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Synchronized Periodic Ca\(^{2+}\) Pulses Define Neurosecretory Activities in Magnocellular Vasotocin and Isotocin Neurons

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The electrical activity of magnocellular neurosecretory cells (NSCs) is correlated with the release rates of neurohypophysial hormones. NSCs may control their secretory activity in a cooperative manner by changing their electrical activity in response to changes in the internal milieu. In the present study, we applied confocal Ca\(^{2+}\) imaging to a sagittally hemisected rainbow trout brain to simultaneously monitor the neuronal activity of a number of NSCs. We found that NSCs in vitro showed synchronized pulsatile elevations of intracellular Ca\(^{2+}\) levels at regular intervals. Double immunostaining of vasotocin (VT) and isotocin (IT) after the confocal imaging clarified that each of the VT and IT neuronal populations showed a distinct pattern of periodic Ca\(^{2+}\) pulses. Simultaneous cell-attached patch recordings ensured that individual Ca\(^{2+}\) pulses were associated with a phasic burst firing. Depolarizing stimuli by increasing the extracellular K\(^+\) concentration from 5 to 7–9 mM reversibly shortened the interpulse intervals in both VT and IT neurons. Interpulse intervals but not durations of pulses were shortened by hypo-osmotic stimuli and prolonged by hyperosmotic stimuli, consistent with the osmoregulatory function of teleost NSCs. We therefore hypothesize that NSCs use intervals of synchronized periodic burst discharges to fit the levels of secretory activity to physiological requirements.

Key words: neurosecretion; vasotocin; magnocellular neuron; rainbow trout; osmoregulation; synchronization; calcium oscillation; phasic burst

The magnocellular neurosecretory system is crucial for homeostatic control of the internal milieu through secretion of neurohypophysial hormones in all vertebrates. The electrical activity of neurosecretory cells (NSCs) determines the amount of hormones released from the terminals in the neurohypophysis. NSCs may regulate their secretory activity by changing their electrical activity in response to the physiological stimuli (Leng et al., 1999). Rat oxytocin (OT) neurons show suckling-induced synchronous burst firing during lactation (Belin and Moos, 1986), whereas vasopressin (VP) neurons show asynchronous phasic firing in response to hypertonic saline injection (Leng and Dyball, 1983).

In the present study, we examined how a population of NSCs responds to osmotic stimuli. We applied confocal Ca\(^{2+}\) imaging to a sagittally hemisected rainbow trout brain to record the neuronal activity of a number of NSCs. In the trout brain, the thin layers of preoptic magnocellular NSCs expand beside the third ventricle (Fig. 1A). The entire population of preoptic vasotocin (VT) and isotocin (IT) neurons spread beneath the ependymal layer can be viewed from the ventricular side of a hemisected brain (Fig. 1B). This superficial localization of the preoptic NSCs is favorable for recording the activity of multiple neurons by confocal Ca\(^{2+}\) imaging. In addition, the hemisected brain preparation maintains the intact neuronal connections, which are usually severed in a slice preparation.

We now provide evidence that trout preoptic VT and IT neuronal populations show distinct patterns of synchronized periodic Ca\(^{2+}\) pulses, which are sensitive to osmotic stimuli. The results indicate that NSCs use the synchronized electrical activity to adjust the neurosecretory activity to physiological requirements.

MATERIALS AND METHODS

Hemisected brain preparation. Immature rainbow trout (Oncorhynchus mykiss) (body weight 50–100 gm) were obtained from a commercial source (Sapporo Nijimasu, Co. Ltd., Sapporo, Japan), and were maintained in a 0.5 ton circular tank at 15°C for more than a week. They were anesthetized by immersion in 0.005% MS-222 (Sigma, St. Louis, MO) buffered with an equal amount of sodium bicarbonate. After decapitation, the brain of each fish was dissected out, trimmed, and hemisected with a razor blade in ice-cold fish saline (in mM: 124 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 1.25 NaH\(_2\)PO\(_4\), 26 NaHCO\(_3\), and 10 glucose). Next, the tissue was maintained in fish saline oxygenated with 95% O\(_2\)/5% CO\(_2\) at 15°C. Before dye loading, the ependymal layer was removed by incubation with 0.1% collagenase (Life Technologies, Rockville, MD) and 0.1% trypsin (Difco, Detroit, MI) in oxygenated fish saline at 15°C for 2 hr, followed by a rinse in a Ca\(^{2+}\)-, Mg\(^{2+}\)-free saline containing 1 mM EDTA for 10 min. The exposed NSCs were loaded with 1 μM Oregon Green BAPTA-1/AM (Molecular Probes, Eugene, OR) in oxygenated fish saline at 15°C for 1 hr.

Confocal Ca\(^{2+}\) imaging. The tissue was placed in a recording chamber continuously perfused (flow rate, 0.7 ml/min) with oxygenated fish saline at 13–15°C. Time-lapse confocal Ca\(^{2+}\) images of NSCs were captured at 0.2 Hz unless otherwise specified using a Bio-Rad MicroRadiance confocal laser system (Bio-Rad, Hercules, CA) mounted on a Zeiss Axio...
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Enzymatic removal of the ependymal layer enabled us to monitor the activity of many NSCs in a single optical section of confocal microscopy images (Fig. 1C) as changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)])). The types of the recorded cells were individually identified by whole-mount double immunostaining of VT and IT (Fig. 1D). Recordings were obtained from 289 VT neurons, 214 IT neurons, and 85 nonimmunoreactive neurons from 21 fish.

Both VT and IT neurons showed spontaneous pulsatile elevations of [Ca\(^{2+}\)] at regular intervals, the lengths of which were specific to each neuronal group. These periodic Ca\(^{2+}\) pulses synchronized well within the same neuronal groups, (i.e., in VT and IT neurons) (Fig. 1E, green and red traces) throughout all of the preoptic nucleus (PON) pars magnocellularis beyond the boundaries of subnuclei. Preoptic neurons that were neither VT nor IT immunoreactive and were mostly smaller than typical NSCs showed asynchronous [Ca\(^{2+}\)], oscillation, [Ca\(^{2+}\)], pulses with irregular intervals, or episodic rises of [Ca\(^{2+}\)] (Fig. 1E, blue traces). Synchronized periodic Ca\(^{2+}\) pulses of VT and IT neurons usually continued during 6–8 hr of experiments without considerable changes. Such Ca\(^{2+}\) pulses could be observed on the following day of brain preparation (n = 21 cells from two fish).

These periodic Ca\(^{2+}\) pulses were abolished in a Ca\(^{2+}\)-free medium (n = 49 cells from six fish) and blocked by Cd\(^{2+}\) (n = 48 cells from three fish).

RESULTS

Synchronized periodic Ca\(^{2+}\) pulses in preoptic NSCs

Figure 1. Synchronized periodic Ca\(^{2+}\) pulses in preoptic NSCs in the rainbow trout brain. A. Frontal section (70 μm) of the preoptic nucleus showing immunostained VT neurons (green) and IT neurons (red) aligned along the third ventricle (3v). B. Planar distribution of the preoptic NSCs viewed from the ventricular side of a hemisected brain. C. Confocal Ca\(^{2+}\) image of preoptic NSCs in a single optical section. D. Identification of the recorded cells by double immunofluorescence of VT (green) and IT (red). Scale bars, 50 μm. E. Spontaneous periodic [Ca\(^{2+}\)] pulses recorded in the preoptic NSCs shown in C and D. VT neurons (v1–v7) and IT neurons (i1–i8) showed synchronous Ca\(^{2+}\) pulses within the same cell groups. Nonimmunoreactive neurons (n1–n4) showed various patterns of [Ca\(^{2+}\)] changes. The sampling rate was changed from 1 to 0.2 Hz at 13 min after the start points of traces.

Scope (Zeiss, Thornwood, NY) with an Olympus 40× water immersion objective (Olympus Optical, Tokyo, Japan). The number of NSCs recorded in a single optical section ranged from 5 to 50 depending on the shapes of the ventricular surface and the degree of enzymatic removal of the ependymal cells. Changes in fluorescence intensity in NSCs were analyzed postexperimentally using computer software [NIH Image/Java and Origin (OriginLab, Northampton, MA)]. Data are presented as relative changes in fluorescence intensity normalized to base line fluorescence (ΔF/F).

Solutions. The salinity and osmolarity of fish saline was adjusted to be at the mean values in plasma of fresh-water-adapted rainbow trout. High KCl solutions were prepared by replacing 2 or 4 mM NaCl with equimolar KCl in fish saline. Ca\(^{2+}\)-free solution was prepared by replacing CaCl\(_2\) with 1 mM EGTA in fish saline. CdCl\(_2\) was dissolved in fish saline at 100 μM. For osmotic stimuli, 10 mM NaCl or 20 mM mannitol was added to or subtracted from the saline. All solutions were bath-applied. They required 2.5 min to be fully replaced over the antecedent solution.

Cell-attached recording. The capacitive currents of NSCs on confocal Ca\(^{2+}\) imaging were recorded with patch pipettes (resistance of 3–7 MΩ) filled with fish saline connected to a patch-clamp amplifier (EPC-2000, Nihon Kohden, Tokyo, Japan). The pipette potential was clamped to 0 mV. Current records were printed out on a thermal array recorder.

Immunohistochemical identification of VT and IT cells. Anti-VP and anti-OT antisera (provided by Dr. S. Kawashima, Zenyaku Kogyo Co. Ltd., Tokyo, Japan) were used to recognize VT and IT, respectively. Cross-reaction was prevented by preincubation with respective heterologous peptides coupled to Sepharose 4B beads (Pharmacia Biotech, Uppsala, Sweden) as reported previously (Hyodo and Urano, 1991). Specific staining with the preabsorbed antiserum was further confirmed by incubation with homologous peptide.

Because we used antisera raised in the same species (i.e., rabbit) for double immunolabeling, we adopted a sequential protocol that uses Fab fragment as the secondary antibody to avoid possible interference (Ne-goescu et al., 1994). After recording, the tissue was fixed in 4% paraformaldehyde in 0.05 M PBS for 12 hr, rinsed in PBS for 12 hr, incubated in methanol for 2 hr, and immersed in a blocking solution containing 0.5% BSA and 2% normal goat serum in 0.1% Triton X-100 in PBS (PBST) for 2 hr. Next, the tissue was sequentially incubated with (1) anti-vasopressin antiserum (1:5000) for 24 hr, (2) fluorescein-labeled anti-rabbit IgG goat Fab fragment (1:100; Jackson ImmunoResearch, West Grove, PA) for 12 hr, (3) anti-oxytocin antiserum (1:5000) for 24 hr, and (4) a mixture of Alexa 488-labeled anti-fluorescein (1:100; Molecular Probes) and Alexa 546-labeled anti rabbit IgG (1:100; Molecular Probes) for 12 hr. Between each step, the tissue was rinsed in PBST for 12 hr. Primary and secondary antibodies were diluted with blocking solution. All steps were carried out at 4°C. The NSCs were visualized by confocal microscopy.
Characterization of periodic Ca$^{2+}$ pulses

The features of periodic Ca$^{2+}$ pulses, such as shapes, durations, and intervals, were stable during the recording sessions but were apparently different in VT and IT neurons. In VT neurons, the Ca$^{2+}$ pulse was composed of 15–20 sec rising and declining phases with an intervening short plateau phase, forming a symmetrical appearance (Fig. 2A). In IT neurons, the Ca$^{2+}$ pulse was composed of the initial rising phase, the subsequent plateau phase, and the latter oscillating phase (Fig. 2B).

Effects of depolarizing stimulation on Ca$^{2+}$ pulses

Depolarizing stimulation by increasing the extracellular K$^+$ concentration ([K$^+$]o) reversibly shortened the intervals of synchro-
nized periodic Ca\(^{2+}\) pulses in both VT and IT neurons. When [K\(^{+}\)], was elevated from 5 to 7 mM, the frequencies of periodic Ca\(^{2+}\) pulses were apparently increased ~1.4-fold in VT neurons and 2.4-fold in IT neurons (Fig. 3A,B). Additional increases in [K\(^{+}\)], up to 9 mM drastically shortened the interpulse intervals in VT neurons, whereas IT neurons showed a sustained Ca\(^{2+}\) rise. The durations of individual pulses were significantly decreased with increases in [K\(^{+}\)], (Fig. 3C).

**Effects of hyperosmotic and hypo-osmotic stimulation on Ca\(^{2+}\) pulses**

A question arising here is whether synchronized periodic Ca\(^{2+}\) pulses respond to osmotic stimuli. Many salmonid species are euryhaline, so that plasma levels of Na\(^{+}\) are adjusted in a range from 130 to 190 mM according to their environmental salinity. When the extracellular Na\(^{+}\) concentration ([Na\(^{+}\)],) was elevated by 10 mM from the plasma Na\(^{+}\) level of freshwater-adapted rainbow trout, frequencies of periodic Ca\(^{2+}\) pulses were reversibly lowered to certain stable levels in both VT and IT neurons (Fig. 4A,C). When [Na\(^{+}\)], was decreased by 10 mM, the frequencies were elevated. The duration of Ca\(^{2+}\) pulses did not show notable changes (Fig. 4B,D). In two of four fish examined, NSCs were sensitive to a 5 mM increase in [Na\(^{+}\)],, equivalent to a 3.4% increase in extracellular fluid osmolarity. Increasing osmolarity with 20 mM mannitol as well as with 10 mM NaCl induced similar changes in the frequencies of Ca\(^{2+}\) pulses in both VT and IT neurons (n = 3 fish) (Fig. 4E).

**DISCUSSION**

In the present study, we found that trout preoptic VT and IT neuronal populations showed distinct patterns of synchronized periodic Ca\(^{2+}\) pulses, which were closely associated with phasic burst firing. Hyperosmotic stimuli lowered and hypo-osmotic stimuli elevated the frequency of Ca\(^{2+}\) pulses in both VT and IT neurons. The results indicate that NSCs use synchronized electrical activity to adjust neurosecretory activity to physiological requirements.

**Electrical activity of trout NSCs**

We are concerned that the neuronal behaviors of NSCs in the present study may not be comparable with those in vivo. Acute in vitro preparations often fail to show neuronal properties known in vivo. Nonetheless, we believe that the synchronized periodic Ca\(^{2+}\) pulses reflect the basal neuronal activity of preoptic NSCs. Our hemisected brain preparations contained the entire forebrain region, including the olfactory bulb, telencephalon, and diencephalon. Thus, the integrity of neuronal connections within and around the PON were well maintained. It is possible that enzymatic removal of ependymal cells affected the electrical activity of NSCs. The degrees of the enzymatic removal varied among preparations. In some cases the ependymal layer remained almost intact, so that only a few NSCs were exposed, whereas in other cases the ependymal layer was completely removed, so that most of NSCs were exposed. In either case, the appearance of Ca\(^{2+}\)
pulses seemed similar, indicating that removal of ependymal cells left the electrical activity of NSCs intact.

Whereas VT and IT NSCs always showed synchronized periodic Ca\textsuperscript{2+} pulses, non-NSCs did not show such Ca\textsuperscript{2+} pulses but showed various patterns of [Ca\textsuperscript{2+}] changes. The synchronized periodic Ca\textsuperscript{2+} pulses usually continued for 6–8 hr of experiments without any changes in their patterns. In addition, the interpulse intervals and durations of Ca\textsuperscript{2+} pulses did not vary among individual fish. Together, synchronized periodic Ca\textsuperscript{2+} pulses certainly reflect the basal neuronal activity of preoptic NSCs in vivo.

There are many differences between the electrical activity of trout NSCs and that seen for mammalian NSCs and other neuroendocrine cells. The electrical activity of preoptic VT and IT neurons is characterized by (1) phasic burst firing with a strict periodicity, (2) synchronization of burst activity within same cell groups, (3) different patterns between VT and IT neurons, and (4) excitation by hypothalamic stimuli.

In rats, VP neurons display phasic burst firing in response to hypertonic saline (Brimbale and Dyball, 1977). The bursts do not occur synchronously (Leng and Dyball, 1983). The intervals and durations of phasic bursts in VP neurons are variable within and between neurons and are much shorter than those in VT neurons. OT neurons in lactating rat show burst discharges in response to suckling (Lincoln and Wakerley, 1974); these bursts occur every 5–10 min, last 1–2 sec, and are synchronized (Belin and Moos, 1986). At all other times, OT neurons show continuous background activity. Gonadotropin-releasing hormone neurons derived from the monkey olfactory placode exhibit [Ca\textsuperscript{2+}], oscillations at an interval that is unique to individual cells (an average of 8 min), and [Ca\textsuperscript{2+}], oscillations in a population of cells synchronized at an interval of 50 min (Terasawa et al., 1999). Thus, synchronized phasic bursts are unique to trout NSCs among hitherto examined systems, although the appearances of phasic bursts are similar between VP and VT neurons.

Interestingly, NSCs freshly isolated from the rat supraoptic nucleus displayed distinct patterns of [Ca\textsuperscript{2+}] changes in response to applied neuropeptides. VP induces a transient rise in [Ca\textsuperscript{2+}], whereas OT induces a sustained rise in [Ca\textsuperscript{2+}]; these bursts occur every 5–10 min, last 1–2 sec, and are synchronized (Belin and Moos, 1986). At all other times, OT neurons display continuous background activity. Gonadotropin-releasing hormone neurons derived from the monkey olfactory placode exhibit [Ca\textsuperscript{2+}], oscillations at an interval that is unique to individual cells (an average of 8 min), and [Ca\textsuperscript{2+}], oscillations in a population of cells synchronized at an interval of 50 min (Terasawa et al., 1999). Thus, synchronized phasic bursts are unique to trout NSCs among hitherto examined systems, although the appearances of phasic bursts are similar between VP and VT neurons.

Mechanism underlying synchronized periodic Ca\textsuperscript{2+} pulses

A lack of correlation of periodic Ca\textsuperscript{2+} pulses between VT and IT neurons is in contrast to good synchronization within each neuronal group. This fact implies that distinct pattern-generating mechanisms govern VT and IT neurons individually, and that these mechanisms have separate signaling pathways to avoid cross talk. Local anatomical and chemical circuits are probably involved in such mechanisms.

In the trout PON, VT neurons are medially localized, whereas IT neurons are laterally distributed (Fig. 1A). In addition, we frequently observed that VT and IT neurons clustered together within the same cell group (Fig. 1A,B). These anatomical arrangements suggest the presence of electrical and/or dendrodendritic synapses within a cluster, in addition to the recurrent axon collaterals shown in the goldfish PON (Kandel, 1964). Actually, the presence of dye coupling between IT neurons was evidenced by injection with biocytin, although none of the VT neurons were transneuronally stained (M. Komatsu and A. Urano, unpublished observations). At present, we believe that dye coupling only between IT neurons is one factor for the separation of VT and IT neurons. A possible presence of dendrodendritic synapses is another factor, because in rats dendritically released VP acts on local VP neurons (Gouzenes et al., 1998) and OT modulates OT neurons (Lambert et al., 1993).

Hypothalamic NSCs in lower vertebrates receive synaptic inputs on dendrites in the lateral neuroepilem part of the PON (Urano, 1988). Because the distribution patterns of NSC dendrites in the trout PON are quite similar to those in other lower vertebrates, synaptic inputs into the neuroepilems of the PON may be involved in the organization of synchronous periodic bursts. In the trout preoptic NSCs, application of glutamate evoked [Ca\textsuperscript{2+}], rises (our unpublished observation). In eel brains, most PON neurons were excited by glutamate, whereas spontaneous phasic neurons were suppressed by GABA (Sugita and Urano, 1985). These results suggest that, in the trout PON, glutamate and GABA act on NSCs, as was reported in the rat hypothalamus, in which glutamatergic and GABAergic inputs constitute most of the excitatory and inhibitory synapses (Decavel and van Den Pol, 1990; van Den Pol et al., 1990). The localization of guanylyl cyclase mRNAs and nitric oxide (NO) synthase in the trout PON neurons (Shi et al., 2000) suggests the involvement of NO signaling in local neuronal communication. A recent study showing the modulatory effects of NO on the electrical activity of the rat NSCs (Ozaki et al., 2000) supports our idea.

Physiological implication

The frequencies of synchronized periodic Ca\textsuperscript{2+} pulses were lowered by hyperosmotic stimuli and elevated by hypo-osmotic stimuli. The results coincide with a previous single-cell study that showed reversible decreases in the discharge rates of goldfish NSCs by perfusion of the gills with diluted seawater (Kandel, 1964). In trout preoptic NSCs, VT gene expression was decreased by hyperosmotic stimuli and elevated by hypo-osmotic stimuli. The results coincide with a previous single-cell study that showed reversible decreases in the discharge rates of goldfish NSCs by perfusion of the gills with diluted seawater (Kandel, 1964). In trout preoptic NSCs, VT gene expression was decreased by hyperosmotic stimuli and elevated by hypo-osmotic stimuli. The results coincide with a previous single-cell study that showed reversible decreases in the discharge rates of goldfish NSCs by perfusion of the gills with diluted seawater (Kandel, 1964). The results coincide with a previous single-cell study that showed reversible decreases in the discharge rates of goldfish NSCs by perfusion of the gills with diluted seawater (Kandel, 1964).

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