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Drosophila Mushroom Body Kenyon Cells Generate Spontaneous Calcium Transients Mediated by PLTX-Sensitive Calcium Channels

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Jiang, Shaojuan Amy, Jorge M. Campusano, Hailing Su, and Diane K. O'Dowd. *Drosophila* mushroom body Kenyon cells generate spontaneous calcium transients mediated by PLTX-sensitive calcium channels. *J Neurophysiol* 94: 491–500, 2005. First published March 16, 2005; doi:10.1152/jn.00096.2005. Spontaneous calcium oscillations in mushroom bodies of late stage pupal and adult *Drosophila* brains have been implicated in memory consolidation during olfactory associative learning. This study explores the cellular mechanisms regulating calcium dynamics in Kenyon cells, principal neurons in mushroom bodies. Fura-2 imaging shows that Kenyon cells cultured from late stage *Drosophila* pupae generate spontaneous calcium transients in a cell autonomous fashion, at a frequency similar to calcium oscillations in vivo (10–20/h). The expression of calcium transients is up regulated during pupal development. Although the ability to generate transients is a property intrinsic to Kenyon cells, transients can be modulated by bath application of nicotine and GABA. Calcium transients are blocked, and baseline calcium levels reduced, by removal of external calcium, addition of cobalt, or addition of *Plectreurys* toxin (PLTX), an insect-specific calcium channel antagonist. Transients do not require calcium release from intracellular stores. Whole cell recordings reveal that the majority of voltage-gated calcium channels in Kenyon cells are PLTX-sensitive. Together these data show that influx of calcium through PLTX-sensitive voltage-gated calcium channels mediates spontaneous calcium transients and regulates basal calcium levels in cultured Kenyon cells. The data also suggest that these calcium transients represent cellular events underlying calcium oscillations in the intact mushroom bodies. However, spontaneous calcium transients are not unique to Kenyon cells as they are present in approximately 60% of all cultured central brain neurons. This suggests the calcium transients play a more general role in maturation or function of adult brain neurons.

INTRODUCTION

Transient increases in intracellular calcium regulate a wide range of cellular processes in developing and mature neurons, from rapid enhancement of neurotransmitter release to long-lasting changes in gene expression (Carey and Matsumoto 1999; Emptage et al. 2001; Spitzer et al. 2004; Syed et al. 2004; Yuste et al. 1992). In *Drosophila*, GAL4 driving expression of the calcium-sensitive luminescent protein, apoaequorin, reveals slow rhythmic oscillations in intracellular calcium levels localized in mushroom bodies, a region of the insect brain required for olfactory associative learning (Rosay et al. 2001). An increase in oscillation amplitude in the mushroom bodies of *amnesiac* memory mutants suggests these slow wave calcium oscillations may contribute to memory consolidation in the fly.

Calcium oscillations are not only present in intact flies, but also occur in isolated brains, indicating these spontaneous events are independent of sensory input (Rosay et al. 2001). Blockade of a variety of voltage-gated ion channels and neurotransmitter receptors modulate the oscillation amplitude and/or frequency. However, because the oscillations reflect synchronized changes in calcium in large populations of mushroom body cells, it has not been possible to determine the contribution of intrinsic membrane properties versus intercellular communication in generation of this activity. Assessment of the biophysical mechanisms involved in generating the oscillations requires the ability to resolve calcium dynamics in single cells.

Cameleons and camgaroos, two other calcium sensors that have also been transgenically expressed in the fly, have provided increased spatial resolution in analysis of calcium dynamics. These have been used to discriminate between odor-evoked responses in pre- and postsynaptic regions, or between acetylcholine (ACh)-evoked responses in different lobes, of the mushroom bodies (Fiala et al. 2002; Yu et al. 2003). However, neither of these calcium sensors has been used to follow intracellular calcium levels at the resolution of individual Kenyon cells in the intact fly brain. In contrast, it has been possible to examine calcium levels in single *Drosophila* neurons using membrane permeant fluorescent dyes. Calcium green-1 reveals calcium responses to odorant stimuli in one or a small number of Kenyon cells in the semi-intact fly (Wang et al. 2001). Fura-2 imaging shows changes in intracellular calcium in response to depolarization and other stimuli, including application of acetylcholine, in “giant neurons” in embryonic *Drosophila* cultures treated with cytochalasin B (Alshuaib et al. 2004; Berke and Wu 2002). However, neither the study of Kenyon cells in the brain nor giant embryonic neurons in culture have reported detection of spontaneous fluctuations in intracellular calcium.

To explore the cellular mechanisms involved in regulating calcium levels in individual mushroom body Kenyon cells, we employed fura-2 imaging and electrophysiological analysis of neurons harvested from the central brain region of late pupal stage *Drosophila*. When grown in dissociated cell culture, these neurons regenerate processes and form functional synaptic connections, and Kenyon cells can be identified by cell-specific expression of green fluorescent protein (GFP) using the GAL4 system (Su and O'Dowd 2003). Here we report that Kenyon cells generate spontaneous, transient increases in intracellular calcium, mediated by *Plectreurys* toxin (PLTX)-sensitive voltage-gated calcium channels. The ability to gen-

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erate transients is up regulated during pupal development, transient frequency is similar to the frequency of calcium oscillations *in vivo*, but spontaneous calcium transients are not specific to Kenyon cells. This suggests that, while the transients are likely to represent cellular events that contribute to calcium oscillations in the mushroom bodies, they may also play a more general role in adult brain neurons.

METHODS

Fly strains

Pupae were obtained from the mating of males from the homozygous enhancer trap line, OK107-GAL4 (Connolly et al. 1996), and females homozygous for a UAS-GFP transgene (both lines kindly provided by Y. Zhong). The OK107-GAL4;UAS-GFP pupae exhibit high levels of GFP expression in the cell bodies and processes of the mushroom body Kenyon cells in late stage pupae (Su and O'Dowd 2003). Kenyon cells in cultures were identified as GFP+ neurons with small soma diameters (3–6 μm).

Primary pupal cultures

Cultures were prepared as described previously with minor modifications noted below. Heads were removed from animals at ~55–78 h after pupation (Bainbridge and Brownes 1981). Immediately after decapitation, heads were placed in sterile dissecting saline (DS) containing (in mM) 137 NaCl, 5.4 KCl, 0.17 NaH_2PO_4 , 0.22 KH_2PO_4 , 33.3 glucose, 43.8 sucrose, and 9.9 HEPES, pH 7.4. The brains were removed, and the optic lobes were discarded. Central brain regions from 5 to 15 animals were incubated in dissecting saline containing 50 U/ml papain activated by L-cysteine (1.32 mM) for 15 min at room temperature. Tissue was washed (3 times with 1.5 ml) with DS and (2 times with 1.5 ml) with *Drosophila*-defined culture medium, DDM2 composed of Ham's F-12 DMEM (high glucose; Irvine Scientific, Santa Ana, CA) supplemented with 1 mg/ml sodium bicarbonate, 20 mM HEPES, 100 μM putrescine, 30 nM sodium selenite, 20 ng/ml progesterone, 50 $\mu\text{g}/\text{ml}$ insulin, 100 $\mu\text{g}/\text{ml}$ transferrin, and 1 $\mu\text{g}/\text{ml}$ of 20-hydroxyecdysone (Su and O'Dowd 2003). Each culture was prepared from a single brain transferred to a 5- μl drop of DDM2 culture medium on a ConA-laminin coated glass coverslip mounted to the bottom of a petri dish. The tissue was mechanically dissociated and the cells were allowed to settle to the substrate for 15 min. Dishes were flooded with 1.5 ml of DDM2 and maintained in a 23°C humidified 5% CO_2 incubator. After 24 h, 0.5 ml of a 3:1 mixture of DDM2 and conditioned Neurobasal medium (cNBM) was added to each dish. cNBM is neurobasal medium + B27 supplements (Life Technologies) conditioned for 24 h by non-neuronal mouse brain feeder cell cultures (Hilgenberg et al. 1999). Cultures were fed every 4–5 days by removing 1 ml of media from the dish and adding 1 ml of the 3:1 mixture of DDM2 and cNBM.

Culture dishes were prepared by punching 9-mm holes in the bottom of 35-mm petri dishes. Round 12-mm-diam glass coverslips were attached to the bottom of the dish with Sylgard. After fabrication, dishes were immersed in 70% EtOH for 5 min and allowed to air dry. Before coating, dishes were exposed to ultraviolet light for a minimum of 15 min. Coverslips were coated with ConA-laminin and used for culturing within 1 mo of coating (Kraft et al. 1998).

Calcium imaging

Cultured neurons were rinsed in a HEPES-buffered salt solution (HBSS) containing (in mM) 120 NaCl, 5.4 KCl, 1.8 CaCl_2 , 0.8 MgCl_2 , 15 glucose, 20 HEPES, 10 NaOH, and 0.001% phenol red, pH 7.2–7.4. They were loaded with calcium indicator dye by incubating cultures in HBSS containing 5 μM fura-2 AM and 0.1% Pluronic F-127 (Molecular Probes) for 40 min, in the dark, at room tempera-

ture. To allow for complete hydrolysis of the AM ester before imaging, cultures were washed three times with HBSS and incubated in fresh HBSS for another 45 min, in the dark, at room temperature. Fresh HBSS was added to the culture dish, and it was transferred to the stage of an inverted Nikon microscope for imaging with a $\times 40$, 1.3 numerical aperture oil immersion objective. Fluorescent illumination was provided by a 150-W xenon arc lamp, and band-specific filters were used for excitation, alternating between 340 and 380 nm. All images were acquired at the emission wavelength 510 nm and recorded with a digital CCD camera (Orca-100, Hamamatsu). The 340/380 ratio images and their pseudocolor representations were generated digitally using Metafluor 4.0 imaging software (Universal Imaging). Data were collected at 5-s intervals.

Estimates of intracellular calcium concentrations were based on the following formula

$$[\text{Ca}^{2+}] = K_d \cdot (R - R_{\min}) / (R_{\max} - R) \cdot F_o / F_s$$

where K_d is 225 nM (dissociation constant for fura-2) and R is the ratio of emission intensity at 510 nm after excitation at 340 and 380 nm (Grynkiewicz et al. 1985). R_{\min} is the ratio in a calcium-free solution, and R_{\max} is the ratio at saturating free Ca (39 μM). F_o and F_s are the emission intensities at 510 nm after excitation at 380 nm in calcium-free and calcium-saturated solutions, respectively. Background levels of fluorescence in calcium-free, fura-free solution were determined at each wavelength and subtracted from signals detected. Values for R_{\min} (0.43), R_{\max} (7.89), and F_o/F_s (8.85) were determined using the Fura-2 Calcium Imaging Calibration Kit (F-6774, Molecular Probes). Because the calibration is based on a cell-free system, the values for intracellular calcium levels reported for the cultured neurons are viewed as estimates that are primarily useful in comparison of changes in calcium levels under different experimental conditions.

Data analysis

Intracellular calcium levels were evaluated by determining the average signal over the soma region of all GFP+ Kenyon cells (7–40) in a single field of view in each OK107-GAL4;UAS-GFP culture. Neurons were included in the data set if a stable baseline, ≤ 150 nM calcium, was recorded for a minimum of 10 min, and an increase in the 340/380 ratio, > 600 nM, was induced by exposure to 5 μM ionomycin at the end of the experiment. A calcium transient was defined as an increase in 340/380 ratio, > 75 nM (> 5 times baseline noise level), that reaches its peak within 20 s and declines by $\geq 50\%$ from the peak within 3 min. In a single field of view, the percentage of active Kenyon cells reflects the number of Kenyon cells exhibiting one or more transients in the first 10-min recording period, divided by the total number of Kenyon cells. The transient frequency in each cell was calculated as the number of transients divided by the time of recording. The amplitude of each transient was measured from baseline to peak, and an average value for each cell was calculated. An average transient frequency and amplitude were determined for each culture. The mean percentage of active cells, transient frequency, and transient amplitude were calculated from three or more cultures (n) at each age/condition in which seven or more individual neurons were analyzed in each culture. Only one field of view was examined/culture and each culture was prepared from the brain of a single animal.

Electrophysiology

To examine calcium currents in isolation, the pipette solution contained (in mM) 120 D-gluconic acid, 120 cesium hydroxide, 20 NaCl, 0.1 CaCl_2 , 2 MgCl_2 , 1.1 EGTA, and 10 HEPES, pH 7.2. The external recording saline was composed of HBSS containing TTX, curare, PTX (TCP). To examine voltage-gated potassium currents in isolation, the pipette contained (in mM) 120 K-gluconate, 20 NaCl, 0.1 CaCl_2 , 2 MgCl_2 , 1.1 EGTA, 4 ATP, and 10 HEPES, pH 7.2. In addition, 2 mM CoCl_2 was added to the external recording saline

(TCP) to block the calcium currents and calcium-activated potassium currents. All data shown are corrected for the 5-mV liquid junction potential generated in these solutions. Data were acquired with a List EPC7 amplifier, a Digidata 1320A D-A converter (Axon Instruments), a Dell (Dimension 4100) computer, and pClamp 8 (Axon Instruments) software. Recordings were made at room temperature.

Drug application

The following drugs were bath-applied in specific experiments as indicated in the results: TTX (1 μ M, Alomone Labs), D-tubocurarine (curare, 20 μ M, Sigma), α -bungarotoxin (α -BTX, 0.1 μ M, Sigma), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 5 μ M, Sigma), D(-)-2-amino-5-phosphonopentanoic acid (APV, 50 μ M, Sigma), picrotoxin (PTX, 10 μ M, Sigma), verapamil (5 μ M, Sigma), omega-conotoxin GVIA (conotoxin, 2 μ M, Sigma), Plectreurys toxin (PLTX-II, 50 nM, Alomone Labs), and cobalt chloride (Co^{2+} , 2 mM, Sigma).

RESULTS

Cultured Kenyon cells generate spontaneous calcium transients

To explore the cellular mechanisms involved in regulation of calcium in neurons from the mushroom body region, intracellular calcium levels were monitored in Kenyon cells grown in primary culture. Each culture was prepared from a single central brain region harvested from a late stage OK107-GAL4; UAS-GFP pupa. The mushroom body Kenyon cells in these cultures were identified as small-diameter (3–6 μ m), GFP+ neurons (Su and O'Dowd 2003).

Fura-2 AM, a ratiometric calcium-imaging dye, was used to monitor calcium levels in the neurons. Fura-2 loaded neurons appeared healthy based on overall morphology and GFP+ Kenyon cells, representing 10–15% of the total population, were clearly identifiable when viewed with fluorescent illumination (Fig. 1A). A pseudo-color representation of the 340/380 ratio image of a single field, at two time-points during a 15-min recording in standard HBSS saline, revealed differences in the intracellular calcium levels in the cell bodies of the two indicated GFP+ Kenyon cells (Fig. 1, B and C). Changes in calcium levels were also observed in the processes of some Kenyon cells. However, due to the small diameter of the neuritic processes in these neurons, analysis of calcium fluctuations was limited to the soma region.

The 340/380 ratios in each cell were converted to estimates of intracellular calcium concentrations based on a standard calcium curve generated in a cell-free system (see METHODS for details). The highly dynamic nature of calcium levels in one Kenyon cell (indicated by red arrow) is shown in a plot of the intracellular calcium concentration as a function of time (Fig. 1D). Simultaneous analysis of a second Kenyon cell (indicated by purple arrowhead) that appeared to have no physical connection with the first, showed that these cells exhibit independent calcium fluctuations that occur with different frequencies and patterns (Fig. 1D). No evidence of synchronized activity between Kenyon cells was observed, even when the cell bodies were in direct physical contact. Of 14 pairs of Kenyon cells that were physically adjacent to each other, transients were observed in one Kenyon cell but not the other in five cases. In the other nine pairs, both Kenyon cells exhibited calcium transients, but there was no synchrony in the timing of the transients (Fig. 1E). Finally, examination of Kenyon cells in

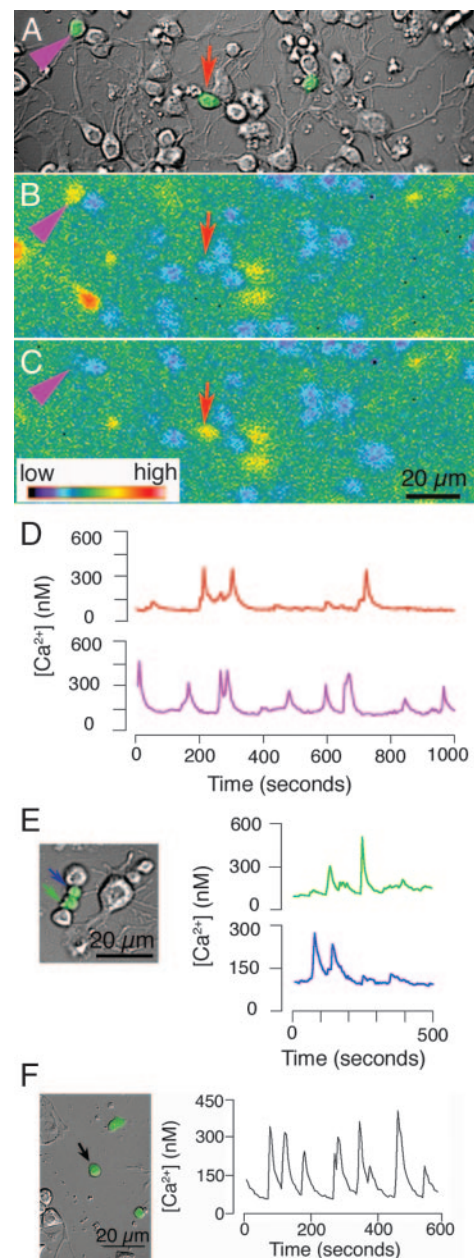


FIG. 1. Spontaneous calcium transients in cultured mushroom body Kenyon cells. *A*: pupal neurons loaded with calcium indicator dye fura-2 exhibit normal morphology. Kenyon cells are identified as small diameter, green fluorescent protein (GFP)+ neurons in a 2-day-old culture prepared from an OK107-GAL4;UAS-GFP pupa 72 h after puparium formation (APF). Fluorescent mask projected on the Nomarski image. *B* and *C*: color-coded, ratiometric images (340/380) of same field of view, examined in standard HBSS recording saline, shows a difference in calcium levels in the soma region of 2 Kenyon cells (arrows, arrowheads) viewed at different times. *D*: intracellular calcium concentration as a function of time, determined over the soma region of the indicated Kenyon cells. *E*: physically connected pair of Kenyon cells exhibit nonsynchronous spontaneous calcium transients. *F*: calcium transients in a physically isolated Kenyon cell that has not yet extended neurites. Values plotted in *D–F* represent the background corrected average 340/380 ratios, converted to estimates of calcium levels based on a standard calcium curve generated in a cell-free system. Calcium levels evaluated in a circular field placed over the soma region of the neurons, sampled at 5-s intervals.

low-density cultures within 1–2 days of plating showed that physically isolated Kenyon cells can also generate calcium transients (Fig. 1F).

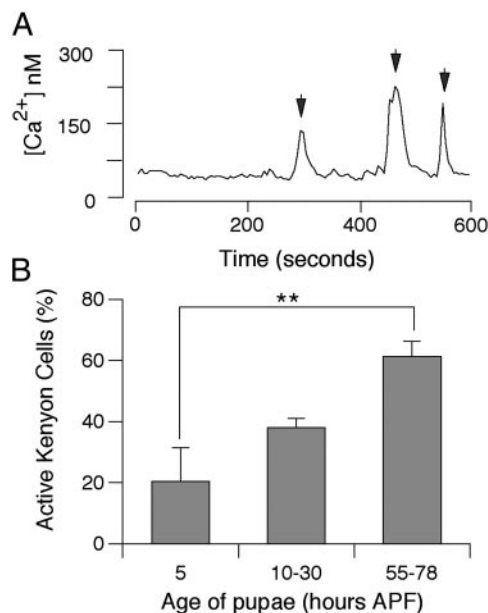


FIG. 2. Percentage of active Kenyon cells is significantly higher in cultures prepared from late vs. early stage pupae. *A*: neurons are classified as active based on the presence of 1 or more transients (indicated by arrows) during the 1st 600 s (10 min) of recording. *B*: number of active Kenyon cells expressed as a percentage of the total number of Kenyon cells in cultures prepared from early (5 h, $n = 5$ cultures), mid- (10–30 h, $n = 3$ cultures), and late (55–78 h, $n = 14$ cultures) stage pupae. $**P < 0.01$, Fisher's PLSD (ANOVA). Data obtained between 1 and 4 days in culture in standard HBSS recording saline. Bars indicate mean \pm SE.

The actual calcium waveforms vary considerably between cells and even within a cell (Fig. 2*A*). For the purposes of this study, a single transient was defined as a rapid increase (< 20 s from baseline to peak) in intracellular calcium, 75 nM or more above the basal calcium level that declines by $\geq 50\%$ within 3 min. A Kenyon cell with one or more transients during the first 600 s of recording was defined as active. The number of active Kenyon cells in each field was expressed as a percentage of all Kenyon cells in each field. In 1- to 4-day-old cultures, made from 55- to 78-h pupae, the majority ($61 \pm 5\%$, $n = 14$ cultures) of Kenyon cells were spontaneously active. While active Kenyon cells were observed in cultures as long as 12 days after plating, the percent that were active decreased with increasing age in culture.

Spontaneous calcium oscillations in the mushroom body region in whole brains first appeared 55–69 h after pupation and persisted into adulthood (Rosay et al. 2001). To determine if the ability of Kenyon cells to generate transients is also developmentally regulated, cultures were prepared from brains obtained from two earlier pupal stages, ~ 5 and 10–30 h after puparium formation (APF). Cultures were observed at 1–4 days in vitro. Calcium transients were present in some Kenyon cells in the cultures prepared from pupae at 5 and 10–30 h APF, showing that Kenyon cells are capable of generating calcium transients before oscillations are observed in the intact mushroom bodies. However, the number of active Kenyon cells, expressed as a percentage of the total number of Kenyon cells, was significantly lower in cultures made from pupae at 5 h compared with 55–78 h APF. The percentage of Kenyon cells active, in cultures made from pupae 10–30 h APF, was intermediate (Fig. 2*B*). This pattern of developmental regula-

tion is consistent with the calcium transients in the cultured Kenyon cells representing cellular events that contribute to generation of the calcium oscillations observed in the intact mushroom bodies.

The majority of the subsequent experiments focused on analysis of Kenyon cells in cultures prepared from late stage pupae (55–78 h APF) at 1–4 days in vitro.

Spontaneous calcium transients are an intrinsic property of Kenyon cells

Addition of 1 μM TTX to the HBSS recording saline, a concentration previously shown to block sodium currents in *Drosophila* neurons (O'Dowd 1995), did not block the calcium transients, showing that they can occur independently of sodium channel activity (Fig. 3*A*). Spontaneous synaptic activity, mediated by nicotinic acetylcholine receptors (nAChRs) and GABA receptors, has also been observed in the cultured Kenyon cells (Su and O'Dowd 2003). This activity is not necessary for generation of the transients either, because calcium transients persist in saline containing curare and PTX at concentrations that completely block the nAChR and GABA receptor-mediated synaptic currents (Fig. 3*B*). Additionally, application of APV and CNQX reveals that endogenous activity of ionotropic glutamate receptors is not required for generation of the calcium transients (Fig. 3*C*). To quantitatively assess the effect of specific blockers on transient activity, the percentage of active cells, transient frequency, and transient amplitude were compared in Kenyon cells examined in different external solutions. No significant differences were apparent between Kenyon cells examined in control saline (HBSS), TTX, TCP, or TCPAC (TTX, curare, PTX, APV, CNQX; Fig. 3*D*). Thus the majority of the calcium transients in the cultured Kenyon cells are independent of sodium-dependent action potentials and neurotransmitter receptor activity.

These data suggest that spontaneous calcium transients are generated in a cell autonomous fashion. However, it was still possible that there were receptors or channels mediating intercellular communication in cultured neurons that have yet to be identified. Therefore to more rigorously test this hypothesis, cultures were imaged at 1–2 days in culture, facilitating analysis of physically isolated Kenyon cells, in some cases before neurite outgrowth. These Kenyon cells were also able to generate spontaneous calcium transients (Fig. 1*F*). Quantitative analysis indicated that the properties of the transients in physically isolated Kenyon cells and transients in Kenyon cells that were in contact with the processes or cell bodies of other neurons were not significantly different (Fig. 3*D*). Together these data show that spontaneous calcium transients can be generated in a cell autonomous manner.

Calcium transients are modulated by nicotine and GABA

Blockade of nAChR and GABA receptor-mediated synaptic transmission did not inhibit generation of calcium transients. However, to determine whether activation of these receptors by exogenous agonists affects intrinsically generated calcium transients, cultures were exposed to nicotine or GABA. Bath application of 0.5 μM nicotine caused a rapid and reversible increase in baseline calcium to levels higher than the peak of the average spontaneous calcium transient (Fig. 4*A*). Few if any transients

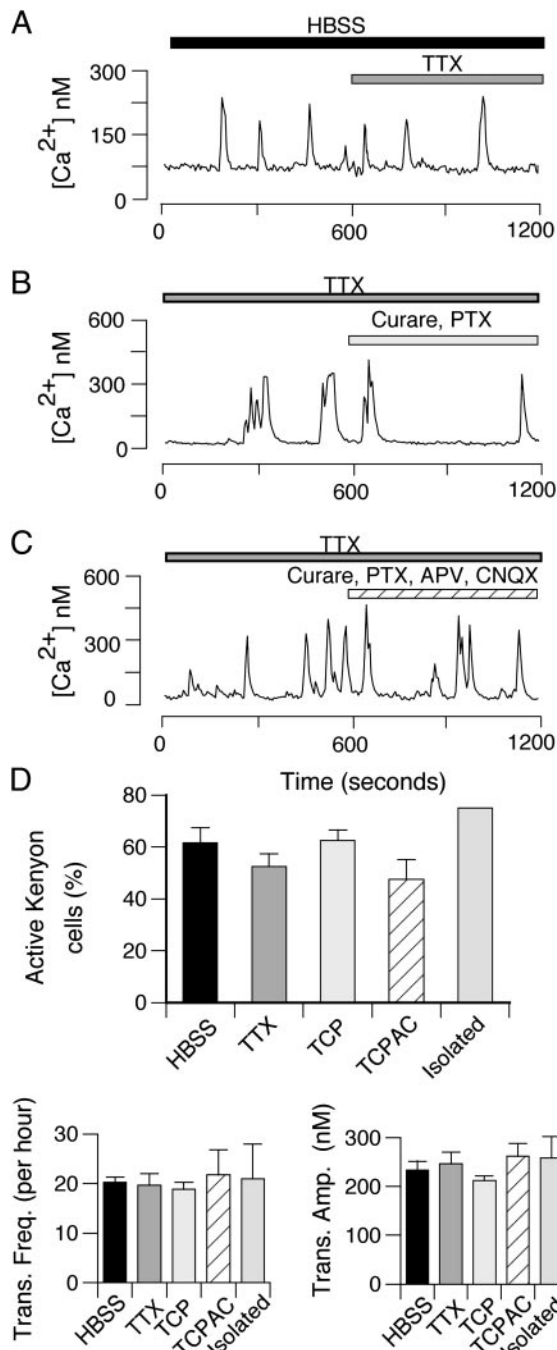


FIG. 3. Spontaneous calcium transients are cell autonomous events. *A*: spontaneous calcium transients observed in a Kenyon cell persist when TTX, at a concentration (1 μ M) that blocks all voltage-gated sodium channels, is added to the recording saline (HBSS). *B*: Kenyon cell calcium transients are not blocked by addition of 20 μ M curare and 10 μ M PTX (antagonists that specifically block nAChRs and GABA receptors, respectively) to a TTX-containing saline. *C*: transients are not blocked by addition of D(-)-2-amino-5-phosphonopentanoic acid (APV; 50 μ M) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 5 μ M), *N*-methyl-D-aspartate (NMDA), and non-NMDA-type ionotropic glutamate receptor antagonists respectively, in addition to TTX-, curare-, and picrotoxin (PTX)-containing saline. *D*: there is no significant difference (ANOVA, $P > 0.27$) in the mean percentage of active neurons, transient amplitude, or transient frequency in Kenyon cells examined in any of the following salines: HBSS ($n = 15$ cultures); TTX ($n = 14$ cultures); TCP (TTX, curare, PTX), ($n = 25$ cultures); TCPAC (TTX, curare, PTX, APV, CNQX), ($n = 4$ cultures). Transients show similar properties in Kenyon cells that are not physically in contact with surrounding cells, (Isolated, $n = 12$ neurons). Bars indicate mean \pm SE.

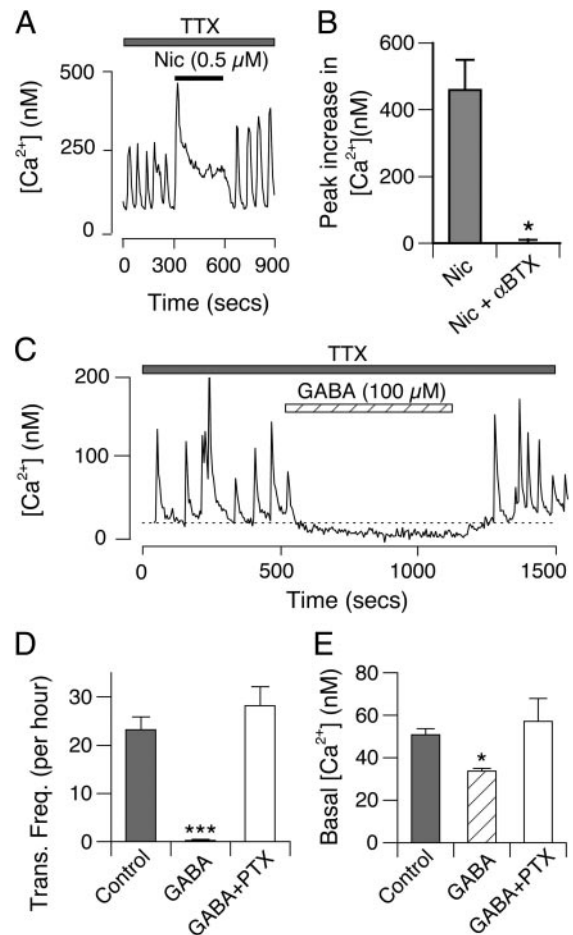


FIG. 4. Nicotine and GABA modulate transients and basal calcium levels. *A*: addition of nicotine (0.5 μ M) induces a large increase in intracellular calcium in a Kenyon cell. Calcium transients are observed before and after, but not during, nicotine exposure. *B*: nicotine-induced increase in intracellular calcium is blocked by α -bungarotoxin. Nic., mean amplitude of peak increase in intracellular calcium induced by 0.5 μ M nicotine ($n = 9$ cultures); Nic + α BTX, mean amplitude of peak increase in intracellular calcium when nicotine is added in the presence of 0.1 μ M α -BTX ($n = 3$ cultures). * $P < 0.05$, Student's *t*-test, unpaired. *C*: bath application of GABA (100 μ M) reversibly blocks the spontaneous calcium transients and reduces basal calcium levels. *D*, *E*: GABA blockade of calcium transients and GABA-induced reduction in basal calcium levels are blocked by 20 μ M PTX. Control, mean prior to GABA application ($n = 11$ cultures); GABA, mean during GABA application ($n = 4$ cultures); GABA + PTX, mean during GABA application in presence of 20 μ M PTX ($n = 4$ cultures). * $P < 0.05$, *** $P < 0.001$, Fisher's PLSD (ANOVA). Bars indicate mean \pm SE.

were seen to rise above the elevated calcium levels in the presence of nicotine (Fig. 4A). The nicotine-induced elevation in intracellular calcium was blocked by preincubation of the culture in 0.1 μ M α -BTX, a nAChR antagonist (Fig. 4B). In contrast, application of exogenous GABA (100 μ M) resulted in a reversible decrease in basal calcium levels and a blockade of the calcium transients (Fig. 4, C–E). The GABA-induced changes were inhibited by preincubation of the cultures in 20 μ M PTX, a GABA receptor antagonist (Fig. 4, D and E). These data show that activation of excitatory receptors increases intracellular calcium levels, whereas activation of inhibitory receptors decreases basal calcium levels and blocks the transients. This suggests that basal calcium levels and calcium transients are regulated by membrane potential.

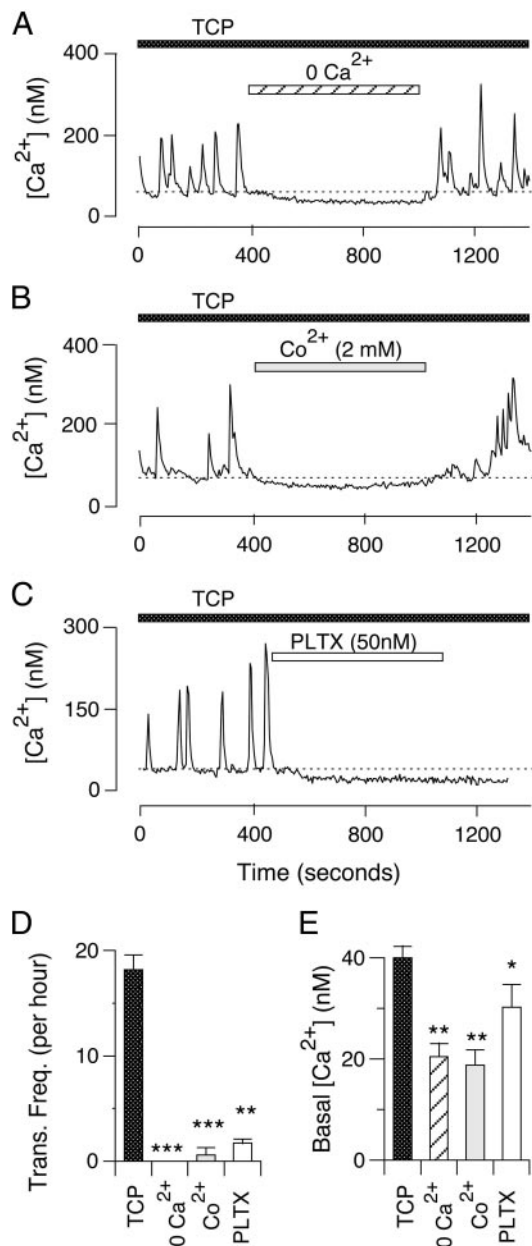


FIG. 5. Spontaneous transients mediated by calcium influx across the membrane through *Plectrurus* toxin (PLTX)-sensitive calcium channels. *A*: removal of calcium from TCP (TTX, curare, PTX) recording saline ($0 Ca^{2+}$) reversibly blocks the calcium transients and reduces basal calcium levels in a Kenyon cell. *B*: addition of 2 mM cobalt (Co^{2+}) also reversibly blocks the calcium transients and reduces the basal calcium levels in a Kenyon cell. *C*: bath application of 50 nM PLTX irreversibly inhibits calcium transients and reduces basal calcium levels. *D* and *E*: mean transient frequency and basal calcium levels are significantly reduced by removal of calcium ($0 Ca^{2+}$, $n = 3$ cultures), addition of 2 mM cobalt (Co^{2+} , $n = 4$ cultures), and addition of PLTX (50 nM, $n = 6$ cultures) to the control recording saline (TCP or HBSS). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Fisher's PLSD (ANOVA). Bars indicate mean \pm SE.

Transients are mediated by PLTX-sensitive calcium channels

To explore the role of calcium channels in generation of the transients, we examined intracellular calcium levels when extracellular calcium (normally 1.8 mM) was removed from the recording saline. This reversibly blocked the spontaneous calcium transients in Kenyon cells (Fig. 5, *A* and *D*). Similarly,

addition of cobalt (2 mM), a general voltage-gated calcium channel blocker previously shown to block calcium currents in embryonic *Drosophila* neurons (O'Dowd 1995), reversibly inhibited spontaneous calcium transients in Kenyon cells (Fig. 5, *B* and *D*). These data show that calcium flux into the cell, through calcium channels, is necessary for generation of the calcium transients. Similar to the effect of GABA, there is also a reversible reduction in the baseline calcium levels after removal of calcium or addition of cobalt (Fig. 5, *A*, *B*, and *E*). This shows that calcium channels are active and regulate basal calcium levels in the absence of external stimulation, even when sodium channels and neurotransmitter receptors are blocked.

The pharmacological profile of the calcium channels underlying the calcium transients was further examined through bath application of several specific calcium channel antagonists. Calcium transient frequency ($19 \pm 1/h$) in control saline (TCP) was not significantly reduced by the vertebrate L-type calcium channel antagonist, verapamil (20 ± 2 transients/h, $5 \mu M$), or the N-type blocker, ω -conotoxin GVIA (24 ± 1 transients/h, $2 \mu M$). In sharp contrast, the insect calcium channel antagonist PLTX blocked the majority of the calcium transients in Kenyon cells within 3 min of application (Fig. 5, *C* and *D*). There was no recovery during a 10-min wash, consistent with the irreversible blockade of calcium currents by PLTX previously reported in embryonic *Drosophila* neurons (Leung et al. 1989). In addition, the basal calcium level was also significantly reduced by PLTX treatment (Fig. 5, *C* and *E*), though the magnitude of the reduction was smaller than that induced by $0 Ca^{2+}$ and 2 mM Co^{2+} . Thus calcium influx through calcium channels sensitive to PLTX but not to vertebrate L- or N-type channel blockers is involved in generation of spontaneous calcium transients in Kenyon cells.

Transients do not require calcium release from intracellular stores

In some vertebrate neurons, spontaneous increases in intracellular calcium are triggered by the influx of calcium across the plasma membrane, but the bulk of the calcium increase is due to release from intracellular stores (Gu et al. 1994; Owens and Kriegstein 1998). To determine if release of calcium from intracellular stores could be detected in Kenyon cells, cultures were exposed to caffeine during blockade of the voltage-gated calcium channels by cobalt. An increase in intracellular calcium typically induced by application of 10 mM caffeine is shown in Fig. 6*A*. Caffeine triggered an increase in intracellular calcium levels in 73% (16/22) of the active Kenyon cells with a mean amplitude of 297 ± 33 nM.

To determine if calcium release from these intracellular stores is involved in generation of the transients in Kenyon cells, the effect of blocking release was monitored. Cultures were pretreated with caffeine to deplete intracellular stores, followed by thapsigargin to block reuptake. Kenyon cells in caffeine/thapsigargin-treated cultures were not only capable of generating robust spontaneous calcium transients (Fig. 6*B*), but the transient frequency was significantly higher in treated versus control cultures (26.5 ± 2.4 and $18.2 \pm 1.4/h$, respectively; $P < 0.01$, Student's *t*-test). In addition, basal

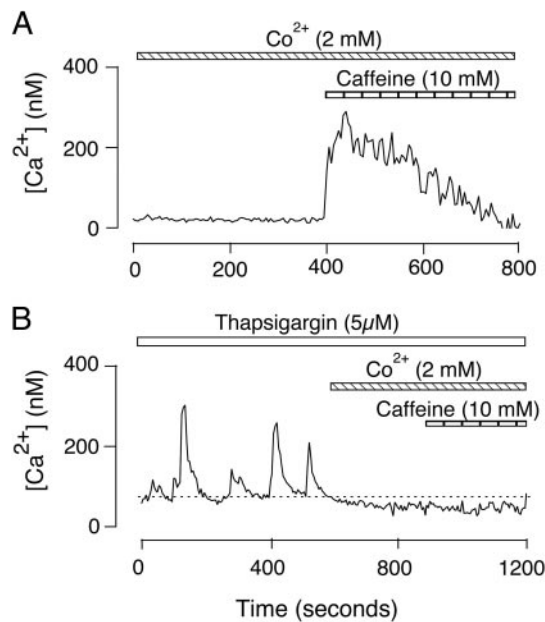


FIG. 6. Release of calcium from intracellular stores is not required for generation of calcium transients. *A*: caffeine (10 mM) induces an increase in intracellular calcium in a single Kenyon cell in the presence of cobalt to block voltage-gated calcium transients. *B*: spontaneous calcium transients recorded in a Kenyon cell in which intracellular calcium stores have been depleted. Addition of 10 mM caffeine at 900 s into the recording saline, in the presence of cobalt to block the transients, did not induce an increase in intracellular calcium, confirming the depletion of intracellular calcium stores. Culture was pre-exposed to caffeine for 1 min and incubated in 5 nM thapsigargin for 45 min to block reuptake of calcium.

calcium levels were also significantly higher in the caffeine/thapsigargin-treated versus control cultures (59 ± 5 and 40 ± 2.3 nM, respectively; $P < 0.01$, Student's *t*-test). These data show that calcium transients do not require calcium release from intracellular stores but are instead up-regulated by depletion of this calcium reservoir.

Voltage-gated calcium currents in Kenyon cells are PLTX sensitive

Based on previous studies in embryonic *Drosophila* neurons (Leung et al. 1989; O'Dowd 1995), the blockade of calcium transients by cobalt and PLTX suggests that the calcium channels involved in regulating the spontaneous transients in Kenyon cells are voltage-dependent. To test this hypothesis directly, whole cell voltage-clamp recordings were employed to examine isolated calcium currents. Potassium currents were blocked using cesium in place of potassium in the internal solution, and TCP was used as the control external recording solution.

A series of step depolarizations, from holding potentials of -75 or -80 mV, elicited a family of inward currents carried through voltage-gated calcium channels in 76% (19/25) of the Kenyon cells examined in control solution (Fig. 7*A*). Bath perfusion of 50 nM PLTX dramatically reduced the inward calcium currents in five of five Kenyon cells (Fig. 7*A*). To estimate the PLTX-induced blockade of the current and minimize the potential contribution of rundown, calcium currents recorded from Kenyon cells in control conditions (TCP) were compared with those preincubated for ≥ 10 min in 50 nM PLTX. All Kenyon cells in which a stable whole cell recording was obtained were included in the analysis. A value of zero was assigned to cells without detectable calcium currents. Peak calcium current amplitudes were normalized to the whole cell capacitance and expressed as current densities to control for variation in size of individual Kenyon cells (range, 1–8 pF).

Comparison of the current density versus voltage curves confirmed that 50 nM PLTX results in a dramatic and significant reduction in peak calcium current (Fig. 7*B*). The nonzero current density voltage curve in 50 nM PLTX reflects the presence of a residual voltage-gated calcium current in 5/12 cells. The blockade of calcium channels was specific in that voltage-gated potassium currents were not significantly altered by perfusion with (Fig. 7*C*), or preincubation (Fig. 7*D*) in, 50 nM PLTX. These data confirm that Kenyon cells express voltage-gated calcium channels, the majority of which are

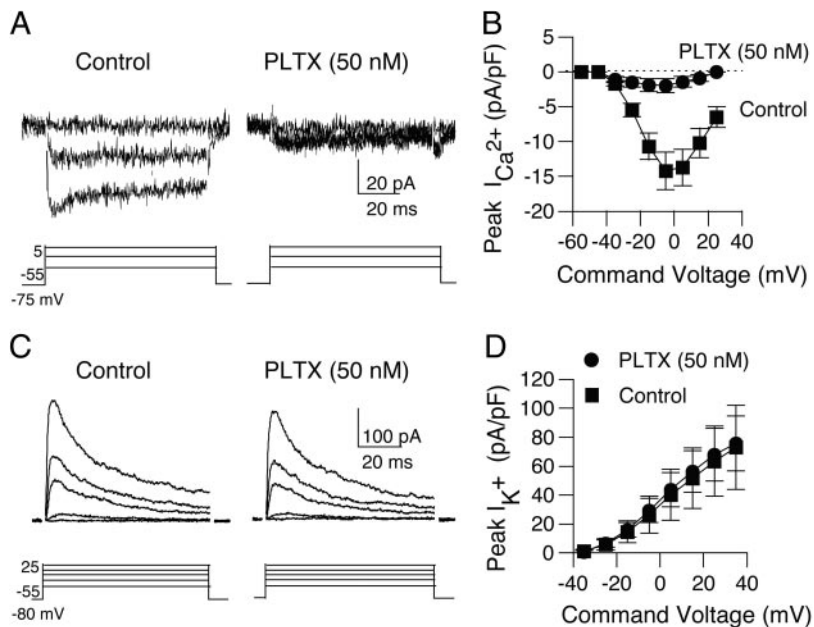


FIG. 7. PLTX-sensitive voltage-gated calcium currents in Kenyon cells. *A*: isolated calcium currents recorded from a Kenyon cell in a 4-day-old culture are dramatically reduced by bath application of 50 nM PLTX. Internal solution contained cesium to block potassium currents and the external solution (Control) was the TCP saline used in the imaging experiments. *B*: peak calcium current density vs. voltage curve in Kenyon cells preincubated/examined in the presence of 50 nM PLTX ($n = 12$ Kenyon cells) vs. control saline ($n = 26$ Kenyon cells). Values in control and PLTX treated cells are significantly different at all voltages at, or above, -25 mV. $P < 0.01$, Student's *t*-test, unpaired. Bars indicate SE. *C*: voltage-gated potassium currents recorded from a Kenyon cell are not altered by bath application of 50 nM PLTX. Internal solution contained potassium and external solution (Control) was TCP containing 2 mM cobalt. *D*: peak potassium current density is similar in Kenyon cells examined in control ($n = 7$ Kenyon cells) and PLTX ($n = 10$ Kenyon cells) containing saline. Bars indicate SE.

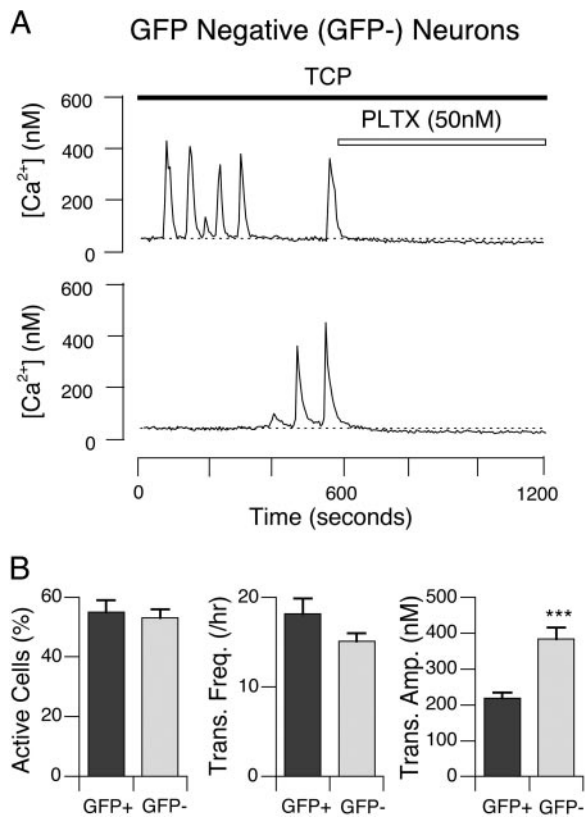


FIG. 8. GFP negative (GFP⁻) neurons in OK107;UAS-GFP cultures also exhibit spontaneous, PLTX sensitive calcium transients. *A*: spontaneous calcium transients in 2 GFP⁻ neurons are irreversibly blocked by bath application of 50 nM PLTX. *B*: percentage of active neurons and mean transient frequency are similar in GFP⁻ and GFP⁺ neurons. The transient amplitude in GFP⁻ neurons is significantly larger than the GFP⁺ neurons. ****P* < 0.001, Student's *t*-test, unpaired. Bars indicate mean \pm SE. *n* = 16 cultures in which the same number of GFP⁻ and GFP⁺ neurons were examined in each culture.

blocked by 50 nM PLTX. In combination with the imaging data, these findings indicate that calcium influx through PLTX-sensitive voltage-gated calcium channels mediates the spontaneous calcium transients and regulates resting levels of calcium in Kenyon cells.

Calcium transients are not limited to Kenyon cells

The calcium oscillations reported in the intact *Drosophila* brain, visualized with apoaequorin imaging, are restricted to the mushroom body region. In contrast, GFP negative (GFP⁻) neurons in the OK107-GAL4;UAS-GFP cultures, representing a variety of neuronal subtypes found within the central brain region but outside the mushroom bodies, also exhibit spontaneous PLTX-sensitive calcium transients (Fig. 8*A*). There was no significant difference in the percentage of active cells or the transient frequency in GFP⁺ Kenyon cells and GFP⁻ neurons examined in the same cultures (Fig. 8*B*). Although it should be noted that the amplitude of the transients in the GFP⁻ neurons was significantly larger than in the GFP⁺ Kenyon cells, the mechanism underlying this difference is unknown. These data show that cell autonomous calcium transients, unlike the synchronous calcium oscillations recorded in the intact brain, are not unique to Kenyon cells.

DISCUSSION

Spontaneous transient changes in intracellular calcium levels have been shown to play an important role in maturation of cells in the nervous system of many animals. Our results show that the principal neurons in *Drosophila* mushroom bodies, Kenyon cells, are capable of generating spontaneous calcium transients in a cell autonomous fashion. The transient increases in intracellular calcium are mediated by calcium flux through a PLTX-sensitive voltage-gated calcium channels and do not require release of calcium from intracellular stores. The frequency of the Kenyon cell calcium transients *in vitro* is similar to the mushroom body calcium oscillations *in vivo*. In addition, the ability of Kenyon cells to generate spontaneous calcium transients is up-regulated during pupal development. These data suggest that cell autonomous calcium transients mediated by PLTX-sensitive calcium channels in the cultured Kenyon cells represent cellular events that contribute to generation of the calcium oscillations in the intact mushroom bodies, activity that has been implicated in memory consolidation (Rosay et al. 2001). However, the presence of calcium transients in the majority of non-Kenyon cells, as well Kenyon cells, suggests they may play a more general role in maturation or function of neurons in the CNS.

Why are the calcium oscillations imaged with apoaequorin localized specifically in mushroom bodies in the whole brain, when the majority of neurons from the central brain region have the ability to generate spontaneous calcium transients? One possibility is that only mushroom body Kenyon cells are synchronously activated in the intact brain preparations described previously (Rosay et al. 2001). The imaging system used to visualize the apoaequorin signal is capable of detecting the calcium oscillations in groups of coordinately activated cells but is not sensitive enough to measure calcium transients in single cells (Davis 2001). It is clear that the calcium oscillations in the intact mushroom bodies are the result of synchronized activation of the Kenyon cell population since drugs that alter electrical excitability or synaptic transmission can modulate the oscillation frequency and amplitude. If neurons in the central complex and antennal lobes generate calcium transients but are not coordinately activated, this could account for the reported absence of calcium oscillations in these regions (Rosay et al. 2001). A second possibility is that synchronized oscillations occur in other neuronal populations in the brain but were not detected due to technical limitations. One potential problem with the apoaequorin assay is that it relies on formation of a complex between transgenically expressed apoaequorin and a cofactor, coelenterazine, present in the recording saline (Rosay et al. 2001). Limited access of the cofactor to neurons in the central complex and antennal lobes could affect the ability to detect synchronous oscillations in these regions. It should be possible to test this hypothesis by imaging whole brains in which specific neuronal groups express camgaros, calcium reporters that do not require cofactors (Yu et al. 2003).

Influx of calcium through voltage-gated channels is necessary for expression of synchronous calcium oscillations *in vivo* and calcium transients *in vitro*, based on blockade of both by removal of external calcium or addition of cobalt. However, drugs that block nAChRs, GABA receptors, and sodium channels alter calcium oscillations *in vivo* (Rosay et al. 2001) but

do not significantly alter the calcium transients recorded in cultured Kenyon cells. This is consistent with calcium transients in cultured cells representing intrinsic events and the oscillations *in vivo* representing coordination of these events, via electrical and synaptic interactions, between cells in the mushroom body network. In addition, verapamil, a vertebrate calcium channel blocker, reduces the amplitude of synchronous oscillations in the brain but does not alter the calcium transients in cultured Kenyon cells. This suggests that verapamil-sensitive calcium channels are important in coordinating activity between cells in the brain but are not primarily responsible for generation of cell autonomous transients, events mediated by PLTX-sensitive calcium channels.

What regulates the activity of PLTX-sensitive voltage-gated calcium channels? In *Xenopus* spinal neurons, low threshold T-type calcium channels that activate near rest are important in triggering increases in intracellular calcium leading to membrane depolarization and activation of high-voltage activated calcium channels underlying spontaneous calcium transients (Gu and Spitzer 1993). Cell autonomous generation of calcium transients in the presence of TTX indicates that the underlying PLTX-sensitive calcium channels actively open and close in the absence of sodium channel activity in Kenyon cells. Removal of calcium or addition of cobalt also reversibly reduces basal calcium levels. This indicates that flux through voltage-gated calcium channels occurs at rest and mediates basal calcium levels. While PLTX-sensitive currents seem to activate at more depolarized levels than classic T-type channels in vertebrates (-40 vs. -60 mV), the resting membrane potential in *Drosophila* Kenyon cells *in situ*, -55 to -60 mV, is also more depolarized than typical for many vertebrate neurons (data not shown). This suggests that an activation threshold for the majority of the voltage-gated calcium currents is within 15 – 20 mV of the mean resting potential. Future studies combining calcium imaging and electrophysiological analysis will be important in exploring the mechanisms underlying spontaneous activation of PLTX-sensitive channels and regulation of intracellular calcium levels in individual cells.

Application of exogenous GABA rapidly and reversibly reduces baseline calcium levels and blocks calcium transients. This is consistent with the observation that GABA activates chloride-conducting channels in *Drosophila* neurons (Lee et al. 2003; Su and O'Dowd 2003). GABA-induced hyperpolarization decreases the PLTX-sensitive calcium channel open probability, reducing basal calcium and blocking transients. Conversely, nicotine, a specific agonist for nAChRs that has previously been shown to increase calcium levels in Kenyon cells cultured from adult crickets (Cayre et al. 1999), also results in a large amplitude increase in basal calcium in *Drosophila* Kenyon cells. This is consistent with nicotine mediating a rapid membrane depolarization, causing opening of voltage-gated PLTX-sensitive calcium channels. The ability to regulate basal calcium levels and calcium transients by synchronous activation of nAChRs or GABA receptors supports the hypothesis that coordination of synaptic input to the Kenyon cells regulates calcium oscillations mediated by PLTX-sensitive calcium channels in the intact mushroom bodies.

In contrast to neurons in the visual system of blowflies (Oertner et al. 2001), caffeine application resulted in an increase in intracellular calcium in the presence of cobalt, showing the presence of significant intracellular calcium stores in *Drosophila* Kenyon cells. Presence of the transients in caf-

feine/thapsigargin-treated cultures indicates that these events do not require release of calcium from intracellular stores. However, unexpectedly, the depletion of calcium from intracellular stores caused a significant increase in the baseline calcium levels and frequency of calcium transients in Kenyon cells. This result is consistent with capacitative calcium entry in which depletion of intracellular calcium stores activates store-operated calcium channels (SOCs) located in the plasma membrane (Putney 2003). Although this has not been described previously in *Drosophila* neurons, a recent study has shown the presence of SOC channels in *Drosophila* S2 cells (Yeromin et al. 2004). Therefore it seems likely that calcium influx through SOC channels, after depletion of intracellular calcium stores with thapsigargin, either directly or indirectly increases the activity of calcium channels that regulate baseline calcium levels and spontaneous calcium transients. These primary *Drosophila* neurons provide an excellent model for future studies aimed at functional and molecular analysis of SOC channels and exploring their role in regulation of neuronal calcium levels.

What voltage-gated calcium channel subtype(s) underlie the spontaneous calcium transients? Calcium channels are multimeric proteins that include a pore-forming α -subunit. Three calcium α -subunit genes have been identified in *Drosophila*: a single homologue in the mammalian L-type (*Dmca1D*), N-type (*Dmca1A*), and T-type (*α -1G*) families (Littleton and Ganetzky 2000). The channels underlying the calcium transients in Kenyon cells are blocked by cobalt but are otherwise pharmacologically distinct from the vertebrate channel subtypes. Although *Drosophila* brain membrane preparations contain high affinity binding sites for vertebrate L-type calcium channel antagonists (Pelzer et al. 1989), the transients are not sensitive to verapamil or ω -conotoxin, an N-type blocker. In contrast, 50 nM PLTX, a toxin that blocks voltage-gated calcium channels in embryonic *Drosophila* neurons (Leung and Byerly 1991), completely blocks the calcium transients and the majority of the voltage-gated calcium currents in Kenyon cells. Previous studies have shown that *Dmca1A*-encoded N-type calcium channels (Smith et al. 1996) are localized in the presynaptic terminals of glutamatergic motor neurons at the *Drosophila* neuromuscular junction (NMJ) (Kawasaki et al. 2000, 2004), where PLTX also blocks glutamate release (Branton et al. 1987). This suggests that *Dmca1A* channels are PLTX-sensitive and therefore may underlie the calcium transients. Analysis of calcium transients and voltage-gated calcium currents in Kenyon cells cultured from animals with mutations in the *Dmca1A* N-type calcium channel gene will be needed to determine if the *Dmca1A* gene encodes the channels underlying the PLTX-sensitive calcium transients in Kenyon cells.

The ubiquitous expression of calcium transients in central brain region neurons from late stage pupae suggests these events may play a general role in the adult CNS. At the *Drosophila* NMJ, PLTX regulates release of glutamate from motor neurons (Branton et al. 1987), perhaps by blocking calcium influx in the presynaptic terminals. Therefore it is possible that PLTX-sensitive calcium channel activity is involved in regulating calcium influx involved in exocytosis at central cholinergic and/or GABAergic synapses that mediate fast transmission in *Drosophila* neurons (Su and O'Dowd 2003). If this is true, the observation that the PLTX-sensitive channels are active even in the presence of TTX to block

sodium channels would explain why cholinergic miniature excitatory postsynaptic current and GABAergic miniature inhibitory postsynaptic current frequencies in cultured *Drosophila* neurons are strongly regulated by external calcium levels (Lee and O'Dowd 1999; Lee et al. 2003). A recent study also links activity of *Dmca1A* calcium channels to regulation of synaptic growth at the *Drosophila* NMJ (Rieckhof et al. 2003). Analysis of cultures made from *Dmca1A* mutants and/or addition of PLTX to the growth media will be used to explore the role of these channels in regulating spontaneous neurotransmitter release and synaptic growth.

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