR did not appear to be dependent on IP_3 -mediated release from intracellular stores, we examined the DAG branch of the PLC pathway using two different PKC inhibitors: bisindolylmaleimide I and chelerythrine chloride. We found that these inhibitors significantly attenuated all types of Hcrt/Orx-evoked Ca^{2+} rises (range of reduction: 100–11%; mean reduction: $54.8 \pm 0.9\%$, $P < 0.05$, 9 DR slices, $n = 20$, 7 LDT slices, $n = 17$; Fig. 10). Since bisindolylmaleimide I also has been re-

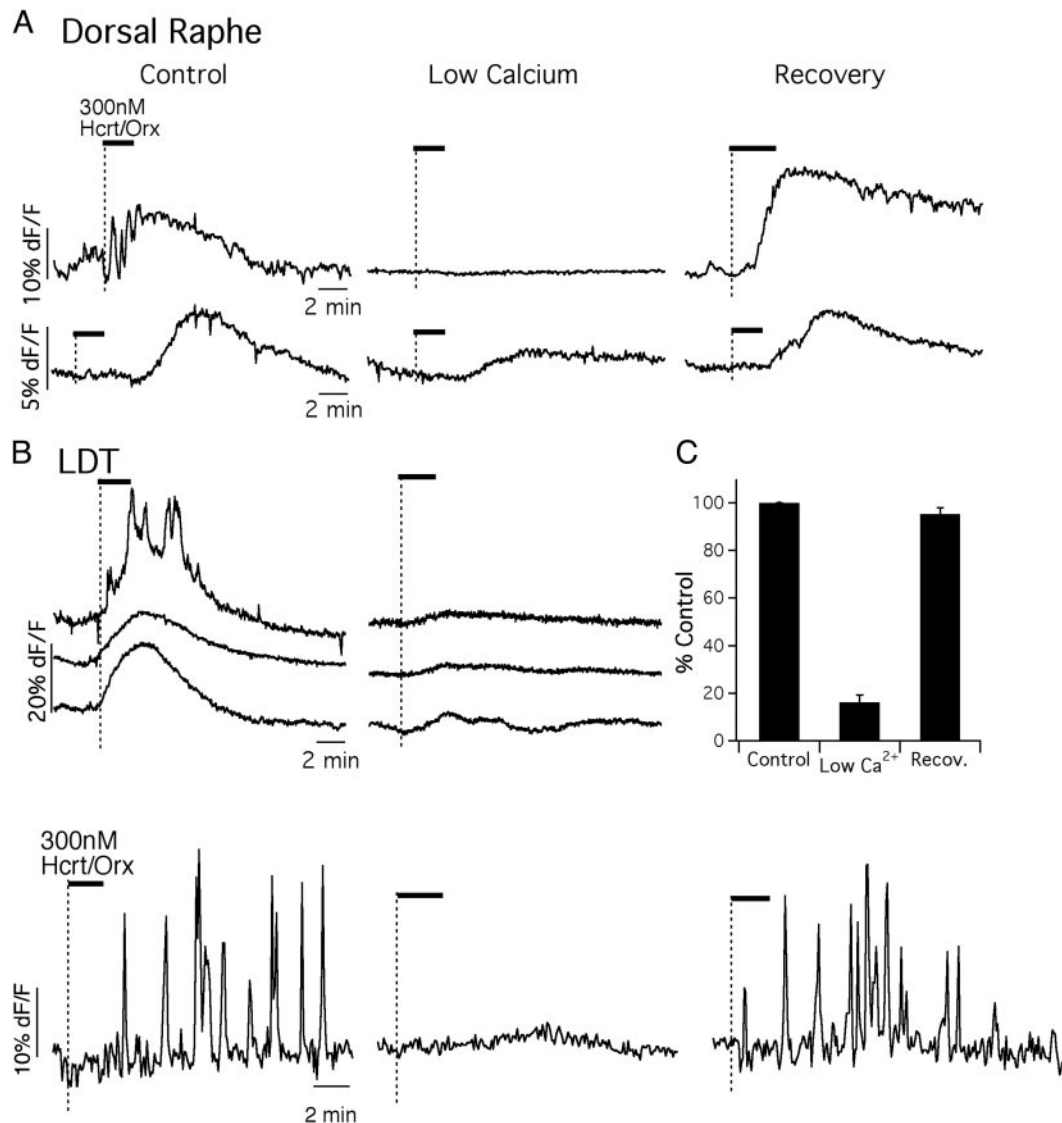


FIG. 7. Hcrt/Orx-induced rises in $[Ca^{2+}]_i$ were dependent on influx of Ca^{2+} from the extracellular space. A: control responses in the DR (left) were significantly attenuated by low Ca^{2+} ACSF (middle). Recovery of responses occurred following return to normal Ca^{2+} ACSF (right). B: Hcrt/Orx-induced responses in LDT cells were also attenuated in low Ca^{2+} ACSF. Data from 2 different slices show that low Ca^{2+} ACSF attenuated responses for all categories of responders. C: summary histogram showing the percent change in fluorescence in control solution, low Ca^{2+} solution, and following washout of low Ca^{2+} solution normalized to responses obtained in control conditions. Data indicate that Hcrt/Orx-induced $[Ca^{2+}]_i$ rises depend on extracellular Ca^{2+} influx.

ported to have weak inhibitory effects on PKA (Toullec et al. 1991), we examined the effect of adenylyl cyclase inhibition by 2'5'dideoxyadenosine (DDA) as a control. In eight cells in which Hcrt/Orx was found to induce rises in $[Ca^{2+}]_i$, responses to Hcrt/Orx were not significantly attenuated in the presence of DDA (plateau response control amplitude, 13.9 ± 4.4 vs. $13.5 \pm 4.4\%$ in DDA, 2 DR slices, $n = 3$, 2 LDT slices, $n = 5$, $P > 0.05$, Fig. 10). Cells exhibiting the spiker response also appeared unaffected. Supporting a role of PKC mechanisms in Hcrt/Orx-induced rises in $[Ca^{2+}]_i$, we found that application of a phorbol ester induced patterns of Ca^{2+} rises similar to those elicited by Hcrt/Orx ($n = 23$; Fig. 10D).

To examine whether actions of Hcrt/Orx on rises in calcium mediated by L-type calcium channels were independent of PKC, we examined the effects of Hcrt/Orx when applied in the presence of nifedipine and bisindolylmaleimide I. Hcrt/Orx applied in the presence of these two inhibitors induced a

response that was reduced by $52.9 \pm 8.4\%$ from control ($n = 15$), which was not different from the reduction induced by application of Hcrt/Orx in the presence of either nifedipine or bisindolylmaleimide I ($P > 0.05$, ANOVA; Fig. 10E). Since the Hcrt/Orx response was reduced by about the same amount with both blockers as with each alone, these data are consistent with the activation of L-channels being dependent on PKC. While we only observed partial inhibition with PKC inhibitors, which may indicate incomplete access to cells in the slice or involvement of non-PKC mechanisms (Smart et al. 1999), collectively, the data suggest involvement of PKC but not PKA in Hcrt/Orx-induced $[Ca^{2+}]_i$ increases,

Hcrt/Orx elevated $[Ca^{2+}]_i$ in neurons rather than glia

To verify that Hcrt/Orx-induced changes in fluorescence arose from neurons rather than glia, we patch-clamped 14

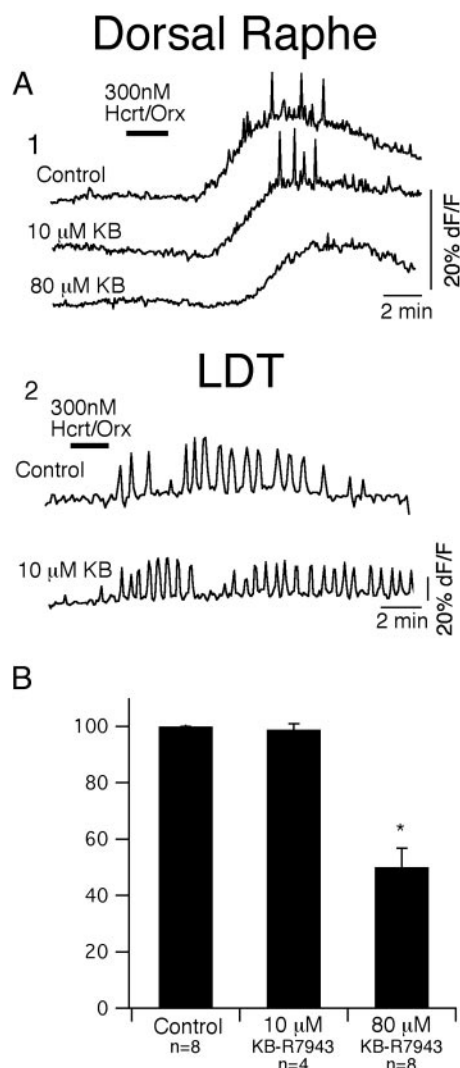


FIG. 8. Selective inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCE) did not attenuate Hcrt/Orexin-induced rises in $[\text{Ca}^{2+}]_i$. *A1*: Hcrt/Orexin responses in control conditions and with 2 concentrations of KB-R7943, an inhibitor of NCE. At 10 μM , a concentration reported to specifically inhibit the NCE, Hcrt/Orexin responses, including "spiker" responses (*A2*), were not significantly different from control. At the higher concentration, which has been reported to have nonspecific effects, Hcrt/Orexin responses were partially inhibited. *B*: histogram summarizes the effect of these two KB-R7943 concentrations on Hcrt/Orexin-induced changes in $[\text{Ca}^{2+}]_i$ that are normalized to control responses.

fura-2AM loaded cells that exhibited Hcrt/Orexin induced transients (4 DR slices, $n = 5$; 7 LDT slices, $n = 9$) and 2 that did not respond. Since fura-2 fluorescence rapidly washed out of recorded cells after establishing the whole cell configuration, we included the dye Alexa-594 in the pipettes so that recorded cells could be visualized while recording. In all cases, depolarizing current steps (100- to 500-ms duration) evoked one or more overshooting action potentials (peak $> +10$ mV), indicating the imaged cells were neurons (Fig. 11).

DISCUSSION

The chief finding of this study is that Hcrt/Orexin produces a dose-dependent elevation of $[\text{Ca}^{2+}]_i$ in significant numbers of neurons in both the LDT and DR—two nuclei thought to play important roles in behavioral state control. Interestingly, we

found for the first time that Hcrt/Orexin elicits Ca^{2+} transients having multiple temporal patterns, ranging from repetitive spikes to smooth plateaus. Neurons responding with these different patterns were encountered in both nuclei at about the same frequency. Moreover, each pattern was independent of SERCA pump-filled stores, depended on extracellular Ca^{2+} , and involved L-type calcium channels. These actions were clearly different from those produced by the metabotropic glutamate receptor agonist t-ACPD that evoked Ca^{2+} transients that were abolished by store depletion. Finally, since these Hcrt/Orexin actions were attenuated by inhibitors of PKC but not by inhibitors of adenylate cyclase, and since concentrations of KB-R7943 that selectively inhibit Na/Ca exchange did not inhibit these responses, our data indicate that each was probably mediated by PKC-sensitive influx of Ca^{2+} across the plasma membrane.

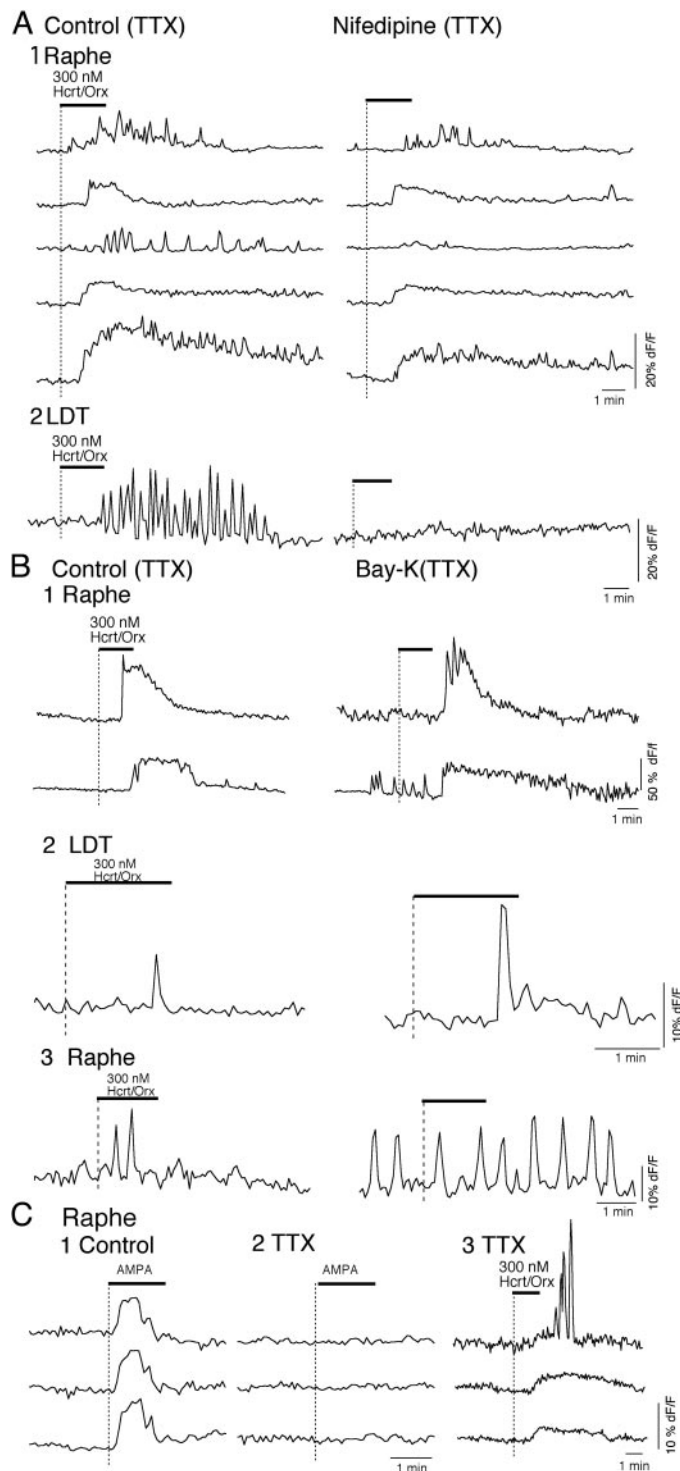
Ca^{2+} Influx versus release from intracellular stores

Orexin receptors (Ox1R and Ox2R) were originally discovered through screening an orphan G protein-coupled receptor library for ligand-evoked cytoplasmic Ca^{2+} transients (Sakurai et al. 1998). Results from subsequent studies with CHO cells transfected with Ox1Rs implicated activation of phospholipase C (PLC) and the IP_3 -mediated release of Ca^{2+} from SERCA pump-dependent intracellular stores that activated a Ca^{2+} influx pathway that was inhibited following thapsigargin-mediated depletion of internal stores, suggesting a role of store-operated channels (Lund et al. 2000; Smart et al. 1999). Subsequently, this influx pathway was pharmacologically distinguished from store-operated channels in CHO cells (Kukkonen and Akerman 2001). In contrast, we found that the Ca^{2+} transients evoked by Hcrt/Orexin in the LDT and DR were blocked by lowering $[\text{Ca}^{2+}]_o$, but were insensitive to depletion of intracellular stores, even at Hcrt/Orexin concentrations that produce robust store-dependent Ca^{2+} transients in Ox1R-transfected CHO cells. The apparent store independence of these transients did not result from a lack of CPA/thapsigargin-sensitive stores since Ca^{2+} mobilization by t-ACPD was blocked by CPA. Thus activation of native OxRs in LDT and DR appears to engage a Ca^{2+} influx pathway without the store-dependent release mechanism observed in transfected cells. This agrees with previous observations in cultured hypothalamic neurons where orexin-B evokes Ca^{2+} transients that are thapsigargin-insensitive but dependent on $[\text{Ca}^{2+}]_o$ (van den Pol 1999). It is also consistent with observations from acutely isolated ventral tegmentum neurons (Uramura et al. 2001) where low concentrations of orexin-A (≤ 10 nM) evoked Ca^{2+} transients that depended on extracellular Ca^{2+} , although in that study, the sensitivity to store depletion was not tested.

Second messengers and effectors

Our results and other published evidence indicate that the Ca^{2+} influx pathway(s) activated by Hcrt/Orexin is partially mediated by activation of PKC. Evidence from CHO-Ox1R cells indicate that inhibition of PLC abolishes Hcrt/Orexin-evoked Ca^{2+} transients (Lund et al. 2000) but that inhibition of IP_3 receptors only inhibited responses that lead to Ca^{2+} release from intracellular stores (Kukkonen and Akerman 2001). Consistent with this, experiments using cultured hypothalamic neu-

rons found that Hcrt/Orex-evoked Ca^{2+} transients were strongly attenuated by compounds inhibiting PKC but not by those inhibiting PKA (van den Pol et al. 1998) and PKC involvement was also found in Hcrt/Orex-induced transients in isolated VTA neurons (Uramura et al. 2001). Our results using PKC and PKA inhibitors in LDT and DR were consistent with these studies. Additionally, we found that a phorbol ester induced patterns of rises in $[\text{Ca}^{2+}]_i$ that were similar to those elicited by Hcrt/Orex. Thus there is an emerging consensus that a Ca^{2+} influx pathway activated by native OxRs involves PKC.



Several effectors might contribute to the observed Ca^{2+} influx. The sodium/calcium exchanger (NCE) appears to mediate the depolarization produced by Hcrt/Orex in septal and arcuate nucleus neurons (Burdakov et al. 2003; Wu et al. 2002) and in the tuberomammillary nucleus (Eriksson et al. 2001) where Hcrt/Orex also elevates $[\text{Ca}^{2+}]_i$ (Willie et al. 2003). Since reverse operation of the NCE, which elevates $[\text{Ca}^{2+}]_i$ in cardiac muscle (Bridge et al. 1990; Wier 1990) and is found in neurons (Li et al. 1994), might account for the Ca^{2+} influx, we examined whether the NCE inhibitor KB-R7943 attenuated Hcrt/Orex-evoked Ca^{2+} transients. At a concentration of 10 μM, which inhibits >80% of the reverse mode operation of the NCE (Iwamoto et al. 1996), the Hcrt/Orex-evoked Ca^{2+} transients were not attenuated, although at 80 μM, these transients were reduced by about 50%. However, since KB-R7943 at ≥ 30 μM also inhibits dihydropyridine-sensitive Ca^{2+} channels and voltage-dependent sodium channels (Iwamoto et al. 1996), this effect of high concentrations of KB-R7943 is unlikely to be specific. Hence, the NCE does not appear to mediate the Ca^{2+} influx evoked by Hcrt/Orex in DR and LDT neurons.

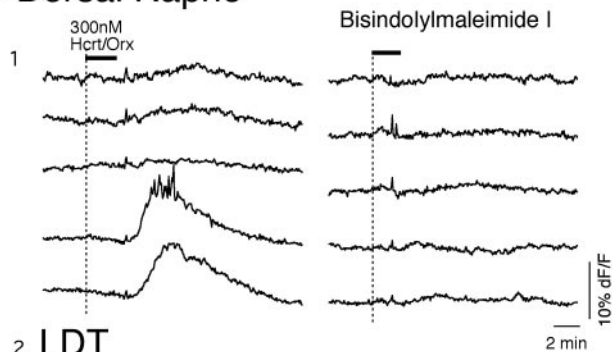
It is more likely that the Ca^{2+} influx is mediated by the opening of ion channels. Accordingly, we observed that Hcrt/Orex-induced Ca^{2+} transients were significantly attenuated by an L-type calcium channel antagonist and enhanced in the presence of an L-type channel agonist. These findings are consistent with results from dissociated VTA neurons (Uramura et al. 2001) and cultured somatotrophs where Hcrt/Orex augments an L-type calcium current (Xu et al. 2002). Nevertheless, blockade of L-type calcium channels did not abolish all Hcrt/Orex responses. While this may have resulted from incomplete inhibition of L-type calcium channels, this seems unlikely since nifedipine abolished the Ca^{2+} transients in many cells, especially those with spiking responses. Hence, it is likely that additional calcium permeable channels are involved in mediating the Hcrt/Orex evoked Ca^{2+} influx in many DR and LDT cells.

Although we did not measure whole cell currents in this study, we previously found that Hcrt/Orex evokes an inward current with an increase in membrane conductance in LDT neurons (Burlet et al. 2002), and it is known to depolarize DR neurons (Brown et al. 2001). Since both these actions appear to involve a nonselective cation current (Brown et al. 2002; Liu et al. 2002; Tyler et al. 2001), it is possible that this current is partly carried by Ca^{2+} ions and may contribute to the nifedipine-resistant responses. Indeed, such a nonspecific cation current was suggested to account for the orexin-evoked Ca^{2+}

FIG. 9. L-type calcium channels contribute to Hcrt/Orex-induced responses in the DR and LDT. **A:** nifedipine (10 μM; in ACSF with TTX) attenuated or abolished both plateau and spiking responses evoked by Hcrt/Orex in the DR (A_1) and LDT (A_2). Spiking responses were more consistently abolished than plateau response (see RESULTS). **B:** Bay-K 8644, an L-type Ca^{2+} channel agonist, augmented Hcrt/Orex-induced Ca^{2+} rises in the DR (B_1) and LDT (B_2). In some cases, Bay-K 8644 alone induced spiking that could obscure Hcrt/Orex-induced responses. **C:** Hcrt/Orex-evoked calcium transients do not result from depolarization alone. Strong depolarization by AMPA (1 μM) induced Ca^{2+} transients (C_1). Following blockade of action potentials with TTX, the AMPA-mediated depolarization failed to elicit Ca^{2+} transients (C_2). Nevertheless, subsequent application of Hcrt/Orex in TTX induced both plateau and spiking Ca^{2+} transients (C_3). Collectively, data suggest that mechanisms in addition to depolarization are required to generate Hcrt/Orex-evoked calcium transients.

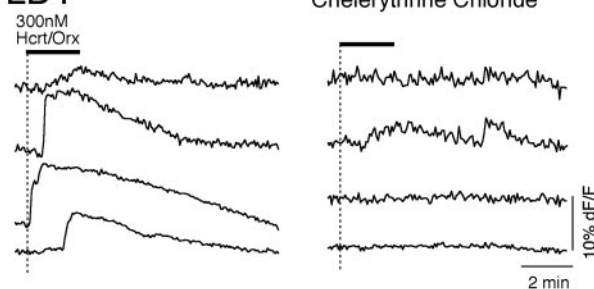
influx in CHO cells (Lund et al. 2000). In contrast, even though large Ca^{2+} transients were evoked in cultured hypothalamic neurons by Hcrt/Orx, no whole cell currents were observed when these cells were recorded under whole cell conditions (van den Pol 1999). A possible explanation of this discrepancy is that recordings were made using a high chloride pipette

A Dorsal Raphe

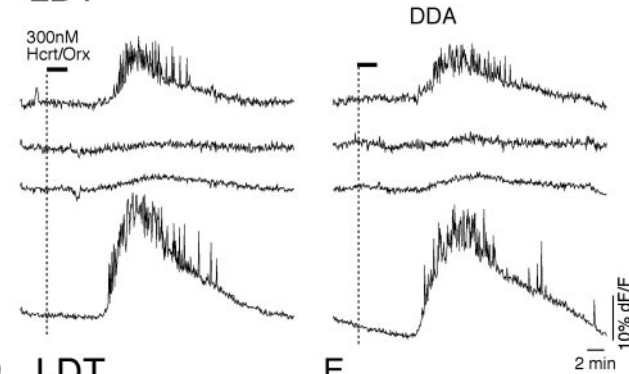


2 LDT

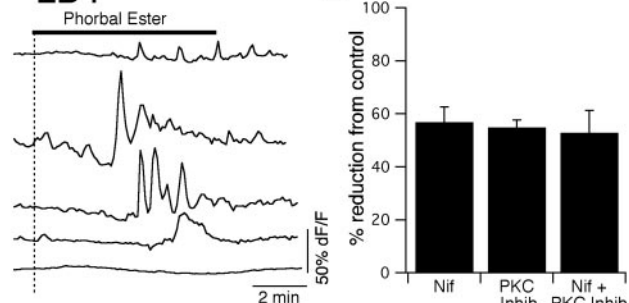
B LDT



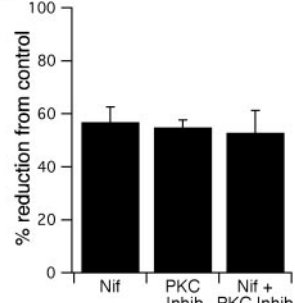
C LDT



D LDT



E



A Dorsal Raphe B LDT

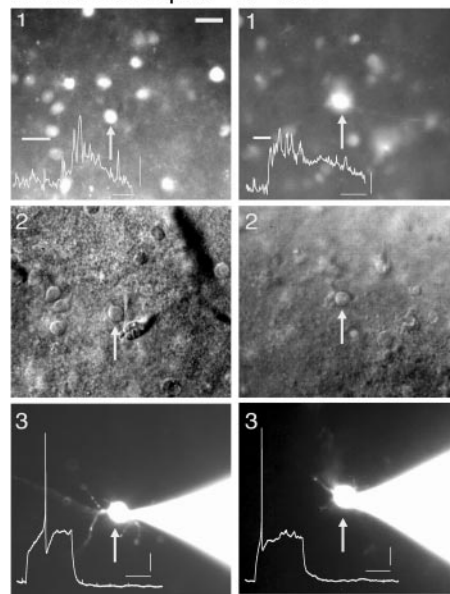


FIG. 11. Hcrt/Orx-evoked calcium transients arose from neurons in the DR and LDT. Fura-2-loaded cells (F_{380}) within the DR (A1) and LDT (B1) from which fluorescence changes were monitored (scale in top right A1 = 20 μm and applies to all panels). Overlaid traces show changes in dF/F induced by Hcrt/Orx in the indicated cells (scale bars = 10% dF/F and 2 min). DIC images of the corresponding cells are shown in A2 and B2 (arrows). Whole cell recordings were obtained from these cells that are shown during the recordings after filling with Alexa-594 (560 ± 27 nm; A3 and B3) from the patch pipette (right). Overlaid traces show action potentials elicited by depolarizing current steps, showing that recorded cells were neurons (scale bars = 10 mV and 250 ms).

solution in the van den Pol study that we found to inhibit the Hcrt/Orx-evoked current (Burlet et al. 2002), probably by uncoupling G protein-coupled receptors (Lenz et al. 1997). Thus accumulating evidence suggests one or more Ca^{2+} -permeable ion channels are involved. Future experiments using simultaneous ion imaging and current recording will be necessary to determine the relative roles played by each possible effector.

Diverse temporal profiles of Hcrt/Orx-evoked Ca^{2+} transients

We also found that Hcrt/Orx evoked Ca^{2+} transients with diverse temporal profiles in the LDT and DR. While a few previous studies of mammalian neurons have reported Ca^{2+} transients evoked by Hcrt/Orx (Uramura et al. 2001; van den

FIG. 10. Inhibition of protein kinase C (PKC) attenuated Hcrt/Orx-induced rises in $[\text{Ca}^{2+}]_i$. A1 and A2: responses to Hcrt/Orx under control conditions (left) were significantly attenuated in the presence of the PKC inhibitor bisindolylmaleimide I (1 μM). B: similarly, Hcrt/Orx responses were attenuated in the presence of the structurally dissimilar PKC inhibitor, chelerythrine chloride (10 μM). Because bisindolylmaleimide I has been reported to also have actions on protein kinase A, responses to Hcrt/Orx were also examined in the presence of an adenylyl cyclase inhibitor, DDA. Hcrt/Orx-induced Ca^{2+} transients were not attenuated in the presence of 50 μM DDA (C). D: a phorbol ester that results in production of PKC-induced patterns of rises in $[\text{Ca}^{2+}]_i$ similar, albeit larger in amplitude, to those elicited by Hcrt/Orx. E: histogram comparing percentage of reduction of changes in dF/F from control in the presence of nifedipine, bisindolylmaleimide I, and a combination of the two. Data indicate a PKC-regulated mechanism is involved in Hcrt/Orx responses and activation of L-type calcium channels probably involves this 2nd messenger.

Pol 1999; van den Pol et al. 1998), none have reported such diverse patterns. Such diverse profiles are common in nonneuronal cells and can arise from multiple mechanisms (Berridge and Dupont 1994). While spiking and oscillating Ca^{2+} transients are often associated with the release of Ca^{2+} from intracellular stores (Berridge and Dupont 1994), such behavior can also be produced by Ca^{2+} influx through membrane channels (Byron and Taylor 1993), as appears to be the case for all the responses we observed. While it is possible that a thapsigargin/CPA and ryanodine-insensitive store (e.g., NAADP-sensitive store) may have contributed (Genazzani et al. 1997; Lee 1997), such a store has not been observed in neurons and is unlikely to account for the immediate attenuation of Hcrt/Orex responses produced by lowering extracellular Ca^{2+} . Furthermore, all response types were sensitive to nifedipine, which reduced or abolished the spiking responses more effectively than the plateau responses. This indicates a role of L-type calcium channels in each response pattern and an even greater role in spiking. Interestingly, these responses did not appear to result simply from membrane depolarization. We found that concentrations of AMPA expected to produce a larger somatic depolarization than Hcrt/Orex did not produce any somatic Ca^{2+} transients in the absence of TTX-sensitive action potentials although Hcrt/Orex did evoke somatic Ca^{2+} transients in the same cells in the presence of TTX. Since intracellular calcium diffusion is highly restricted (Connor and Nikolakopoulou 1982), these transients most likely arose from calcium permeable channels, including L-type calcium channels located at the soma. Since the AMPA-mediated depolarization failed to activate these channels, additional modulation by Hcrt/Orex receptor activation may be necessary. Our finding that PKC inhibition was not additive with L-channel inhibition is consistent with the possibility that Hcrt/Orex receptor activation of PKC promotes L-channel activity in LDT and DR neurons. Ca^{2+} entry through L-type calcium channels may be of considerable functional significance since it can preferentially couple to immediate early gene transcription (Deisseroth et al. 2003). Moreover, the particular temporal dynamics of the Ca^{2+} signals appears to be important in determining which transcription pathways become activated (Dolmetsch et al. 1997). Thus one intriguing possibility is Hcrt/Orex can engage different transcription pathways within subpopulations of LDT and DR neurons depending on the temporal profile of the evoked Ca^{2+} response.

Neuronal heterogeneity of the LDT and DR

Both the LDT and DR contain neurons expressing different transmitter phenotypes. Evidence from whole cell recordings in the LDT (Bulet et al. 2002) and DR (Liu et al. 2002) indicate that both the principal neurons (cholinergic in LDT and serotonergic in DR) and the other neurons, presumably including GABAergic neurons, are excited by Hcrt/Orex. A different situation has been observed in some other structures. For example, in the basal forebrain, Hcrt/Orex excites cholinergic neurons but not GABAergic neurons (Eggermann et al. 2001) and in the arcuate nucleus, Hcrt/Orex excites a population of electrophysiologically distinct GABA neurons (Burdakov et al. 2003). While we were unable to identify the histochemical nature of the neurons in our study, we expect that both principal and other neurons were imaged in the LDT and DR.

Given previous electrophysiological findings, it is therefore likely that all types of neurons in these structures exhibit Ca^{2+} transients elicited by Hcrt/Orex. Thus Hcrt/Orex may provide similar biophysical signals to both the principal and GABAergic cells of these nuclei that also appear to be involved in behavioral state control (Maloney et al. 1999). Nevertheless, future simultaneous recording and imaging studies will be necessary to explicitly examine this point. Such studies should also prove useful in determining if Ca^{2+} signals with particular temporal profiles are associated with specific transmitter phenotypes.

Metabotropic glutamate receptors in the LDT

Another new observation was that the activation of metabotropic glutamate receptors with t-ACPD evokes rises in $[\text{Ca}^{2+}]_i$ via store-mediated release in LDT neurons. These data indicate for the first time that metabotropic glutamate receptors (mGluRs) are present in LDT and that they are capable of mobilizing Ca^{2+} from intracellular stores—presumably via production of IP_3 (Sugiyama et al. 1987). LDT neurons receive fast glutamatergic synaptic input (Sanchez and Leonard 1996), which is important for generating REM sleep and wakefulness (Datta et al. 2001). Moreover, glutamatergic input can both be inhibited (Arrigoni et al. 2001) and enhanced (Bulet et al. 2002), suggesting that regulation of excitatory synaptic pathways are important in the state-dependent control of these and other neurons (Peever et al. 2003). The fact that Hcrt/Orex enhances synaptic release of glutamate also suggests that Hcrt/Orex might regulate $[\text{Ca}^{2+}]_i$ both by enhancing synaptic activation of mGluRs and by directly elevating $[\text{Ca}^{2+}]_i$ by receptor stimulated influx. Such a dual action could enhance the temporal concordance of these two Ca^{2+} signals. This may be of considerable functional interest since the coincidence of Ca^{2+} transients arising from mGluR activation and either action potentials (Nakamura et al. 1999) or NMDA receptors (Nakamura et al. 2002) can result in very large propagating Ca^{2+} waves. Depending on when Hcrt/Orex release occurs in these nuclei (Estabrooke et al. 2001; Kiyashchenko et al. 2002; Tortorolo et al. 2001), temporal concordance of these calcium signals might occur at different times since presumed cholinergic LDT and serotonergic DR neurons have different firing patterns across the sleep-wake cycle. During waking, firing in both groups is high, while during REM sleep, firing in a subpopulation of presumed cholinergic neurons is high but the serotonergic neurons are silent (Saper et al. 2001). Future studies will be necessary to examine both the interactions and biological consequences of such calcium signals in these neurons which appear so critical for sleep and wakefulness.

ACKNOWLEDGMENTS

We thank Drs. C. Tyler, N. Ross, and W. Ross for many useful discussions and comments on a previous version of this manuscript.

GRANTS

This study was supported by National Institutes of Health Grants NS-10877 to K. A. Kohlmeier and NS-27881 and HL-64150 to C. S. Leonard.

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