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Flubendiamide, the first phthalic acid diamide insecticide, impairs neuronal calcium signalling in the honey bee's antennae

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ABSTRACT

Calcium is an important intracellular second messenger involved in several processes such as the transduction of odour signals and neuronal excitability. Despite this critical role, relatively little information is available with respect to the impact of insecticides on the dynamics of intracellular calcium homeostasis in olfactory neurons. For the first time here, physiological stimuli (depolarizing current or pheromone) were shown to elicit calcium transients in peripheral neurons from the honey bee antenna. In addition, neurotoxic xenobiotics (the first synthetic phthalic diamide insecticide flubendiamide or botanical alkaloids ryanodine and caffeine) do interfere with normal calcium homeostasis. Our *in vitro* experiments show that these three xenobiotics can induce sustained abnormal calcium transients in antennal neurons. The present results provide a new insight into the toxicity of diamides, showing that flubendiamide drastically impairs calcium homeostasis in antennal neurons. We propose that a calcium imaging assay should provide an efficient tool dedicated to the modern assessment strategies of insecticides toxicity.

1. Introduction

Diamides are a new family of insecticides that have been increasingly used in the recent years (Casida, 2015; Lahm et al., 2009; Sattelle et al., 2008; Sparks and Nauen, 2015). This family of insecticides includes anthranilic diamides (chlorantraniliprole and cyantraniliprole, for instance) and phthalic diamides (flubendiamide, for instance). Flubendiamide was first approved in the US in 2008 for the control of lepidopterous pests on corn, cotton, grapes (US Environmental Protection Agency, 2008) but the molecule was withdrawn in 2016 due to environmental concerns (US Environmental Protection Agency, 2016). In Europe, approval for flubendiamide runs from 2014 to 2024 in three countries for use on crops, including tomato and bell pepper against lepidopterous pests (European Commission, http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database). We have recently shown that the anthranilic diamide chlorantraniliprole activates ryanodine receptors (RyRs) and disturbs calcium homeostasis in the honey bee muscle. This molecule was also more toxic when applied on antennae (Kadala et al., 2019), suggesting that these organs (the 'nose' of the honey bee) are extremely sensitive to diamides. Antennal neurons are critical for the detection of environmental clues such as odours and pheromones (Alaux et al., 2010; Slessor et al., 2005), or mechanical inputs (Esslen and Kaissling, 1976; Gascuel et al., 1994). We find it necessary to investigate their sensitivity to flubendiamide, a representative molecule of the diamides family.

Antennal olfactory receptor neurons (ORNs) carry olfactory receptors that allow the insect to identify odorant information. In these neurons, the chemical information is transduced into an electrical signal which is amplified thanks to voltage-gated ion channels, and then propagates to higher stages of the olfactory system (Kaissling, 1986). The mechanisms underlying this signal transduction involve the mobilization of calcium and have been investigated mostly in vertebrates like salamander (Leinders-Zufall et al., 1998; Leinders-Zufall et al., 1997) and cat (Gomez et al., 2005), and in some invertebrates. In insects, the binding of an odour or a pheromone to the olfactory receptor triggers intracellular events causing an increase in intracellular calcium concentration and surface membrane depolarization. This calcium influx can also trigger the release of calcium from endoplasmic reticulum stocks through ryanodine receptors (for review, see Berridge et al., 2003; Jacquin-Joly and Lucas, 2005). Calcium thus plays an essential role in the ability of insects to detect olfactory cues. In the honey bee, intracellular calcium variations have been recorded in mushroom bodies (Bicker, 1996; Szyszka et al., 2008), in photoreceptor cells (Walz et al., 1995) in antennal lobes (Deisig et al., 2006; Galizia et al., 2000) and in muscle cells (Collet, 2009). We used calcium imaging to monitor neuronal activity following *in vitro* exposure to physiological stimuli

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(depolarizing solution, pheromone 9-ODA) and to xenobiotics, including flubendiamide and natural alkaloids (caffeine and ryanodine). Caffeine and ryanodine are known to bind to ryanodine receptors (Usachev et al., 1993; Walz et al., 1995) and have been shown to have insecticidal properties (Araque et al., 2007; Nathanson, 1984). Their ability to disturb neuronal calcium homeostasis in the antennae is explored here.

Xenobiotics were able to induce sustained calcium transients in the honey bee antennal neurons. Ryanodine (an old and now withdrawn insecticide) is a stronger effector of calcium transients than caffeine. The insecticide flubendiamide is very potent at inducing sustained calcium release from internal stores, leading to high elevation of the cytoplasmic concentration that may induce Ca^{2+} cytotoxicity. Our study suggests that neuronal intracellular calcium monitoring should be included in the modern assessment strategies of insecticides toxicity.

2. Materials and methods

2.1. Isolation of antennal olfactory receptor neurons

We performed our experiments on neurons from prepupae antennae (9 to 11 days of development) of domestic honey bees *Apis mellifera*. All stages of dissection were performed under conditions of maximal sterility (microdissection instruments autoclaved and solutions filtered with 0.2 μm filter). Prepupae were immersed 10 seconds in a solution of 70% ethanol and then briefly rinsed in sterile distilled water. For dissection, each prepupa was immersed in a Ca^{2+}-and Mg^{2+}-free Tyrode (see Solutions section). The larvae moult, which surrounds and protects the prepupa, was gently opened at the front of the head, antennae were detached, and the tracheas were removed. Antennae were then stored in the Ca^{2+}- and Mg^{2+}-free Tyrode solution. Only the last nine antennal segments, which contain the olfactory receptor neurons, were kept. Antennae were then placed in an Eppendorf tube containing 0.2 ml Ca^{2+}- and Mg^{2+}-free Tyrode and were shredded with microscissors. Enzymatic dissociation was then performed by adding 0.4% trypsin to the antennae at 37 °C for 12 minutes under gentle agitation. The enzymatic reaction was stopped by adding 0.5 ml of culture medium (see Solutions section). After centrifugation (0.3 g; 3 minutes), the supernatant was aspirated and the pellet was re-suspended in the culture medium (15 μl per antenna). A 50-μl droplet was then placed in the center of each Petri dish whose bottom had previously been coated with poly L-lysine. After 40 minutes (time required for sedimentation and adhesion of neurons to the bottom of the Petri dish), a glass slide was placed on the droplet, and based on two small blocks of glass attached to the bottom of the box with a non-cytotoxic silicone grease. The Petri dishes were then inverted (upside down) to achieve the "hanging drop column method". Intact neurons adhered to the substrate of the dish whereas debris and dead cells fell on the glass slide. The Petri dishes were then placed in an incubator at 29 °C (high humidity). Cultures were used for experiments on the following 1–3 days after plating. Prior to recordings, the glass slide was removed and the culture medium was gently replaced with 3 ml of standard extracellular solution.

2.2. Intracellular calcium imaging

Cells were loaded in the dark with the membrane permeant Ca^{2+} indicator fluo-3-AM (2 μM; Molecular Probes, Eugene, OR, USA) for 60 minutes at room temperature. A 2 mM stock solution of fluo-3-AM was prepared in DMSO solvent and was diluted 1:1000 in standard extracellular solution, in order to load neurons. Final DMSO concentration did not exceed 0.1%. The neurons were placed on an inverted microscope stage (DMIRB, Leica Microsystems, Wetzlar, Germany). The excitation light for fluo-3 originated from a beam of light (from a 100-watts mercury bulb) passed through a 450–490 nm bandpass filter. Fluorescence was collected with an x20 objective, using a dichroic mirror and a longpass filter (wavelength > 515 nm). Fluorescence intensity was detected with a fast cooled CCD camera (Qicam CCD, 12-bits, Qimaging, Surrey, BC, Canada) in 4x4 binning mode. Series of frames ('sequences', hereafter) were acquired with QCapture Pro software at a frequency of 1 Hz with a chip exposure time of 50 ms. To reduce photo-bleaching and photodynamic damages to neurons, we used a shutter that was synchronized with frame acquisition and opened each time for 150 ms only.

Offline analyses of sequences were done with ImageJ (NIH, USA) and OriginPro 7.5 (OriginLab Corp., Northampton, MA, USA) softwares. Fluorescence intensity was measured on each individual cell by measuring the average pixel intensity values in defined regions of interest (ROI) on each frame of the sequence. Background fluorescence was subtracted using series of cell-free ROI. Variations in cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) are presented as relative changes in fluorescence intensity (ΔF/F0), F0 being the baseline fluorescence, and ΔF the change in fluorescence from baseline (hereafter described as ΔF/F). In order to take into account actual calcium responses (*i.e.* well above the baseline fluorescence signal noise), only signals above a threshold value were considered for statistical analysis. This threshold value, calculated as mean + 2SD of basal fluorescence, was 0.09 and we took a security margin by setting it at 0.1 ΔF/F. Some neurons were consecutively tested with two different stimuli and in this paper, only the very first stimulus was considered for analysis. This allows us to avoid any adaptation effect due to a prior stimulation. Moreover, if a neuron was subjected to consecutive perfusions of the same compound during the same sequence acquisition, then only the perfusion yielding the largest amplitude was taken into account for statistical analysis. GraphPad Prism (version 6 for Windows, GraphPad Software, La Jolla California USA) was used for statistical analyses. Amplitudes of calcium transients were compared with the Mann-Whitney test. Averaged data are given as mean ± S.E.M.

2.3. Solutions

The culture medium consisted in L15 medium (Invitrogen™, Carlsbad, CA, USA) with L-glutamine, supplemented with 1% penicillin/streptomycine, 10% FBS, 3.3 mM L-proline, 5.5 mM D-glucose and 75 mM Sucrose (pH 7.2; 400 mOsm/l). The standard extracellular (Tyrode) solution contained (in mM) 140 NaCl, 5 KCl, 0 or 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 100 sucrose (pH 7.2; 400 mOsm/l). A depolarizing potassium chloride solution was also prepared and contained (in mM) 70 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 250 sucrose (pH 7.2; 400 mOsm/l). Caffeine (20 mM) was dissolved in Tyrode. The stock-solution (1 M) for the pheromone compound (E)-9-oxo-2-decenoic acid (9-ODA; CAS number 2575-01-1) was prepared in DMSO and diluted right before use in Tyrode at a final concentration of 1 mM. Ryanodine (CAS number 15662-33-6) and flubendiamide (CAS number 272451-65-7) were prepared as stock-solutions and were diluted later in Tyrode at 5 μM and 3 μM, respectively. The final concentration of DMSO in these solutions did not exceed 0.1%. Except in experiments with flubendiamide, tetrodotoxin (TTX, 1 μM) was added to the bath to exclude contribution of voltage-gated sodium channels to the neurons activity.

Cells were continuously perfused with extracellular solution and on average, 39 neurons were recorded per sequence. We used a valve-operated perfusion system that was made of polyethylene tubes, each connected on one end to a syringe. The other ends of the tubes were gathered together in a common outlet (manifold). The exhaust of that tube can be seen at the top-left side of Fig. 1A. The perfusion system was fed by 2-psi pressure providing a flow rate of 350 μl/min with a dead volume of 15 μl upon activation of the perfusion. Pinch valves were computer-operated (Valvelink 8, Automate Scientific Inc., San Francisco, CA, USA). This system allows for repeated perfusions of localized and separate areas of the Petri dish while limiting cross-contamination. Two separate areas were generally perfused and recorded in each Petri dish. A continuous peristaltic aspiration system was

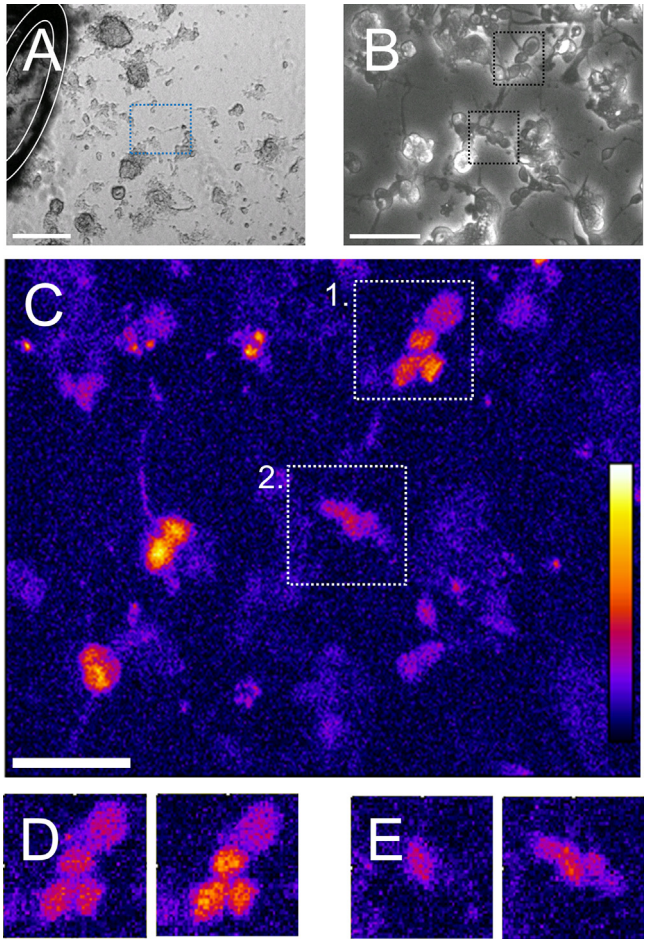

Fig. 1. Monitoring of cytoplasmic free calcium levels in dissociated antennal neurons. A, Bright field image of the bottom of a Petri dish covered with antennal cells seen at low magnification. The tip of the perfusion system allowing for extracellular solution exchange (*top-left*) and the fluorescence measurement field (*blue rectangle*) are annotated. B, Measurement field described in A, seen at higher magnification under phase contrast mode. Two groups of neurons are marked with dotted lines. C, Measurement field in fluorescence mode. Colour scale: 0–1 ΔF/F. D, The group of neurons in the C1 area is presented before (left) and during an increase in fluorescence in three neurons (right). Increase in fluorescence levels indicates a $[Ca^{2+}]_i$ increase. E, Another example, taken from area C2. Scale bars: A, 200 μm; B and C, 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

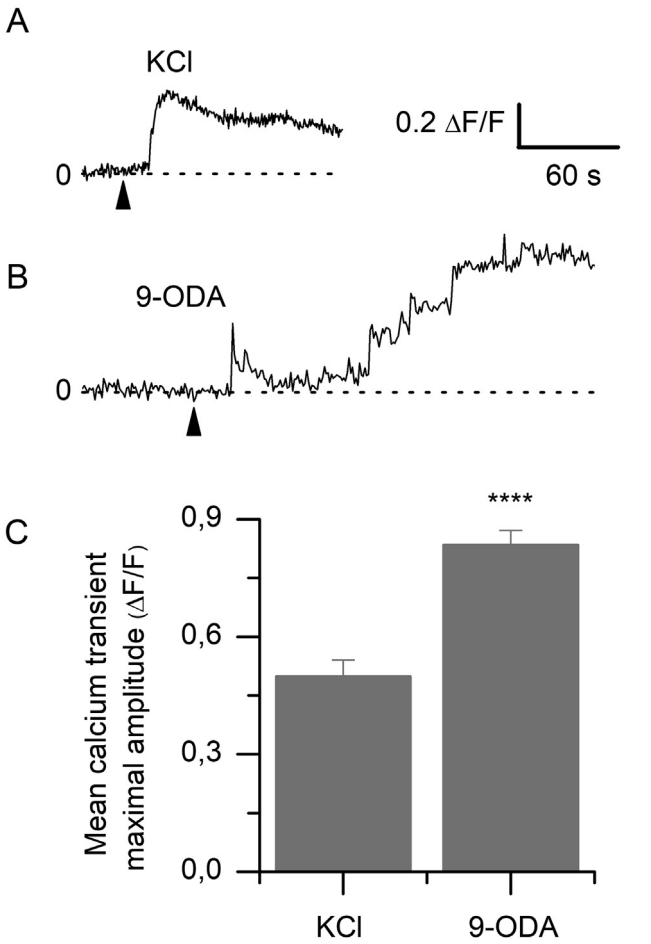

Fig. 2. Antennal neurons responded to physiological stimuli. Examples of calcium signals recorded in the presence of KCl (70 mM + 1 μM TTX, A), or in the presence of 9-ODA (1 mM + 1 μM TTX, B). Arrowheads indicate the beginning of the perfusion. C, Mean calcium transient maximal amplitude. 9-ODA induced a significantly stronger calcium increase than KCl.

operated throughout the duration of experiments to keep the volume of the extracellular solution at a constant level.

3. Results

3.1. Monitoring of intracellular calcium in neurons

The calcium indicator Fluo-3-AM was chosen because of its specificity to calcium and its large fluorescence ratio (Minta et al., 1989) which allows for the detection of calcium transients in small cells. Fluorescence measurements were made from antennal neurons that were discriminated based on morphology. These are small cells with a roundish or oval cell body (5–10 μm; Fig. 1B) with thin processes (Gascuel et al., 1994). *In situ* sensillae quantifications and neuronal counts suggest that olfactory receptor neurons account for nearly 72% of total antennal neurons in worker bee antennae (Esslen and Kaissling, 1976). No attempt was made to distinguish olfactory receptors neurons from other neuronal types. Fig. 1C shows the measurement fields (C1 and C2) in fluorescence mode. Fig. 1D and 1E show the same measurement fields before (left) and as intracellular calcium increases (right).

We performed two sets of experiments to monitor calcium transients in antennal neurons. In the first set, we tested the ability of the honey bee antennal neurons to respond to physiological stimuli including the queen pheromone compound 9-ODA and the depolarization-inducing KCl solution. Then in the second set of experiments, we explored calcium transients caused by molecules known as agonists of ryanodine receptors, including ryanodine, caffeine, and flubendiamide.

3.2. Calcium transients induced by physiological stimuli

Some antennal neurons exhibited sustained calcium transients in the presence of physiological stimuli KCl (Fig. 2A) and 9-ODA (Fig. 2B). These transients demonstrate that *in vitro* antennal neurons are able to respond to physiological stimuli.

According to the Nernst equation and assuming an intracellular potassium concentration of 140 mM, perfusion with an extracellular solution containing 70 mM KCl theoretically brings the neuronal membrane potential to −17 mV, which is above the activation threshold of voltage-gated calcium channels. Under our recording conditions, calcium elevations were 0.50 ± 0.04 ΔF/F in amplitude (n = 69 neurons from 5 sequences, Fig. 2C). These calcium responses to KCl probably reveal the contribution of extracellular calcium through

the activation of voltage-gated calcium channels (CaVs), which in turn, can activate intracellular calcium release channels.

The molecule 9-ODA is one of the components of the queen mandibular pheromone and it is involved in attracting drones over large distances (Jarriault and Mercer, 2012). Application of 9-ODA can induce an electrical response in honey bee receptor neurons (Laurent et al., 2002) but the mechanism underlying this response is yet to be fully elucidated. The contribution of calcium to this process is examined here. The mean calcium amplitude was higher with 9-ODA (0.83 ± 0.04 ΔF/F, n = 237, 24 sequences) as compared to KCl ($p < 0.0001$, Fig. 2C). It is now well established that calcium entry from the extracellular environment plays an important role in the transduction of the olfactory signal (for example, see Jacquin-Joly and Lucas, 2005; Wicher, 2015). Calcium signal seen in the presence of 9-ODA may reflect calcium entry through the plasma membrane and a release of calcium from intracellular stocks following the cascade of events from the transduction process.

3.3. Ryanodine and caffeine- induced calcium transients

Ryanodine and caffeine bind to ryanodine receptors and they have proven insecticidal activity in a number of models including *Drosophila melanogaster*, *Musca domestica*, and *Manduca sexta* (Araque et al., 2007; Jefferies et al., 1992; Nathanson, 1984). Here, we used these molecules to investigate the involvement of ryanodine receptors in the honey bee neurons' calcium transients. Antennal neurons responded to ryanodine 5 μM (+1 μM TTX) and caffeine 20 mM (+1 μM TTX) with clear increase in intracellular calcium and examples of calcium recordings are shown in Fig. 3A and B. Even at a concentration 4000 fold lower, ryanodine induced calcium elevations that were significantly higher than caffeine's. The mean calcium amplitude was 0.45 ± 0.06 ΔF/F (n = 30, 4 sequences) with ryanodine and 0.27 ± 0.04 ΔF/F (n = 20, 4 sequences) in the presence of caffeine ($p < 0.0099$, Fig. 3C). Addition of the calcium channel blocker, cadmium (100 μM) in the bath solution had no significant effect on calcium amplitude in the presence of ryanodine (data not shown). This result supports the idea of an internally driven calcium release.

3.4. Flubendiamide-induced calcium transients

In a previous report, we have shown that chlorantraniliprole (which is a diamide as flubendiamide), is able to induce cytotoxic calcium transients and quick contracture in the honey bee muscle cells at nanomolar to micromolar concentrations (Kadala et al., 2019). Here, we investigated the ability of flubendiamide to alter calcium homeostasis in antennal neurons. Perfusion of neurons with flubendiamide 3 μM (in a standard bath solution without TTX) evoked calcium transients (Fig. 4A) with an average amplitude of 0.50 ± 0.05 ΔF/F (n = 73, 10 sequences, Fig. 4B). Consequences on the honey bee olfaction are discussed later on in this report.

4. Discussion

We report the first exploration of intracellular calcium transients in the honey bees' antennal neurons and the incidence of the insecticide flubendiamide and other ryanodine receptor modulators on these transients. The ability of our *in vitro* preparation to respond to physiological stimuli (9-ODA and depolarization) and to xenobiotics demonstrates the suitability of such preparation for calcium imaging studies. Most of the previous calcium imaging experiments in the honey bees were performed on isolated cells, slices or whole structure from neural tissues such as mushroom bodies (Haehnel et al., 2009), lateral horn (Roussel et al., 2014); antennal lobes (Carcaud et al., 2018; Galizia and Kimmerle, 2004; Sandoz, 2006) or compound eyes (Walz et al., 1995). Calcium imaging experiments were also conducted in the honey bee muscle (Collet, 2009; Kadala et al., 2019).

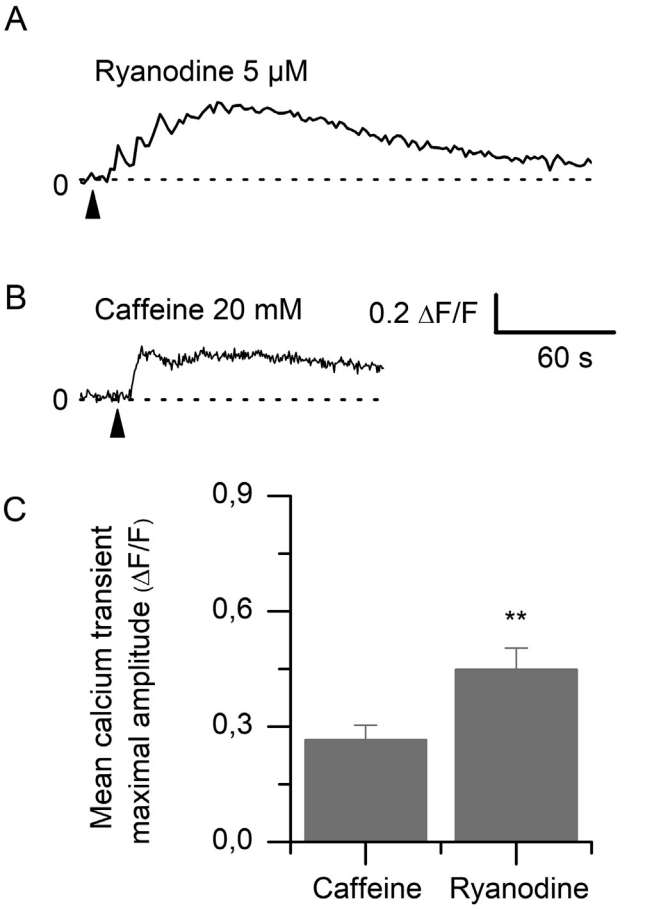

Fig. 3. Calcium activity in the presence of ryanodine and caffeine. Examples of intracellular calcium variations recorded after extracellular application of ryanodine 5 μM (A) or caffeine 20 mM (B) in the presence of TTX. Arrowheads indicate the beginning of the perfusion. C, Mean calcium transient maximal amplitude. Ryanodine induced a significantly stronger calcium increase than caffeine.

Two major sources for cytoplasmic calcium increases exist in the honey bee antennal neurons: Ca^{2+} from the extracellular space and Ca^{2+} stored in the endoplasmic reticulum. Our results show that Ca^{2+} transients can be induced by plasma membrane depolarization and by a pheromone (Fig. 2A–B). Depolarization activates surface membrane voltage-gated Ca^{2+} channels in antennal neurons (Laurent et al., 2002), and these channels provide a major entry route for neuronal Ca^{2+}, as in other bee structures involved in olfaction (mushroom body and antennal lobes neurons) or motor control (Rousset et al., 2017). Such influx of calcium from the extracellular compartment has been monitored in the honey bee skeletal muscle fibers (Collet, 2009), and the study suggests that extracellular calcium strongly influences the amplitude of the intracellular calcium signal. The role of olfactory receptors in the calcium entry from the extracellular compartment is disputed in the scientific community. One hypothesis proposes that olfactory receptors can act as ion channels and allow for a direct influx of calcium (Sato et al., 2008; Touhara and Vosshall, 2009). Another hypothesis supports a more canonical mechanism (inspired by mammalians) where olfactory receptors function at least partially as G-protein-coupled receptors (Wicher et al., 2008). So far, our results with 9-ODA cannot support one hypothesis or another.

The functional role for the external source of calcium is complex insofar as this calcium can in turn trigger calcium release from intracellular stocks (for review, Berridge et al., 2003; Clapham, 1995). Another way to increase calcium in the cytoplasm is indeed to draw it from intracellular stocks, one of which is the endoplasmic reticulum

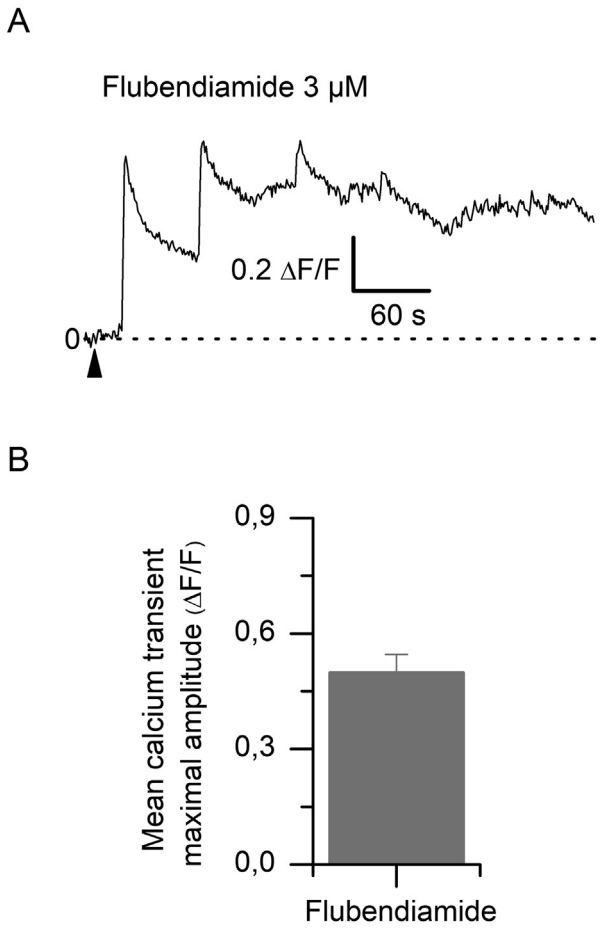

Fig. 4. Flubendiamide-induced calcium increases in antennal neurons. A, An example of intracellular calcium variations recorded after extracellular application of flubendiamide 3 µM. The arrowhead indicates the beginning of the perfusion. The mean calcium transient maximal amplitude is displayed in panel B.

(ER). Ryanodine receptors (RyRs) cover the surface of the ER and their activation can cause a release of calcium into the cytoplasm. This has been shown in the cockroach dorsal unpaired median neurons (Messutat et al., 2001) and in the salamander ORNs (Zufall et al., 2000). In our recordings, the 9-ODA and KCl induced strong calcium increases (Fig. 2). From a toxicological point of view, an *in vitro* assay dedicated to the study of calcium homeostasis alterations by xenobiotics requires the use of physiological stimuli to trigger calcium variations efficiently. To this end, KCl-induced depolarizations and pheromone application both appear suitable. Calcium pathways could be explored in more detail by using floral odorants such as linalool (Laurent et al., 2002), or general blockers of plasma-membrane calcium pathways, such as Cd^{2+} (Collet, 2009; Laurent et al., 2002), as well as more specific blockers of voltage-gated calcium channels such as nifedipine, verapamil, or mibefradil (Collet, 2009; Rousset et al., 2017; Schäfer et al., 1994), blockers of cyclic nucleotides activated channels such as diltiazem (Brown et al., 2006), blockers of the transduction cascades involving Phospholipase C or Adenylate Cyclase pathways (Fleischer and Krieger, 2018), or blockers of store-operated calcium entry (Zufall et al., 2000).

Alkaloids ryanodine and caffeine both target RyRs and in our recording conditions, ryanodine triggers larger Ca^{2+} transients even at a concentration 4000 fold smaller than caffeine (Fig. 3C). RyRs from the honey bee antennal neurons are far more sensitive to ryanodine than caffeine, in line with the EC_{50} of these two compounds being in the micromolar and in the millimolar range, respectively (Lee et al., 2002; Meissner, 2017; Sutko et al., 1997). Experiments on another insect, the tobacco budworm *Heliothis virescens*, have even shown a sensitivity to ryanodine at concentrations in the nanomolar range (Scott-Ward et al., 2001). Future experiments in neurons from the honey bee will define their sensitivity (EC_{50}) to ryanodine in more detail than the present study. Currently available data suggest that caffeine can bind to RyRs in the resting closed state and cause quantal release of calcium (Kong et al., 2008). Ryanodine on the other hand, binds to RyRs only in their open state and holds them in that state by either stabilizing a sub-conductance open state of the channels or destabilizing their closed state (Masumiya et al., 2001), leading to a sustained calcium increase in the cytoplasm (Collet and Jacquemond, 2002).

The insecticide flubendiamide induced strong calcium transients in the honey bee antennal neurons (Fig. 4). Previous studies show that flubendiamide binds to RyRs and keeps them open, allowing for a robust release of calcium into the cytoplasm (Ebbinghaus-Kintscher et al., 2006; Isaacs et al., 2012; Masaki et al., 2007; Qi and Casida, 2013; Qi et al., 2014). However, RyRs are not the only targets for flubendiamide as it also increases Ca^{2+}-ATPase activity in lepidopterans (Masaki et al., 2007). Moreover, we have previously shown that chlorantraniliprole which is another molecule from the same family as flubendiamide can block CaVs in the honey bee antennal lobe neurons and muscle fibers at high concentrations (3–200 µM), thus reducing calcium entry into the cytoplasm (Kadala et al., 2019). Although Ebbinghaus-Kintscher and colleagues (2006) showed in their study that compounds II and III from the same phthalic acid family as flubendiamide could trigger calcium increase independently from extracellular calcium, they did not ascertain that these compounds were not blocking CaVs *per se*. Therefore, flubendiamide may exert a complex cytotoxic action by, on one hand, limiting calcium entry into the cytoplasm through the blockade of voltage-gated calcium channels and by increasing calcium pumping through Ca^{2+}-ATPases. On the other hand, it may also promote calcium entry into the cytoplasm by holding ryanodine receptors in an open state at nanomolar to micromolar concentrations. The calcium signal that we observed in the presence of flubendiamide (Fig. 4A) may be the result of these opposing effects but this notion needs to be explicitly investigated. A recent study confirmed that flubendiamide has a much broader spectrum of action than defined by the manichean IRAC classification (https//irac-online.org): it also impedes mitochondrial function by interfering with complex I and F0/F1-ATPase activity (Nareshkumar et al., 2017). Flubendiamide mitochondrial action may also participate in triggering cytotoxic calcium anarchic variations since these organelles are also source of cytoplasmic Ca^{2+} (Giorgi et al., 2018). There is also the actual, yet untested possibility that flubendiamide binds directly to olfactory receptors, causing a direct influx of calcium from the exterior environment to the cytoplasm. Studies on mosquito have indeed suggested that the insecticide DEET might bind to the olfactory receptor or its co-receptor in order to exert its repellency (Ditzen et al., 2008; Tsitoura et al., 2015).

Exposure to flubendiamide may, for instance cause the antennal neurons to improperly detect and encode the olfactory information. Our previous study on chlorantraniliprole clearly identified a much stronger toxicity of diamides when applied to antennae (Kadala et al., 2019). Considering the crucial role that chemical communication plays in the honey bee hives (Alaux et al., 2010; Slessor et al., 2005), failure of exposed honey bees to detect and interpret chemical signals correctly may result in a disruption of the colony social dynamics. Although we currently lack data on the functional consequences of an impairment of antennal neurons' calcium homeostasis on the honey bees at individual and colony levels, studies on other insects can provide us with some clues. For example, experiments conducted on another hymenoptera, *Trichogramma brassicae* show that when males are treated with a sublethal dose of pyrethroid deltamethrin, they appeared more receptive to female sex pheromone but on the other hand, untreated males were less receptive to sex pheromone extracted from deltamethrin-exposed females (Delpuech et al., 1999). In another study with *Trichogramma Chilonis*, males exposed to beta-cypermethrin at a sublethal dose spent

less time in areas marked with *Trichogramma Chilonis* female pheromones and mated significantly less than untreated males (Wang et al., 2017).

In this study, we have shown the ability of an *in vitro* honey bee antennal neurons preparation to respond to physiological stimuli. We have also demonstrated that calcium signals in the honey bee antennal neurons are altered by the insecticide flubendiamide. To fully understand the interaction of flubendiamide with ryanodine receptors and other possible molecular targets, a deeper analysis of the calcium signals will be necessary, especially with regard to their kinetics. Additionally, behavioural consequences of alterations induced by flubendiamide insecticide at the individual or colony levels also remain to be investigated in the honey bee. Our study supports that an *in vitro* preparation and a calcium imaging assay can be used as a probing tool to assess the action of insecticides on pollinators and other important insects, and that neuronal intracellular calcium monitoring should be included in the modern assessment strategies of insecticides toxicity.

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Authors contributions

AK and CC performed the calcium imaging experiments, analysed the data and wrote the manuscript. MC performed cell culture experiments and critically read the manuscript. All authors approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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