Distribution and Activation of Intracellular Ca$^{2+}$ Stores in Cultured Olfactory Bulb Neurons

GREG C. CARLSON, MELISSA L. SLAWECKI, ERIC LANCASTER, AND ASAIF KELLER

Department of Anatomy and Neurobiology and The Neuroscience Program, University of Maryland School of Medicine, Baltimore, Maryland 21201

Carlson, Greg C., Melissa L. Slawecki, Eric Lancaster, and Asaf Keller. Distribution and activation of intracellular Ca$^{2+}$ stores in cultured olfactory bulb neurons. J. Neurophysiol. 78: 2176–2185, 1997. The presence and distribution of intracellular Ca$^{2+}$ release pathways in olfactory bulb neurons were studied in dissociated cell cultures. Histochemical techniques and imaging of Ca$^{2+}$ fluxes were used to identify two major intracellular Ca$^{2+}$ release mechanisms: inositol 1,4,5-triphosphate receptor (IP$_R$)-mediated release, and ryanodine receptor-mediated release. Cultured neurons were identified by immunocytochemistry for the neuron-specific marker β-tubulin III. Morphometric analyses and immunocytochemistry for glutamic acid-decarboxylase revealed a heterogeneous population of cultured neurons with phenotypes corresponding to both projection (mitral/tufted) and intrinsic (peri-glomerular/granule) neurons of the in vivo olfactory bulb. Immunocytochemistry for the IP$_R$, and labeling with fluorescently-tagged ryanodine, revealed that, irrespective of cell type, almost all cultured neurons express IP$_R$ and ryanodine binding sites in both soma and dendrites. Functional imaging revealed that intracellular Ca$^{2+}$ fluxes can be generated in the absence of external Ca$^{2+}$, using agonists specific to each of the intracellular release pathways. Local pressure application of glutamate or quisqualate evoked Ca$^{2+}$ fluxes in both soma and dendrites in nominally Ca$^{2+}$-free extracellular solutions, suggesting the presence of IP$_R$-dependent Ca$^{2+}$ release. These fluxes were blocked by preincubation with thapsigargin and persisted in the presence of the glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione. Local application of caffeine, a ryanodine receptor agonist, also evoked intracellular Ca$^{2+}$ fluxes in the absence of extracellular Ca$^{2+}$. These Ca$^{2+}$ fluxes were suppressed by preincubation with ryanodine. In all neurons, both IP$_R$- and ryanodine-dependent release pathways coexisted, suggesting that they interact to modulate intracellular Ca$^{2+}$ concentrations.

INTRODUCTION

Neuronal mechanisms controlling cytoplasmic calcium concentrations ([Ca$^{2+}$]) modulate a number of neuronal activities, including neurotransmitter release (Katz 1969), control of membrane excitability (Llinás 1990) and receptor function (Jaffe et al. 1994), differential gene expression (Sheng and Greenberg 1990), and neuronal migration and growth (Komuro and Rakic 1995). Increases in [Ca$^{2+}$], occur by extracellular Ca$^{2+}$ influx through transmembrane channels and intracellular release of Ca$^{2+}$ sequestered in intracellular organelles. Because intracellular stores, such as the endoplasmic reticulum (ER), can sequester high concentrations of Ca$^{2+}$, these stores serve not only to buffer free intracellular Ca$^{2+}$, but can produce rapid, local Ca$^{2+}$ transients in response to specific stimuli (Clapham 1995; Simpson et al. 1995). Intracellular stores release Ca$^{2+}$ through at least two pathways, each mediated by a specific family of receptors. In one pathway, Ca$^{2+}$ entry through the plasma membrane activates ryanodine receptors in the ER that mediate Ca$^{2+}$ release from this organelle (Galione 1992). This process often is referred to as Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) (Ehrlich et al. 1994; Henzi and MacDermott 1992; Irving et al. 1992; O’Neil et al. 1990). The other pathway involves activation of plasma membrane receptors—such as the glutamate-sensitive metabotropic receptors—that activate, through intermediary G proteins, phospholipase C, leading to the generation of inositol 1,4,5-triphosphate (IP$_3$). IP$_3$ then binds to an IP$_3$ receptor-Ca$^{2+}$ channel complex on the ER, inducing Ca$^{2+}$ release (Putney 1990; Simpson et al. 1995).

Although modulation of [Ca$^{2+}$], in general has been shown to regulate numerous neuronal functions, in some cases, these activities may be specifically dependent on Ca$^{2+}$ release from intracellular stores (reviewed in: Simpson et al. 1995). These include the modulation of neuronal excitability (Abdul-Ghani et al. 1996; Brorson et al. 1991; Kawai and Watanabe 1989), presynaptic transmitter release (Blaustein et al. 1980; Peng 1996), long-term depression and potentiation (Behnisch and Reymann 1995; Kasono and Hirano 1995; Kohda et al. 1995; Reyes and Stanton 1996), and neuronal development (Levick et al. 1995; Stewart et al. 1995). Therefore, studies of Ca$^{2+}$-dependent phenomena in neurons must consider the presence of intracellular release pathways.

The present study was designed to identify the types of intracellular Ca$^{2+}$ release mechanisms in cultured neurons of the rat olfactory bulb. Functional imaging of Ca$^{2+}$ fluxes and immunocytochemical techniques revealed that both IP$_R$- and ryanodine-dependent release mechanisms are expressed in a heterogeneous population of these neurons. Some of these results were published previously in abstract form (Carlson et al. 1996).

METHODS

Cell cultures

Dissociated cell cultures were prepared from 3-day-old Wistar rat pups. Olfactory bulbs were dissected into culture medium containing minimal essential medium supplemented with L-alanyl-L-glutamine (MEM α medium, GIBCO), 10% fetal calf serum (GIBCO), 5% horse serum (GIBCO), and 0.6% glucose. The meninges were removed, and the bulbs were dissociated both enzymatically (papain; 20 U/ml, Worthington) and by gentle mecha-
Ca\textsuperscript{2+} stores in cultured olfactory bulb neurons

Immunocytochemistry

Cell cultures were fixed in methanol at −20°C. Unless otherwise indicated, all incubations were performed at room temperature. Several rinses in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) were performed between each of the incubation steps. The following antibodies were used: mouse anti-β tubulin-III (1:50; Sigma); sheep anti-glutamic acid decarboxylase (GAD1440-4; 1:2400; gift of Dr. Kopin, NINDS); rabbit anti-IP\textsubscript{3}R (AP2A, 10 μg/μl). The IP\textsubscript{3}R antibody generously was provided by Dr. Alan Sharp, who has characterized this antiserum (Sharp et al. 1993).

Preincubation for double labeling of β tubulin-III and GAD was performed for 1 h in PBS containing 5% normal donkey serum. The cultures then were incubated overnight at 4°C in PBS containing the two primary antibodies and donkey serum. The secondary antibody mixture of rhodamine-conjugated donkey anti-mouse and fluorescein-conjugated donkey anti-sheep (both at 1:100; Jackson ImmunoResearch) solution in PBS then was applied to the cultures for 1 h.

For β tubulin-III/IP\textsubscript{3}R double-labeling experiments, cultures were preincubated for 1 h in PBS containing 2.5% normal donkey serum, 2.5% normal goat serum, and 0.2% Triton-X. The cultures were incubated overnight at 4°C in PBS containing the two primary antibodies and donkey serum. The cultures then were incubated in a secondary antibody mixture of biotinylated goat anti-rabbit (1:200; Vector Laboratories) and rhodamine-conjugated donkey anti-mouse (1:100; Jackson ImmunoResearch) for 1 h. A 1 h incubation in fluorescein-conjugated avidin (1:100; Vector Laboratories) diluted in PBS followed. To further increase the IP\textsubscript{3}R signal, a 1 h incubation in biotinylated goat anti-avidin (1:100; Vector Laboratories) was performed, and a second incubation in fluorescein-conjugated avidin (1:100; Vector Laboratories) completed the procedure.

A similar procedure was used for GAD/IP\textsubscript{3}R double labeling, except that Triton-X was not used, the blocking serum consisted of 2.5% normal donkey serum and 2.5% normal goat serum, anti-GAD was used instead of the β tubulin-III antibody and detected with rhodamine-conjugated donkey anti-sheep secondary antibody (1:100; Jackson ImmunoResearch).

After the final washes, coverslips were mounted on glass slides in Prolong antifade reagent (Molecular Probes) and examined with a confocal scanning light microscope (Zeiss LSM410). Digitized images were stored on a personal computer.

Control experiments were performed by omitting the primary or secondary antibody; staining was not detected in these controls.

Ryanodine staining

Ryanodine receptor binding sites were localized in coverslips of live cells placed in a recording chamber that was mounted on an inverted microscope. The cultures were incubated for 15 min in monosubstituted BODIPY FL-X ryanodine derivative (500 nM; Molecular Probes). Digital images of BODIPY FL-X stained cells and brightfield Nomarski images were collected simultaneously with a confocal scanning light microscope (Olympus Fluoview) and stored on a personal computer.

Image analysis

All digitized images were analyzed on a PowerMacintosh 9500, using IPLabs (Signal Analytics) and Photoshop (Adobe). Image manipulations were restricted to linear level adjustments and cropping.

Functional Ca\textsuperscript{2+} imaging

For detecting Ca\textsuperscript{2+} fluxes, cultured cells were incubated with the Ca\textsuperscript{2+} indicator fluo-3 AM (20 μM; Molecular Probes) for 0.75–1 h at 37°C. The cultures were placed in a recording chamber (500 μl volume) mounted on an inverted microscope (Olympus IX70) and perfused with artificial cerebrospinal fluid (ACSF) at 2 ml/min. ACSF contained (in mM) 124 NaCl, 26 NaHCO\textsubscript{3}, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, 3.2 KCl, 1.2 MgSO\textsubscript{4}, 2.4 CaCl\textsubscript{2}, and 20 glucose. To prepare nominally Ca\textsuperscript{2+}-free ACSF (0 Ca\textsuperscript{2+}), the Ca\textsuperscript{2+} was replaced with equimolar Mg\textsuperscript{2+}, and 1 mM ethylene glycol-bis[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid was added.

The following drugs were applied through the perfusate (2 ml/min): 10 μM 6-cyano-7-nitroquinolinoline-2,3-dione (CNQX), 10 μM thapsigargin, and 10 μM ryanodine. Stimulation of visually identified neurons was accomplished by pressure application of agonists through the tip of a large patch pipette (7–9 m diam) placed within 40 μm of the selected cells. Agonists were applied at the following concentrations: glutamate, 10 or 20 μM; quisqualate, 10 μM; and caffeine, 10 mM, with final concentrations in 0 Ca\textsuperscript{2+} ACSF. All drugs were obtained from Research Biochemicals International, except for glutamate and caffeine, which were obtained from Sigma. Pressure pulses, 30 ms to 3 s in duration, were applied to the back of the pipette with a Picospritzer (General Valve).

Fluorescent signals were collected through either a ×40 (N.A. 1.35) or ×60 (N.A. 1.40) objective (Olympus) using a dichroic mirror and filter set that generate an excitation wavelength centered on 490 nm and limited emission to between 518 and 542 nm. To reduce photobleaching, neutral density filters were used, and a shutter prevented illumination of the samples except during data acquisition.

Fluorescent images were collected with an intensified, cooled charge-coupled device (CCD; Princeton Instruments). Individual frames were obtained by integrating the fluorescent signal on the CCD chip for 10–20 ms, and frames were collected every 250 ms. The images were digitized at 16 bits and captured with the software WinView (Princeton Instruments) on a Pentium based personal computer. Analyses were performed in IPLabs (Signal Analytics) on a Power Macintosh 9500.

Changes in fluorescence were determined by calculating peak fluorescence changes in the form of ΔF/ΔF\textsubscript{o}, where F\textsubscript{o} is the average fluorescence value of a region of interest over 10 frames directly preceding drug application and ΔF is the difference between the fluorescence value of a region of interest and the baseline signal (F\textsubscript{o}). This ratio is reported as percent change; unless otherwise indicated, these ratios were calculated for the somatic region of the cell. Cells were considered to have responded to drug application if a change of >1% relative to baseline. This value was arbitrary, but none of the cells that failed to respond showed a >1% change in fluorescence over baseline. All group data are expressed as means ± SE.

Estimates of [Ca\textsuperscript{2+}], from fluorescent values were calculated as described by Kao (1994), using the divalent cation ionophore 4-bromo-A23187 (10 μM; Sigma), in the presence of 4 mM Mn\textsuperscript{2+} in 0 Ca\textsuperscript{2+}, and by permeabilizing the cells with digitonin (2 mM, Sigma).

RESULTS

Characterization of cultured neurons

Dissociated cultures of the olfactory bulb contained a heterogeneous population of cells with respect to size and mor-
One class of cells appeared phase-bright in phase contrast microscopy, had a large, clear nucleus with a prominent nucleolus, and extended processes. These features are characteristic of neurons in primary dissociated cultures (Banker and Goslin 1991). To confirm that these cells were neurons, they were stained for the neuron-specific marker β-tubulin-III (Sullivan et al. 1986). All cells identified as neurons using phase-contrast microscopy stained positively for β-tubulin-III (Fig. 1A). The neurons typically developed on top of a confluent, monolayer of glial cells.

Previous studies suggest that morphological criteria can be used to identify two classes of neurons in dissociated olfactory bulb cultures: projection (mitral/tufted) and intrinsic (periglomerular/granule) cells (e.g., Trombley and Westbrook 1990). To determine whether these classes of neurons exist in our culture system, quantitative morphometric approaches and immunocytochemical techniques were used to characterize the cultured neurons. The area of the somata of β-tubulin-III labeled cells, measured from two-dimensional confocal images, ranged from 38 to 252 μm² (70.3 ± 3.6 μm²). Most neurons were classified as multipolar (58/104; 56%) or bipolar (24%), based on the patterns of processes emanating from their somata. A smaller number of neurons (20%) had a distinct apical dendrite that tapered gradually from its origin at the parent soma and sometimes terminated in a dendritic “tuft” characteristic of mitral/tufted cells (Fig. 1A). Although these neurons resemble mitral and tufted cells, the size of their somata did not differ significantly from that of the multipolar or bitufted cells. It was thus not possible to determine, using morphological criteria alone, whether the different classes of neurons in dissociated cultures correspond to the two major groups of cells in the olfactory bulb in vivo: projection (mitral/tufted) and intrinsic neurons.

Because many types of intrinsic neurons in the olfactory bulb—including periglomerular and granule cells—contain the neurotransmitter γ-aminobutyric acid (GABA) (see Shipley and Ennis 1996), we further characterized the cultured neurons by immunocytochemical localization of GAD, the rate-limiting enzyme in GABA synthesis (Fig. 1B). Double labeling for GAD and β-tubulin-III revealed that GAD is expressed in the vast majority of cultured neurons (292/306; 95%). The proportion of GAD(+) neurons in our cultures is similar to the ratio of intrinsic to projection neurons in the olfactory bulb in vivo: projection (mitral/tufted) and intrinsic neurons.

**FIG. 1.** Morphology and neurochemistry of cultured olfactory bulb neurons. A: digitized phase-contrast image showing a pyramidal-shaped neuron (p) having an apical dendrite that terminates in a tuft and a multipolar neuron (m). B–E: pseudocolor confocal images of cultured olfactory bulb neurons. B: cells stained for neuronal marker β-tubulin-III (red). Bipolar cell (right) also stains for glutamic acid-decarboxylase (GAD; green), whereas presumptive mitral/tufted cell is GAD(−). C: almost all neurons, identified by their β-tubulin-III staining (red), also stained for inositol 1,4,5-triphosphate receptor (IP₃R; green). Nonneuronal cells, presumably astrocytes, were also IP₃R(−). D and E: double-labeling for both IP₃R (green) and GAD (red) demonstrated that most, but not all (E) IP₃R(+) neurons are GAD(+). F: combined Nomarski-optics and fluorescence confocal image demonstrating binding of labeled ryanodine (green) in a cultured neuron. Note that astrocytes are not labeled. Scale bars represent 20 μm.
neurons in the olfactory bulb in vivo (see Shepherd 1990). Most neurons that did not express GAD had pyramidal shaped somata that were, on average, larger than those of GAD(+) cells (area: 91.0 ± 19.0 μm² vs. 50.2 ± 2.7 μm²; n = 306). Although some neurons with large somata (area ≥55 μm²) were GAD(+), a significant proportion (25%) of these large neurons were GAD(−) and therefore classified as presumptive mitral/tufted cells. Therefore, in the Ca²⁺ imaging experiments (described below) we selected both large and small neurons in an attempt to include in the analyses both presumptive projection and intrinsic neurons.

To determine whether different classes of cultured neurons express IP₃R, we immunolabeled neurons using an antibody to IP₃R that was previously characterized by Sharp et al. (1993). IP₃R labeling was present in both glia and in neurons, where labeling was most dense in the somata and proximal dendrites. To determine whether IP₃R is expressed in specific cell types, we double labeled cell cultures using antibodies to both β tubulin-III and IP₃R (Fig. 1C). IP₃R was expressed in 91% of the neurons (94/103). There were no differences in either morphology or size of IP₃R(+) and IP₃R(−) neurons, suggesting that all classes of cultured olfactory bulb neurons express IP₃R. To further test whether IP₃R is expressed preferentially in specific cell types, double-labeling using antibodies to GAD and IP₃R was performed (Fig. 1, D and E). The vast majority of GAD(+) cells examined (123/125; 98.4%) also stained for IP₃R, indicating that essentially all cultured GABAergic neurons express IP₃R. A small proportion (4/87; 4.6%) of the IP₃R(+) neurons did not stain for GAD, suggesting that excitatory neurons also express IP₃R.

To determine the expression patterns of the ryanodine receptor in cultured neurons, in vitro cell cultures were treated with a monosubstituted BODIPY FL-X ryanodine derivative (500 nM). This resulted in labeling in the somata and dendrites of all neurons examined (Fig. 1F). When BODIPY FL-X ryanodine was coapplied with 10 μM unlabeled ryanodine, no staining was observed. These findings indicate that all types of cultured olfactory bulb neurons express ryanodine-binding sites, presumably representing ryanodine receptors. Unlike the IP₃R, which also was expressed by glia, no ryanodine labeling was detected in the astrocyte layer of the cultures (Fig. 1F).

In summary, all classes of neurons express IP₃R and ryanodine binding sites, and these are distributed in both the somata and dendrites. These findings imply that CICR and IP₃-sensitive intracellular release pathways are present in both the soma and dendrites of these neurons. This hypothesis was tested directly with the use of Ca²⁺ imaging approaches.

Intracellular Ca²⁺ responses

We examined the distribution of intracellular Ca²⁺ release pathways in cultured olfactory bulb neurons using drug application combined with functional Ca²⁺ imaging. To identify changes in intracellular Ca²⁺ concentrations ([Ca²⁺]), we bulk loaded the olfactory bulb cultures with the fluorescent Ca²⁺ indicator fluo-3 AM and recorded changes in fluorescence. To generate grouped data, uncorrected fluorescence data were normalized to changes in fluorescence as percent change over baseline (ΔF/F₀). To describe changes in peak responses of an individual cell under different conditions, percent differences in uncorrected fluorescence peak amplitudes were calculated. This allowed comparisons of responses between cells and the use of consistent criteria for determining the presence of evoked Ca²⁺ fluxes. Because Ca²⁺ release from intracellular stores is sensitive to [Ca²⁺], we also performed a semiquantitative calibration (Kao 1994), which revealed resting [Ca²⁺], levels that ranged between 65 and 80 nM (74 ± 1 nM; n = 12), values that are consistent with those reported for other types of CNS neurons (Regehr et al. 1989; Shmigol et al. 1994).

We first tested the response of cultured olfactory bulb neurons to glutamate—the primary excitatory neurotransmitter in the bulb (Ennis et al. 1996; Trombley and Westbrook 1990). After a brief pressure application of glutamate (30–300 ms, 10 or 20 μM) near the soma, olfactory bulb neurons in normal ACSF responded with a robust increase in [Ca²⁺], in both their somata (mean peak ΔF/F₀ = 159 ± 26%; n = 6) and dendrites (mean peak ΔF/F₀ = 89 ± 9%; n = 7 dendrites from 3 cells; Fig. 2C, left). In all cells, the initial response in Ca²⁺-containing ACSF was immediate, occurring within the first 250 ms. The total duration and rate of decay of these responses showed a variety of kinetics, both between cells and among trials from the same cell. Often there was a long-lasting plateau in the response, as shown in Fig. 2C. Other types of responses were relatively rapid and monophasic or long-lasting with multiple peaks. In the presence of Ca²⁺-containing ACSF, the origin of these responses could not be determined because glutamate can activate a number of different receptors. Therefore, the Ca²⁺ fluxes in Ca²⁺-containing ACSF could be mediated by a number of Ca²⁺ sources. These include activation of Ca²⁺-permeable NMDA receptors or other trans-membrane ionotropic receptors, leading to voltage-dependent calcium channel gating, as well as release from intracellular Ca²⁺ stores.

To test if cultured olfactory neurons can generate Ca²⁺ fluxes that are dependent only on intracellular Ca²⁺ stores, glutamate was applied after 5 min of superfusion with nominally Ca²⁺-free ACSF (0 Ca²⁺). In averaged group data of normalized responses, only a slightly smaller change in fluorescence was seen in 0 Ca²⁺ compared with responses in normal ACSF (mean peak ΔF/F₀ = 152 ± 25%; n = 6; in dendrites 85 ± 15%; n = 7; Fig. 2C, right). This similarity in ΔF/F₀ values in 0 Ca²⁺ and in normal ACSF did not imply that nearly the same levels of [Ca²⁺] were reached under these two conditions because ΔF/F₀ normalizes the change in fluorescence to the initial baseline, and the baseline fluorescence was always lower in 0 Ca²⁺. This was represented when the peak fluorescence responses in normal ACSF and in 0 Ca²⁺ were compared for each individual cell: the peak response was always less in 0 Ca²⁺ (83 ± 6% of the ACSF response).

These results indicate that olfactory bulb neurons can generate Ca²⁺ fluxes in the absence of external Ca²⁺, suggesting that these Ca²⁺ fluxes originate from intracellular Ca²⁺ stores.

IP₃-sensitive stores

To test for the presence of IP₃-dependent intracellular Ca²⁺ release, we used quisqualate, an agonist of the type 1
and 5 metabotropic glutamate receptors (mGluR1,5) (Aramori and Nakanishi 1992; Sladecek et al. 1985; Tanabe et al. 1992). Activation of mGluR1,5 has been shown to evoke the production of IP3 in neurons through a G-protein–mediated process linked to phospholipase C (Kirischuk et al. 1995; Seymour-Laurent and Barish 1995; Verkhratsky and Kettenmann 1996). Therefore, quisqualate can be used to assay Ca2+ release from IP3-sensitive Ca2+ stores. Because quisqualate is also a potent and selective agonist of the ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors (Mayer and Westbrook 1987), as well as a mGluR1,5 agonist, Ca2+ transients evoked in normal ACSF could arise from both transmembrane sources and intracellular stores. This allowed us to use two approaches to isolate IP3-dependent Ca2+ release from intracellular stores. First, by lowering external [Ca2+] to limit transmembrane Ca2+ influx, and second, by blocking the AMPA receptor-mediated responses to suppress ionotropic receptor activation and the concomitant changes in membrane potential, thereby limiting Ca2+ fluxes through both Ca2+-permeable AMPA channels and voltage-activated Ca2+ channels.

In normal ACSF, quisqualate application produced a variable rise of [Ca2+], in all neurons (mean peak ΔF/Fo = 198 ± 16%; n = 40), similar to that seen after glutamate application (Figs. 3A and 4A). In 0 Ca2+, intracellular Ca2+ fluxes occurred in nearly all neurons tested (35/40; mean peak response ΔF/Fo = 77 ± 12%; Figs. 3B and 4B). Consistent with the assumption that this response was due to activation of IP3 receptors via mGluR1,5 activation, similar results also were obtained in normal ACSF when the AMPA receptor antagonist CNQX was applied to suppress the ionotropic actions of quisqualate, as shown in Fig. 3D (peak ΔF/Fo = 56 ± 21%; n = 6). Similar to the responses after glutamate application, the quisqualate-evoked Ca2+ fluxes occurred simultaneously in both dendrites and somata. Because Ca2+ diffusion is restricted (Allbritton et al. 1992), this finding suggests that IP3-sensitive stores are distributed in both these compartments (Fig. 2).

To confirm that Ca2+ fluxes in the presence of 0 Ca2+-represented release from intracellular stores, thapsigargin was bath-applied. Thapsigargin causes depletion of intracellular stores by inactivating the ATPase responsible for loading Ca2+ stores against the Ca2+ concentration gradient (Thastrup et al. 1990). Pretreatment of the cultures with 10 μM thapsigargin for 20–30 min completely suppressed quisqualate-induced responses in 0 Ca2+, whereas Ca2+ fluxes still could be evoked by quisqualate in normal ACSF (n = 3).

Each cell from which Ca2+ imaging data were obtained also was included in anatomic analyses to determine its size and morphology, and, whenever possible, its GAD and IP3R immunoreactivity (e.g., Fig. 4B). There was no correlation between the amplitude and kinetics of the responses of individual neurons to glutamate or to quisqualate and their characterization as presumptive projection or intrinsic neurons.

**Responses in glia**

In nonneuronal cells, glutamate or quisqualate application rarely evoked Ca2+ fluxes (4 cells in 53 image fields), despite the presence of putative astrocytes in each field, adjacent to neurons in the field that were activated in 0 Ca2+, and despite the fact that nonneuronal cells stained positive for IP3R (Figs. 1C and 4B). The failure to evoke Ca2+ fluxes in these putative glial cells is consistent with the reported...
Caffeine has been shown to activate these stores while suppressing IP_{3}-induced Ca^{2+} release (Henzi and MacDermott 1992; Irving et al. 1992; Parker and Ivorra 1990) and therefore is a commonly used agonist to isolate the presence of ryanodine-sensitive stores (Ehrlich et al. 1994; Irving et al. 1992; O’Neil et al. 1990). To select viable neurons, caffeine was applied to cells that showed a quisqualate or glutamate response. Caffeine application triggered a monophasic rise in [Ca^{2+}]_{i} (Fig. 5A), and this response was evoked in almost all cells tested (n = 12/13, peak ΔF/F_{i} = 113 ± 25%). As illustrated in Fig. 5A, caffeine-induced Ca^{2+} fluxes also were evoked in 0 Ca^{2+} with kinetics similar to responses evoked in normal ACSF, but the amplitude of the responses in 0 Ca^{2+} were smaller (n = 9/11, peak ΔF/F_{i} = 74 ± 19%; P = 0.049, single factor analysis of variance).

To confirm that the caffeine-induced response was due to activation of ryanodine-sensitive stores, ryanodine was bath-applied in normal ACSF to suppress release from ryanodine-sensitive stores (Irving et al. 1992). Although ryanodine is an agonist of ryanodine receptors at low concentrations (nanomolar), it acts as an antagonist at higher concentrations (Ehrlich et al. 1994; Henzi and MacDermott 1992). As shown in Fig. 5B, bath application of 10 μM ryanodine for 20 min completely suppressed caffeine-induced Ca^{2+} fluxes in both normal ACSF and 0 Ca^{2+} (n = 4). As evidence that Ca^{2+} stores in these cells remained viable after this treatment, partial restoration of this response was produced after a 40-min washout of ryanodine in normal ACSF (Fig. 5B). Caffeine application failed to evoke Ca^{2+} fluxes in all of the nonneuronal cells examined, suggesting that these cells may lack a comparable ryanodine-sensitive release mechanism.

As with the glutamate- and quisqualate-mediated responses, caffeine application evoked Ca^{2+} fluxes in both the soma and proximal dendrites of these neurons. Furthermore, every cell that responded to caffeine also showed IP_{3}-sensitive release (n = 12). There was no correlation between the responses of individual neurons to various antagonists and their characterization as presumptive projection or intrinsic neurons. Thus both forms of intracellular Ca^{2+} release are present in a heterogeneous population of cultured olfactory bulb neurons and are colocalized in the soma and the dendrites.

**DISCUSSION**

The aim of this study was to identify the mechanisms of Ca^{2+} release from intracellular stores in cultured olfactory bulb neurons. As demonstrated above, olfactory bulb cultures contain a heterogeneous population of neurons, including presumptive projection and intrinsic neurons. Consistent with the relative proportions of these cell types in vivo, the vast majority of the neurons are GABAergic, presumably representing periglomerular and granule cells. These findings are in agreement with previous descriptions of cultured olfactory bulb neurons (Trombley and Westbrook 1990).

The results demonstrate that an IP_{3} and a CICR agonist can both generate Ca^{2+} release from intracellular stores in the somata and dendrites of a heterogeneous population of neurons. Although the presence of CICR was not directly tested, the ability of caffeine to generate release from these

![FIG. 3. Release of Ca^{2+} from intracellular stores evoked by IP_{3}R activation after quisqualate application. Changes in fluorescence plotted as a function of time calculated for soma of a cultured neuron. Quisqualate (10 μM) was pressure applied for 300 ms (A). In normal ACSF (A), fluorescence changes represent Ca^{2+} transients through both plasma membrane and from intracellular stores. Intracellular release component is revealed in 0 Ca^{2+} (B) and also is demonstrated by effects of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; D).](image-url)
stores and of ryanodine to antagonize release at micromolar concentrations is a defining pharmacological characteristic of the receptors involved in CICR (Ehrlich et al. 1994; Henzi and MacDermott 1992; Irving et al. 1992; O’Neil et al. 1990). The changes in [Ca\(^{2+}\)] recorded in the somatic and dendritic regions of the cells are likely to represent Ca\(^{2+}\) release from localized intracellular stores, because Ca\(^{2+}\) diffusion is known to be highly restricted (Allbritton et al. 1992). This suggests that both IP\(_3\)- and ryanodine-dependent Ca\(^{2+}\) release mechanisms exist in both the soma and dendrites of cultured olfactory bulb neurons. Support for the presence of these Ca\(^{2+}\) release pathways also is derived from immunocytochemical findings of IP\(_3\)R expression and ryanodine binding in almost all of the neurons examined, irrespective of their morphology, size, or GAD immunoreactivity. Thus we demonstrate that cultured olfactory bulb neurons support both IP\(_3\)- and ryanodine-sensitive Ca\(^{2+}\) release and that these two sources of Ca\(^{2+}\) are colocalized in both somatic and dendritic compartments.

The functional imaging methods used in this study limited us to a qualitative description of both these release pathways. Fluo-3 responds to changes in [Ca\(^{2+}\)], nonlinearly, and it is difficult to control for differences in [fluo-3], that would affect calibration of [Ca\(^{2+}\)], especially at higher [Ca\(^{2+}\)]. Furthermore, when peak [Ca\(^{2+}\)] levels were estimated they often indicated levels that could saturate fluo-3, rendering it impossible to accurately estimate the amplitudes and kinetics of evoked Ca\(^{2+}\) fluxes (Kao 1994). The results do show that many of these response are large, generating >10-fold changes in [Ca\(^{2+}\)].

We found no correlation between response amplitudes or kinetics and the size or morphology of the cells. The variability of the responses may be attributed to a number of sources. These include variations in the effective concentration of the pressure-applied agonists, differences in intracellular fluo-3 fusion is known to be highly restricted (Allbritton et al. 1992) . This suggests that both IP\(_3\) - and ryanodine-dependent concentrations, and the physiological status of the cells.

**IP\(_3\)-mediated Ca\(^{2+}\) release**

The presence of IP\(_3\)-dependent stores was demonstrated by the ability of mGluR\(_{1,5}\) receptors to generate intracellular Ca\(^{2+}\) fluxes in nominally Ca\(^{2+}\)-free extracellular solutions. In the in vivo olfactory bulb, IP\(_3\)-dependent Ca\(^{2+}\) release may be evoked by activation of various metabotropic receptors. These include serotonin receptors type 2, muscarinic receptors type 1, \(\alpha_1\)-adrenoceptors (reviewed in Shipley et al. 1996) as well as mGluR (van den Pol 1995). Thus the intracellular Ca\(^{2+}\) release demonstrated here may be activated in vivo by olfactory nerve inputs, intrinsic glutamatergic activity, and several of the afferent systems projecting to the olfactory bulb.

Our finding that IP\(_3\)-sensitive Ca\(^{2+}\) stores are expressed in a heterogeneous population of neurons is consistent with other studies of cultured olfactory bulb neurons. Tani et al.
CA 2+ STORES IN CULTURED Olfactory BULB NEURONS 2183

(1992) reported that cultured mouse olfactory bulb interneurons respond to serotonin with an increase in [Ca 2+]i in the absence of extracellular Ca 2+. Similarly, putative mitral/tufted cells produce Ca 2+ fluxes in 0 Ca 2+ after activation of mGluRs (Geiling and Schild 1996; van den Pol 1995). Geiling and Schild (1996) report that in cultures of the Xenopus laevis tadpole olfactory bulb, glutamate-dependent Ca 2+ release from intracellular stores is present only in projection neurons. In contrast, our study in the rat demonstrates that IP3-dependent release occurs in all types of cells. The disparity in the results may reflect a species difference or different culture conditions. Alternatively, these differences may be due to the developmental stage of the cells studied—in contrast to the Xenopus tadpole data, our findings are based on studies of cultures prepared from postnatal rats. Support for this hypothesis is derived from a recent study in our laboratory that demonstrated a developmental shift in IP3, R expression in the rat olfactory bulb in vivo (Slawecki et al. 1996). Specifically, we find that during the first postnatal week, IP3, R is expressed exclusively in mitral cells (consistent with Dent et al. 1996) and that IP3, R expression in intrinsic neurons gradually develops during the second and third postnatal weeks concomitant with a reduction in IP3, R expression in mitral cells. By the fourth postnatal week, an adult pattern of IP3, R expression develops in which the receptor is expressed preferentially in GABAergic periglomerular cells and in a distinct population of interneurons in the granule cell layer (Slawecki et al. 1996).

If the IP3, R in vivo is expressed preferentially in subclasses of olfactory bulb neurons, why do essentially all cultured bulbar neurons express IP3, R-mediated Ca 2+ release? One possibility is that the developmental cues found in vivo may be lacking in culture. Although olfactory bulb cultures can produce a diverse population of cell types with many of the characteristic neurotransmitters, receptors, and synaptic interconnections described in the intact olfactory bulb (present study and see Kim 1972; Trombley 1994; Trombley and Westbrook 1990; van den Pol 1995), they lack the appropriate peripheral inputs that may function to regulate the development of these cells into their mature in vivo phenotype. This possibility suggests that expression of IP3, R is activity dependent. The developmental shifts in IP3, R expression described above (Slawecki et al. 1996) support this hypothesis. This hypothesis also is supported by reports of activity-dependent down regulation of IP3, R expression (Wojcikiewicz 1995; Wojcikiewicz and Oberdorf 1996).

**Calcium-induced calcium release**

The ability of ryanodine to block caffeine-evoked Ca 2+ fluxes in both normal ACSF and in the absence of external Ca 2+ suggests that caffeine triggers Ca 2+ release from ryanodine-sensitive intracellular stores. However, peak responses in Ca 2+ -free ACSF averaged only 60 ± 24% of the caffeine induced responses in normal ACSF, and a few cells did not respond at all in 0 Ca 2+. It is possible that caffeine may activate another source of Ca 2+ in the presence of extracellular Ca 2+. Alternatively, these results are consistent with findings that Ca 2+ release from ryanodine-sensitive stores is sensitive to Ca 2+ depletion and cytoplasmic [Ca 2+] (Shmigol et al. 1994). Thus incubation in 0 Ca 2+ may decrease the
[Ca\textsuperscript{2+}], available in the CICR pool for caffeine-dependent release or suppress the responses by modulating the sensitivity or activity of ryanodine receptors.

Activation of ryanodine-dependent CICR in bulbar neurons in vivo may be triggered by a number of mechanisms that evoke increases in [Ca\textsuperscript{2+}]. These mechanisms are likely to include both trans-membrane Ca\textsuperscript{2+} fluxes and Ca\textsuperscript{2+} release from IP\textsubscript{3}-dependent stores. It is therefore possible that some portion of the IP\textsubscript{3}-mediated responses described in the present study may include CICR, triggered by Ca\textsuperscript{2+} efflux from IP\textsubscript{3}-sensitive stores. Because both IP\textsubscript{3} and ryanodine-dependent stores are modulated by [Ca\textsuperscript{2+}], in a biphasic manner (Simpson et al. 1995), Ca\textsuperscript{2+} release from each of these stores can activate, facilitate, or suppress release from the other. Therefore, coactivation of these stores along with transmembrane Ca\textsuperscript{2+} fluxes may interact to produce complex variations in [Ca\textsuperscript{2+}], responses to both ionotropic and metabotropic receptor activation.

The authors are grateful to Drs. Mordecai P. Blaustein, Mathew Ennis, Joseph P. Kao, and Daniel Weinreich for valuable comments on this manuscript.

This work was supported in part by National Institute of Neurological Disorders and Stroke Grants NS-31078 and NS-35360.

Address for reprint requests: A. Keller, Dept. of Anatomy and Neurobiology, University of Maryland School of Medicine, 685 W. Baltimore St., Baltimore, MD 21201.

E-mail: akeller@umabnet.ab.umd.edu

Received 28 January 1997; accepted in final form 29 April 1997.

REFERENCES


Henzl, V. and MacDermott, A. B. Characteristics and function of Ca\textsuperscript{2+} and inositol 1,4,5-releasable stores of Ca\textsuperscript{2+} in neurons. Neuroscience 46: 251–273, 1992.


Kohda, K., Inoue, T., and Mikoshiba, K. Ca\textsuperscript{2+} release from Ca\textsuperscript{2+} stores, particularly from ryanodine-sensitive Ca\textsuperscript{2+} stores, is required for the induction of LTD in cultured cerebellar purkinje cells. J. Neurophysiol. 74: 2184–2188, 1995.


O'Neill, S. C., Donoso, P., and Eisein, D. A. The role of [Ca\textsuperscript{2+}], and Ca\textsuperscript{2+} sensitization in the caffeine contracture of rat myocytes: measurement of [Ca\textsuperscript{2+}] and [caffeine]. J. Physiol. (Lond.) 425: 55–70, 1990.


WOJCIECHOWICZ, R. J. H. Type I, II, and III inositol 1,4,5-trisphosphate receptors are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. *J. Biol. Chem.* 270: 11678–11683, 1995.