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Water Taste Transduction Pathway Is Calcium Dependent in *Drosophila*

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Abstract

In mammals, detection of osmolarity by the gustatory system was overlooked until recently. In insects, specific taste receptor neurons detect hypoosmotic stimuli and are commonly called "W" (water) cells. W cells are easy to access in vivo and represent a good model to study the transduction of hypoosmotic stimuli. Using pharmacological and genetic approaches in *Drosophila*, we show that tarsal W cell firing activity depends on the concentration of external calcium bathing the dendrite. This dependence was confirmed by the strong inhibition of W cell responses to hypoosmotic stimuli by lanthanum (IC_{50} = 8 nM), an ion known to inhibit calcium-permeable channels. Downstream, the transduction pathway likely involves calmodulin because calmodulin antagonists such as W-7 (IC_{50} = 2 μM) and fluphenazine (IC_{50} = 30 μM) prevented the activation of the W cell by hypoosmotic stimuli. A protein kinase C (PKC) may also be involved as W cell responses were blocked by PKC inhibitors, chelerythrine (IC_{50} = 20 μM) and staurosporine (IC_{50} = 30 μM). It was also reduced when expressing an inhibitory pseudosubstrate of PKC in gustatory receptor neurons. In the rat, the transduction pathway underlying low osmolarity detection involves aquaporin and swelling-activated ion channels. Our study suggests that the transduction pathway of hypoosmotic stimuli in insects differs from mammals.

Key words: calmodulin, hypoosmotic, insect, osmolarity, PKC, transduction

Introduction

Hydro-mineral balance is a major concern for air-living organisms. Therefore, they have to adapt their fluid consumption according not only to its palatability but also to its osmolarity to maintain hydro-mineral homeostasis. This ability exists both in insects (Wolbarsht 1957; Evans and Mellon 1962a) and in mammals (Gilbertson et al. 2006) that use taste cells to detect hypoosmotic stimuli. Although receptors to most taste modalities have been identified in mammals and insects (Hallem et al. 2006; Scott 2005; Vosshall and Stocker 2007; Bachmanov and Beauchamp 2007), the molecular elements underlying water detection in taste cells remain elusive. In rats, recordings from the chorda tympani have shown that osmolarity can modulate taste perception (Lyall et al. 1999) and most of their taste cells are sensitive to osmolarity (Gilbertson 2002). The transduction of hypoosmotic stimuli likely involves aquaporin and swelling-activated ion channels (Watson et al. 2007).

Whereas in mammals, taste involves mainly sensors localized in the mouth, insect taste bristles are distributed all over the body. It allows them to obtain information about potential food even before engaging any ingestion activities. Most of these taste bristles house 4 gustatory receptor neurons (GRNs) that respond to specific stimuli (Singh 1997). One class of these GRNs is called the W cell (for water). It was discovered in flies (Wolbarsht 1957; Evans and Mellon 1962a; Rees 1970) and subsequently in other insect species (Fujishiro et al. 1984; Schoonhoven and van Loon 2002). Behavioral and genetic studies show that W cells are involved in water detection. In thirsty flies, their activation triggers the extension of the proboscis and drinking (Evans 1961; Evans and Dethier 1961; Dethier 1976; Inoshita and Tanimura 2006). W cells are excited by hypoosmotic stimuli and inhibited by increasing concentrations of various tastants like salts (Evans and Mellon 1962b; Rees 1970; Goshima et al. 1997), sugars (Wieczorek 1980), and amino acids (Ishikawa 1967; Panzuto and Albert 1998). This broad sensitivity suggested that W cells monitor the osmotic pressure of tastants. However, W cells are not strict osmotic receptor neurons. Electrolytes are more effective than other substances to inhibit their activity (Rees 1970), and bitter substances are also

known to inhibit W cells at very low concentration (Meunier N, Marion-Poll F, Rospars JP, et al. 2003). In addition to hypoosmotic stimuli, W cells are stimulated by anesthetics in *Phormia* (Dethier and Goldrich-Rachman 1976) or by sugars like D-fucose in *Protophormia* (Wieczorek and Köppl 1978; Wieczorek 1980) suggesting that they also have chemoreceptor functions. One of the clearest evidence of its mixed sensitivity comes from experiments where taste sensilla were exposed to pronase, which suppressed the responses to water but not to D-fucose (Wieczorek et al. 1988).

In this work, we examined the transduction pathway involved in the hypoosmotic response of *Drosophila* W cells in tarsal sensilla using pharmacological and genetic approaches. The peculiar structure of taste organs in insects allows the application of drugs and the recording of GRN electrical activity in vivo (Amakawa and Ozaki 1989). In *Drosophila*, the W cell is easier to record than in many other insects. Indeed, when using the tip-recording method (Hodgson et al. 1955), its extracellularly recorded action potentials have a larger amplitude than action potentials generated by other GRNs (Fujishiro et al. 1984; Meunier N, Marion-Poll F, Lansky P, et al. 2003). Our observations indicate that calcium plays a major role in the response to hypoosmotic stimuli. Increasing extracellular calcium concentration enhanced the response of the W cell, whereas extracellular calcium chelators (ethylenediaminetetraacetic acid [EDTA] or ethyleneglycol-bis(aminoethylether)-tetraacetic acid [EGTA]) completely suppressed it. The involvement of extracellular calcium was consistent with the inhibition of the W cell by lanthanum which is a broad inhibitor of calcium-permeable channels (Halaszovich et al. 2000). The transduction pathway of hypoosmotic stimuli detection also involves calmodulin because their responses were strongly depressed by 2 calmodulin antagonists, W-7 and fluphenazine. The calcium/calmodulin pathway may act along with protein kinase C (PKC) because the responses of the W cell were reduced by pharmacological PKC inhibitors (staurosporine and chelerythrine) or by genetically expressing an inhibitory pseudosubstrate of PKC in GRNs. These observations suggest that taste response to water in *Drosophila* tarsal sensilla is performed through different mechanisms than those found in mammals.

Materials and methods

Chemicals

Drugs and chemicals were obtained from Sigma (France, Saint-Quentin Fallavier), except for chelerythrine that was obtained from Alomone (Israel, Jerusalem). Stock solutions were prepared in advance and stored at –20 °C. All stimuli or drugs were prepared as dilutions in 1 mM KCl and kept at 4 °C for less than 1 week. Dimethyl sulfoxide (DMSO) was used to solubilize 1,2-dioctanoyl-sn-glycerol (DOG), U73122, and staurosporine, and the final DMSO dilution was ≤0.1%.

Flies

Canton Special (CS) flies were used as wild type. The P[GAL4] strain *MJ94/FM7* (Joiner and Griffith 1999) was kindly provided by R. Stocker. *UAS-PKCi* (Broughton et al. 1996) was obtained from the *Drosophila* Stock Center (Bloomington, IN). Stocks were maintained at 25 °C on a standard cornmeal agar medium. Flies aged 2–5 days were fed on fresh medium 1 day before experiments. We pooled recordings from females and males as we observed no difference between genders for the W cell responses.

Electrophysiology

GRNs recording technique

A decapitated fly was secured to a flat support with insect pins and tape and electrically grounded via a glass capillary filled with Ringer's solution inserted into the thorax. All recordings were performed from tarsal sensilla "5b" of the front leg which house a W cell but also S/L1/L2 cells sensitive, respectively, to sugars, to salts at low concentrations, and to bitter compounds/salts at high concentrations (Meunier et al. 2000; Meunier N, Marion-Poll F, Rospars JP, et al. 2003). To stimulate a sensillum, its tip was covered for less than 2 s with a recording electrode containing an electrolyte (1 mM KCl) and the stimulus (Hodgson et al. 1955). Consecutive stimuli were applied at least 1 min apart to avoid sensory adaptation.

The recording electrode (a glass capillary with a tip diameter of 20 μm) was connected to a TastePROBE amplifier (Marion-Poll and Van der Pers 1996). The electric signals were amplified (×1000) and filtered (bandpass filter: 1–2800 Hz, eighth order Bessel) using a CyberAmp 320 amplifier (Axon instrument, Sunnyvale USA). Contacting a taste bristles with the stimulus electrode triggered a 2-s data acquisition bout (sampling rate 10 kHz, 16 bits; DT9803, Data Translation, (Marlboro, USA)) that was stored on a computer.

Data analysis

Spikes were detected and sorted using dbWave according to previous methods (Marion-Poll 1996; Meunier N, Marion-Poll F, Lansky P, et al. 2003). We evaluated the action potential frequency by counting spikes during the first second of stimulation. The statistical significance of the results (treatment vs. control) was assessed by Student's *t*-tests.

Effect of stimulation on the water cell firing

Each stimulation protocol on a sensillum started with 1 mM KCl as a reference for the W cell activity and sensilla which responded with less than 10 spikes/s were discarded. Then, we applied different stimulation sequences, either to characterize the W cell as activated by hypoosmotic stimuli: KCl (1, 5, 10, 25, and 50 mM; $n \geq 15$), choline chloride (ChCl; 1, 5, 10, 25, and 50 mM; $n \geq 9$), or in order to study the W cell transduction pathway: $CaCl_2$ (0.01, 0.1, and 1 mM; $n \geq 24$),

EGTA (0.03, 0.1, 0.3, and 1 mM; $n \geq 8$), lanthanum (0.1, 1, 10, and 100 nM; $n \geq 13$), W-7 (0.06, 0.6, 1, 6, and 60 μM; $n \geq 7$), chelerythrine (1, 10, and 30 μM; $n \geq 8$), 8-Br-cAMP (100 μM; $n = 6$), 8-Br-cGMP (100 μM; $n = 12$), DOG (100 μM; $n = 7$), and of U73122 (1 μM; $n = 9$). On average 2, sensilla were recorded per fly, and the effects of each drug were measured on at least 4 flies.

Control stimulation on salt responses

In order to evaluate if our observations were specific to the W cell, we stimulated 5b sensilla with 500 mM KCl in the presence or absence of a subset of the drugs used in these experiments (Table 1). At this concentration, KCl elicits responses mainly from the L1 cell, a GRN sensitive to salts (Meunier N, Marion-Poll F, Lansky P, et al. 2003). Each stimulation protocol started with 500 mM KCl and was followed by 1 mM $CaCl_2$ ($n = 12$), 1 mM EGTA ($n = 8$), 600 μM W-7 ($n = 8$), 30 μM chelerythrine ($n = 8$), or 1 μM lanthanum ($n = 12$) mixed with 500 mM KCl.

Results

Responses of 5b sensilla to increasing concentrations of KCl and choline chloride

We stimulated tarsal terminal sensilla 5b with concentrations of KCl and ChCl ranging from 1 to 50 mM. All action potentials recorded were of comparable amplitude and occurred at regularly spaced intervals. This indicates that only one GRN was active at these concentrations (Figure 1A). As the concentration of salts increased, the spike frequency decreased, which is typical for the W cell (Figure 1B). A complete inhibition of the W cell was observed for concentrations higher than 50 mM of KCl or ChCl. The inhibition of the W cell was observed for concentrations of KCl or ChCl higher than 5 mM (10 mosmol/l). In the following experiments aimed to decipher the W cell transduction pathway, we used compounds at osmolarity lower than the inhibitory threshold (≤5 mosmol/l). Any inhibitory effect observed was thus independent of inhibition by osmolarity. We used 1 mM KCl as the control hypoosmotic stimuli of the W cell in the following experiments.

At higher concentrations of KCl (500 mM), a second cell fired at a rate of 50–90 Hz (Table 1: L1/Control); this type of cell had been formerly described as the GRN sensitive to salts at low concentration named L1 cell (Meunier N, Marion-Poll F, Lansky P, et al. 2003).

The firing response of the W cell to hypoosmotic stimuli depends on the concentration of Ca^{2+} in the sensillar lymph

In order to determine if extracellular calcium is involved in the W cell activity, we stimulated sensilla 5b with 1 mM KCl in the presence of different concentrations of $CaCl_2$ (Figure 2A). Responses of the W cell to hypoosmotic stimuli increased with the concentration of extracellular $CaCl_2$: adding 1 mM $CaCl_2$ in the stimulating electrode increased the W cell activity by approximately 70%. By contrast, the response of the L1 cell to 500 mM KCl was not affected by 1 mM $CaCl_2$ (Table 1).

Calcium chelators decrease the firing of the W cell

We then used increasing concentrations of calcium chelators to examine whether the reduction of free external calcium modifies the W cell activity. EGTA at 1 mM almost completely silenced the W cell when stimulated with 1 mM KCl (Figure 2B). The IC_{50} of EGTA was 100 μM. On the contrary, the response of the L1 cell to 500 mM KCl was not affected by 1 mM EGTA (Table 1). EDTA had a similar inhibitory effect to EGTA on the activity of the W cell (IC_{50} = 90 μM, data not shown).

Lanthanum blocked the W cell activity

The modulation of the W cell activity by extracellular calcium level suggested that a calcium inward current is involved in its transduction pathway. Lanthanum is a broad inhibitor of calcium-permeable channels (Halaszovich et al. 2000). The W cell activity was strongly inhibited by

Table 1 Modulation by $CaCl_2$, EGTA, lanthanum, W-7, and chelerythrine of the responses of the W cell to a hypoosmotic stimulus (1 mM KCl) and of L1 cell to 500 mM KCl

Cell	Treatment	$CaCl_2$, 1 mM	EGTA, 1 mM	Lanthanum, 1 μM	W-7, 600 μM	Chelerythrine, 30 μM
W	Control	26 ± 1	29 ± 4	23 ± 1	26 ± 3	24 ± 4
	Treated	44 ± 1*	4 ± 1*	6 ± 1*	0 ± 1*	7 ± 1*
		($n = 23$)	($n = 8$)	($n = 22$)	($n = 7$)	($n = 8$)
L1	Control	92 ± 5	76 ± 7	61 ± 7	79 ± 6	50 ± 5
	Treated	87 ± 5	76 ± 6	76 ± 6	76 ± 3	40 ± 3
		($n = 12$)	($n = 8$)	($n = 8$)	($n = 8$)	($n = 8$)

Values are means of firing frequency of action potentials during the first second of stimulation (mean ± standard error of the mean). *$P < 0.01$, significantly different from control values (Student's *t*-test).

lanthanum with an IC_{50} of 8 nM (Figure 2C). The addition of 1 μM lanthanum had no statistically significant effect on the response of the L1 cell to 500 mM KCl (Table 1).

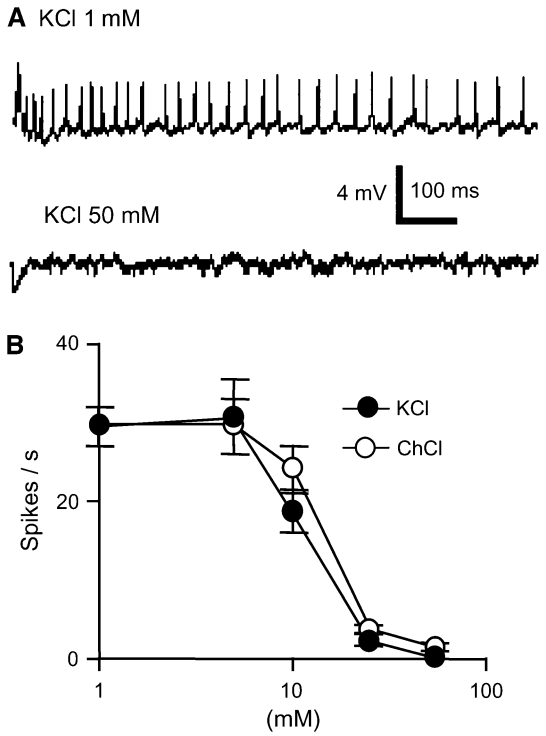

Figure 1 Properties of the W cell in *Drosophila* tarsal sensilla. **(A)** Typical recordings from a tarsal sensillum to a hypoosmotic stimulus (1 mM KCl) versus a higher osmolarity stimulus (50 mM KCl). At 50 mM, KCl did not elicit response from other GRNs. **(B)** Increasing the concentration of KCl or ChCl decreased the response of the W cell which is a characteristic of the water taste. In the following experiments, 1 mM KCl was used as the control hypoosmotic stimuli of the W cell. $N \geq 9$ and $n \geq 15$ for the ChCl and KCl increasing concentration, respectively. Error bars = standard error of the mean.

Calmodulin is involved in the W cell transduction pathway

Calmodulin is one of the transduction proteins whose activity depends on an increase of intracellular calcium concentration. To check whether calmodulin is involved in the transduction of hypoosmotic stimuli in the W cell, we used W-7, an antagonist of this protein. W-7 decreased the response of the W cell to 1 mM KCl (Figure 3A). The IC_{50} of W-7 was 2 μM. Another known inhibitor of calmodulin, fluphenazine, also decreased the response of the W cell to 1 mM KCl (IC_{50} = 30 μM, data not shown). As for $CaCl_2$ and EDTA, we observed no statistically significant effect of W-7, even at 600 μM, on the responses of the L1 cell to 500 mM KCl (Table 1).

PKC is involved in the W cell transduction pathway

PKC is known to be involved in calcium-related transduction pathways. We used 2 different PKC inhibitors, chelerythrine and staurosporine, to check if PKC is involved in the responses of the W cell to hypoosmotic stimuli. Both compounds reduced the responses of the W cell to 1 mM KCl. We found an IC_{50} of 20 μM for chelerythrine (Figure 3B) and of 30 μM for staurosporine (data not shown). The addition of 30 μM chelerythrine to 500 mM KCl did not statistically modify the response of the L1 cell (Table 1).

In addition, we aimed to reduce the activity of PKC in GRNs using a genetic approach. Using the Gal4-UAS system, we expressed ectopically a pseudosubstrate of PKC (PKCi) that inhibits different classes of PKCs (Broughton et al. 1996). The expression of the PKCi peptide was driven with the *MJ94* P[gal4] enhancer trap line in all GRNs (Joiner and Griffith 1999; Gendre et al. 2004). We recorded female progeny of a cross between female *MJ94/FM7* and male *UAS-PKCi*. Whereas the response to 500 mM KCl was not affected, the response of the W cell to 1 mM KCl was statistically reduced by 30% when PKCi was expressed in GRNs (Figure 4).

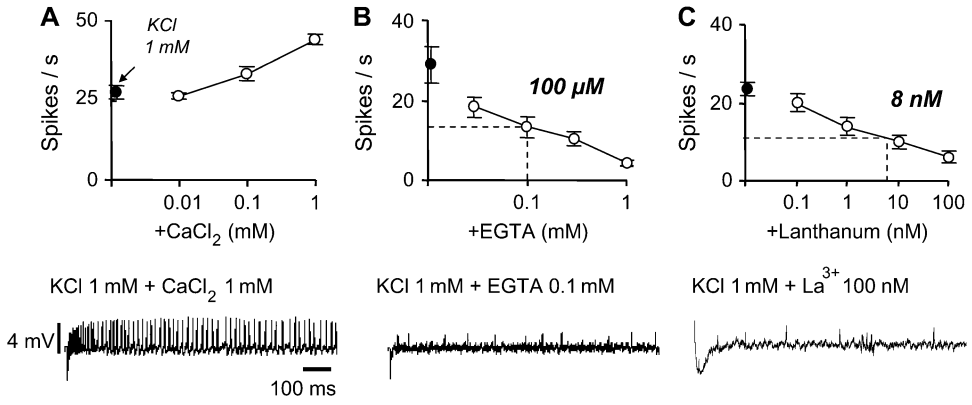

Figure 2 The activity of the W cell to hypoosmotic stimuli (1 mM KCl) depends on a calcium influx. For all graphs, the full circle indicates the activity of the W cell before treatment. A typical recording of 1 s is presented below each graph. **(A)** Increasing the extracellular concentration of calcium enhanced the activity of the W cell. $N \geq 24$. **(B)** On the contrary, chelating the extracellular calcium with EGTA reduced the W cell activity. $N \geq 8$. **(C)** Lanthanum, a broad inhibitor of calcium-permeable channels, also decreased the response of the W cell to hypoosmotic stimuli. $N \geq 13$. All compounds were mixed with 1 mM KCl. IC_{50} values are indicated in italics. Error bars = standard error of the mean.

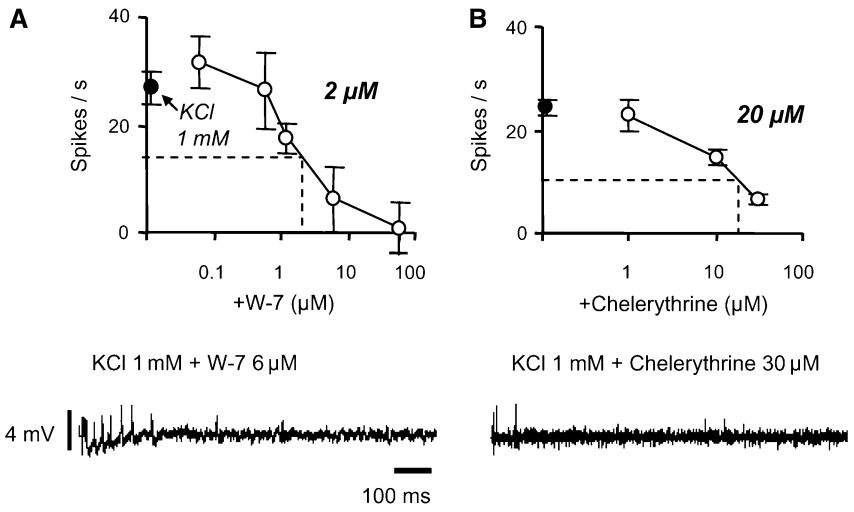

Figure 3 Calmodulin and PKC are necessary for the W cell activity. For all graphs, the full circle indicates the activity of the W cell before treatment. A typical recording of 1 s duration is presented below each graph. Inhibitions of W cell responses were observed with W-7 **(A)** ($n \geq 7$) and chelerythrine **(B)** ($n \geq 8$) suggesting that calmodulin and PKC are involved in the W cell transduction of hypoosmotic stimuli. All compounds were mixed with 1 mM KCl. IC_{50} values are indicated in italics. Error bars = standard error of the mean.

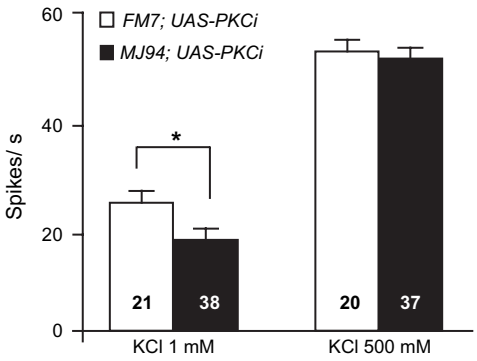

Figure 4 Genetic inhibition of PKC decreased the response of the W cell to hypoosmotic stimuli (1 mM KCl). Directed expression of an inhibitory pseudosubstrate of PKC in all GRNs (*MJ94*; *UAS-PKCi*) decreased the response to hypoosmotic stimuli but not to salts when compared with control progeny (*FM7*; *UAS-PKCi*). The number in each box represents the number of flies used for each genotype coming from the same crossing (mean ± standard error of the mean). $*P < 0.05$, significantly different from control values (Student's *t*-test).

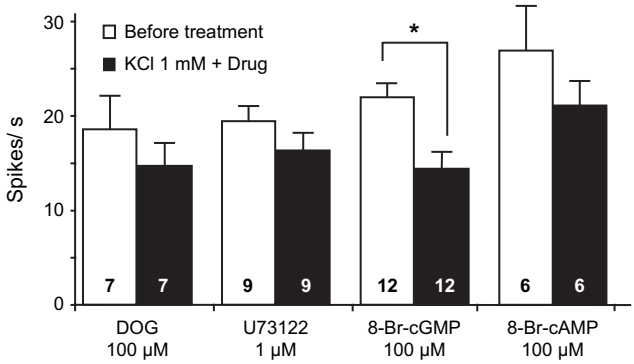

Figure 5 Second messengers of known GPCR-based pathways do not activate the W cell. The number in each box represents the number of sensilla recorded for each drug. Responses of the W cell to 1 mM KCl were compared in the presence versus in the absence of each drug tested. Mean ± standard error of the mean. $*P < 0.05$, significantly different from control values (Student's *t*-test).

Cyclic nucleotides and diacylglycerol are not directly involved in the activation of the W cell

In order to determine whether the calcium inward current could be downstream of G protein–coupled receptors (GPCRs), we used several analogs of second messengers to mimic a possible GPCR activation (Figure 5). Treatments with 100 µM 8-Br-cAMP or 100 µM 8-Br-cGMP, which are membrane-permeable analogs of cAMP and cGMP, respectively, or with 100 µM DOG, a diacylglycerol analog, did not increase the firing of the W cell. On the contrary, we found that 8-Br-cGMP slightly but significantly decreased the response of W cell to 1 mM KCl. U73122, an inhibitor of phospholipase C which is responsible for diacylglycerol and inositol triphosphate production, did not alter the response of the W cell to hypoosmotic stimuli.

Discussion

Taste studies on *Drosophila* using pharmacological approach

We show here that the W cell activity is calcium dependent by the use of a combination of pharmacological, genetic, and electrophysiological methods. The use of a pharmacological approach to elucidate taste transduction pathways has been repeatedly used in larger flies to study sugar detection (Amakawa et al. 1990; Ozaki and Amakawa 1992; Liscia et al. 2002; Seno et al. 2005) but never in

Drosophila. In *Drosophila*, the spike amplitude and shape of one GRN recorded with the tip-recording method varies according to the composition of the recording electrode (Fujishiro et al. 1984; Meunier N, Marion-Poll F, Lansky P, et al. 2003), and we found similar results in this study (Figure 2). Thus, in order to perform pharmacological study, it may be very difficult to decipher which GRN is affected if more than one is activated. In this study, we always observed only one train of action potential of similar shape and amplitude for all recordings except those with high concentrations of salts. Accordingly, even if the shape and amplitude of the action potentials were modulated while adding different drugs with 1 mM KCl, all spikes must originate from the W cell.

The IC_{50} obtained in our experiments were lower than those obtained on larger flies (Table 2). For example, W-7 was effective to modulate the response from the sugar-responding GRN and the W cell in the blowfly at concentrations ranging from 100 to 500 μM (Liscia et al. 2002), whereas we obtained strong inhibitions of the W cell for concentrations 100 times lower. Similarly, we found an IC_{50} for EGTA of 100 μM, whereas concentrations used in the blowfly to modulate responses to sugars were in the millimolar range (Ozaki and Amakawa 1992). The main difference may be based on the smaller size of the sensilla in *Drosophila* allowing a faster diffusion of drugs from the tip of the taste sensillum to the GRN than in larger flies. It can also be that the W cell is simply more sensitive to those drugs in *Drosophila* than other GRNs in larger flies.

The W cell is inhibited by bitter compounds at very low concentration (Meunier N, Marion-Poll F, Rospars JP, et al. 2003). By using a pharmacological approach to study the taste transduction pathway, one cannot exclude that the drug by itself is detected as a bitter compounds. We never observed an activation of the bitter compound-sensitive GRN with the addition of the drugs used in our study. However, for some molecules, the inhibition of the W cell can occur with concentrations lower than those required to activate the bitter-sensitive GRN (Meunier N, Marion-Poll F, Rospars JP, et al. 2003). In order to avoid such false results, we systematically used at least 2 different molecules affecting the same cellular target.

Table 2 Summary of IC_{50} values and effects of the different pharmacological agents used to study the response of the W cell to a hypoosmotic stimulus (1 mM KCl)

Treatment	IC_{50}/effect
EGTA (extracellular calcium chelator)	100 μM
Lanthanum (broad calcium channel inhibitor)	8 nM
W-7 (calmodulin inhibitor)	2 μM
Fluphenazine (calmodulin inhibitor)	30 μM
Chelerythrine (PKC inhibitor)	20 μM
Staurosporine (protein kinase inhibitor)	30 μM
1 μM U73122 (phospholipase C inhibitor)	No effect
100 μM 1,2-dioctanoyl-sn-glycerol (diacylglycerol analog)	No effect
100 μM 8-Br-cAMP (cAMP analog)	No effect
100 μM 8-Br-cGMP (cGMP analog)	Slight inhibition

Except EGTA, EDTA, and lanthanum, all compounds tested are cell permeable.

Importance of the calcium pathway in hypoosmotic detection

Several lines of evidence demonstrate the importance of calcium in hypoosmotic detection for *Drosophila*. Calcium is known to stimulate GRNs responding to salts in various insects at concentration higher than 100 mM (Schnuch 1996). By contrast, we found that for tarsal taste sensilla of *Drosophila*, the firing response of the W cell to hypoosmotic stimuli depends on the concentration of calcium in the sensillar lymph. Even if calcium is known to change the configuration of numerous proteins, a modulation of extracellular concentration of calcium leading to unspecific alteration of GRNs activity is rather unlikely considering that responses to salts were not sensitive to the same change of external calcium concentration. Which role could the calcium play in the transduction of the hypoosmotic stimuli in the W cell? The W cell response to hypoosmotic stimuli was decreased by adding calcium chelators, and it was increased when the external calcium concentration was raised. Thus, a calcium entry is probably involved. This idea is supported by the strong inhibitory effect of lanthanum, which broadly inhibits calcium-permeable channels. Lanthanum is known to block other currents such as potassium currents for concentration higher than 100 μM (Alshuaib and Mathew 2005), but we found a very low IC_{50} for this inorganic ion as in previous studies (Block et al. 1998). In agreement with this hypothesis, the sole presence of calcium in the sensillar lymph would not be sufficient to stimulate the W cell as a calcium channel needs to be opened first. This is in accordance with previous studies using side wall recordings showing that the W cell starts firing after being stimulated with hypoosmotic stimuli (Fujishiro et al. 1984).

The specificity of the calcium pathway is also supported by the effect of calmodulin antagonists such as W-7 and fluphenazine. These results are in good agreement with two studies focused on the elucidation of the sugar detection transduction pathway which pointed out that W-7 also reduces the W cell activity in *Phormia regina* (Liscia et al. 2002; Seno et al. 2005). One can ask about the specificity of those drugs as calmodulin antagonists are also known to inhibit calcium channels (Ehrlich et al. 1988; Khan et al. 2001). Our IC_{50} are, however, lower than in those studies, 6 versus 100 μM for W-7 and 30 versus 75 μM for fluphenazine. Furthermore, we certainly underestimated the IC_{50} of the drugs tested in our study as we applied them from

the recording pipette, and they were then diluted in the sensillum lymph before reaching their target. As calmodulin requires binding calcium in order to be activated, the calmodulin dependency of the W cell reinforces the idea that calcium is essential in the W cell transduction pathway.

Finally, the key role of calcium in water taste is also supported by the pharmacological and the genetic inhibition of PKC. Staurosporine, a broad protein kinase inhibitor, and chelerythrine, a specific inhibitor of PKC, suppressed the W cell response to 1 mM KCl. These results are in good agreement with the effects of the expression of the PKC inhibitory pseudosubstrate in GRNs using the Gal4/UAS system. We observed in this study that the inhibition was only partial but this result is consistent with previous observations. Indeed, the expression of this pseudosubstrate using the Gal4/UAS system originally inhibited ~20% of endogenous PKC in head extract (Broughton et al. 1996) and reduced partially the PKC-dependent mobility of cells during ovarian morphogenesis (Cohen et al. 2002).

A model of transduction pathway in the W cell

The inhibition of calmodulin or PKC completely suppressed the response of the W cell to hypoosmotic stimuli. As calmodulin activation depends on an increase in intracellular calcium concentration, the calcium influx should be among the earliest step of the W cell activation but it is most likely not enough by itself to depolarize the cell and trigger action potentials. Thus, another channel may be involved in the W cell response (Figure 6).

This model is also consistent with the kinetics of the inhibition of the W cell response to hypoosmotic stimulus by various drugs. Effects of drugs can be classified in two broad categories. Those supposed to act on the modulation of the initial calcium current (calcium, EGTA, and lanthanum; Figure 2) modulate the response of the W cell without delay and steadily, whereas those acting on intracellular targets (W-7 and chelerythrine; Figure 3) modulate the response overtime. This last delayed inhibition can be explained by the time required for the drug to enter the GRN. One critical feature lacking in this model is the origin of the opening of the calcium channel.

Ligands of gustatory receptors (GRs) such as sugars, bitter compounds, and pheromones begin to be unraveled in insects, but receptors to hypoosmotic stimuli remain unknown (Ebbs and Amrein 2007). One classical pathway for such detection could involve GPCRs. Neither 8-Br-cAMP nor 8-Br-cGMP nor DOG, a diacylglycerol analog, which should mimic the cAMP, cGMP, and the lipidic branch of the phospholipase C pathways, respectively, did activate the W cell. We even found that 8-Br-cGMP rather inhibited the W cell. It may be that cGMP is involved in the W cell inhibition rather than its activation by low osmolarity stimuli. These observations do not necessary mean that no GRs are involved in the activation of the W cell as recent data indicate that the *Drosophila* ORs, which are very similar to GRs, not only may be coupled to G proteins but also may function as ion channels as well (Kain et al. 2008; Sato et al. 2008; Wicher et al. 2008) as previously suggested for GRs (Murakami

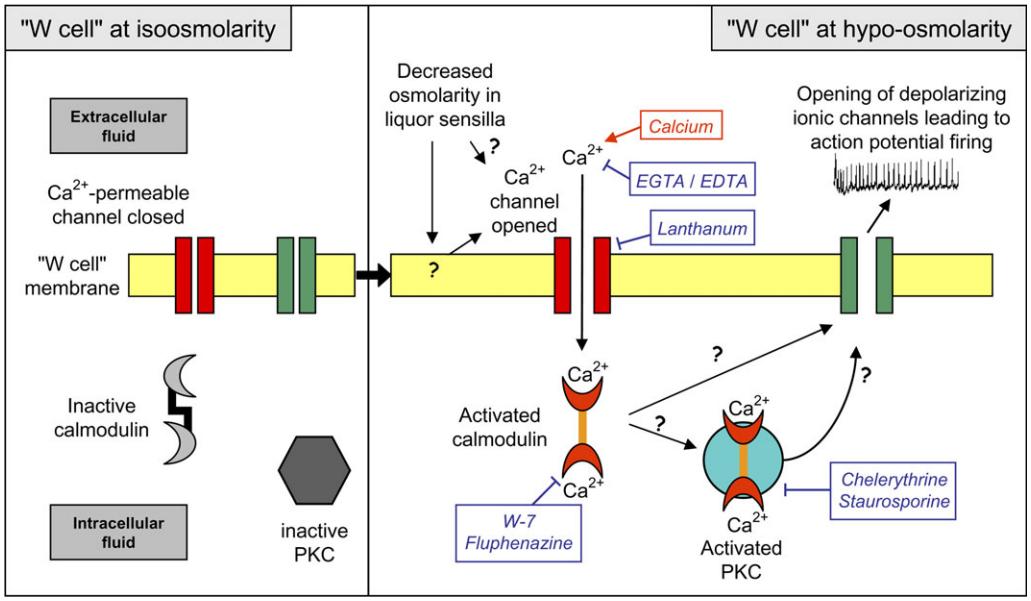

Figure 6 Proposed model of the transduction pathway in the W cell. Responses to hypoosmotic stimuli were correlated to extracellular calcium concentration, and they were reduced in the presence of lanthanum, a broad blocker of calcium-permeable channels. Therefore, the response of the W cell to a low extracellular osmolarity likely involves an inward calcium current. The origin of the opening of this calcium channel needs to be clarified. Such a current most probably cannot depolarize the neuron enough to trigger action potentials because the inhibition of either calmodulin or PKC blocked the W cell firing response. As calmodulin and PKC are also related to the calcium transduction pathway, we propose that their activation by the intracellular calcium increase opens other ionic channels that in turn depolarize the W cell to trigger the firing of action potentials.

and Kijima 2000). Thus, it could be that GRs sensitive to hypoosmotic stimuli would lead to calcium influx.

The transduction pathway of hypoosmotic stimuli by taste receptor cells in mammals involves first aquaporin leading to the activation of swelling-activated chloride channels (Watson et al. 2007). We cannot exclude that a similar mechanism is involved in the first step leading to a calcium influx. However, our finding that calcium, calmodulin, and PKC are involved in the W cell activation suggests that the mechanisms of detection of hypoosmotic stimuli by taste receptor cells differ between mammals and insects. Further experiments will be required to decipher the nature of W cell receptors.

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