INTRACELLULAR-MESSENGER-MEDIATED CATION CHANNELS IN CULTURED OLFACTORY RECEPTOR NEURONS

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Summary

After 2–3 weeks in culture, pupal olfactory receptor neurons from the antennae of male *Manduca sexta* respond to their species-specific sex pheromone by opening cation channels. These pheromone-dependent cation channels are the only channels previously found in cultured olfactory neurons that promote inward currents at membrane potentials more negative than the resting potential. The pheromone-dependent currents depend on external Ca\(^{2+}\) concentration. They are inwardly rectified with 10\(^{-7}\) mol l\(^{-1}\) external Ca\(^{2+}\) and linear with 6 mmol l\(^{-1}\) external Ca\(^{2+}\). This paper shows that perfusion of cultured olfactory receptor neurons with GTP\(_\gamma\)S, ATP, inositol 1,4,5-trisphosphate or 10\(^{-6}\) mol l\(^{-1}\) Ca\(^{2+}\) elicits cation currents resembling the pheromone-dependent cation currents in expressing inward rectification with 10\(^{-7}\) mol l\(^{-1}\) external Ca\(^{2+}\) and being linear at external Ca\(^{2+}\) concentrations of 2 \(\mu\)mol l\(^{-1}\) or more. Stimulation with protein kinase C also elicits cation currents that share properties with the pheromone-dependent cation currents. All agent-induced cation currents appear to depend either directly or indirectly on Ca\(^{2+}\) concentration.

Introduction

*Manduca sexta* females attract their conspecific mates through the release of a unique blend of sex pheromones (Starratt *et al.* 1979; Tumlinson *et al.* 1989). The males detect these pheromones with specialized olfactory receptor neurons (ORNs) which innervate long, male-specific sensilla trichodea on the male antennae (Sanes and Hildebrand, 1976a,b; Schweitzer *et al.* 1976; Keil, 1989; Christensen *et al.* 1989; Kaissling *et al.* 1989). After stimulation of pheromone-sensitive ORNs, sensillar potentials, which exhibit several different time constants and which show adaptation after a strong pheromone stimulus, can be recorded extracellularly (Schneider, 1962; Schneider and Boeckh, 1962; Zack-Strausfeld, 1979; Kaissling and Thorson, 1980; Kaissling, 1987; Zack-Strausfeld and Kaissling, 1986; Kaissling *et al.* 1987; Vogt, 1987). Since the small ORNs are tightly covered by supporting cells and lie beneath a thick cuticle (Keil and

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Steinbrecht, 1987; Keil, 1989), they are relatively inaccessible for intracellular or patch-clamp recordings. Therefore, a primary cell culture system consisting of differentiating, immunocytochemically identifiable ORNs from third-stage pupae of male *M. sexta* was developed (Stengl and Hildebrand, 1990). Within 2–3 weeks in culture, the ORNs differentiate morphologically and physiologically. They express at least three different potassium channels and a tetrodotoxin (TTX)-blockable sodium channel, but no voltage-gated Ca\(^{2+}\) channels (Zufall et al., 1991c). They respond to their species-specific sex pheromone blend by opening nonspecific cation channels (Stengl et al., 1989, 1992a,b). These pheromone-dependent cation channels do not discriminate between Na\(^+\) and K\(^+\). Their current reverses around 0mV, irrespective of the main external anion, and shows pronounced inward rectification with external Ca\(^{2+}\) buffered to 10\(^{-7}\) mol l\(^{-1}\) (Stengl et al., 1992b). These cation channels were the only channels observed in cultured ORNs to carry inward currents at potentials more negative than the resting potential, with ‘normal’ ionic gradients (see Materials and methods). Previous evidence has suggested that the pheromone-dependent cation channels were second-messenger-dependent (Stengl et al., 1992b).

After exposure to pheromone, cells of insect antennae exhibit slow and long-lasting increases in cyclic GMP concentration (Ziegelberger et al., 1990) and antennal extracts show G-protein-dependent rapid and transient increases in inositol 1,4,5-trisphosphate (Ins\(\text{P}_3\)) concentration (Boekhoff et al., 1990; Breer et al., 1988, 1990). Hence, it was postulated that a G-protein-dependent phosphoinositidase C generating the two second messengers Ins\(\text{P}_3\) and diacylglycerol (Berridge, 1987; Gilman, 1987; Berridge and Irvine, 1989) was involved in the generation of the rising phase of the receptor potential, while a guanyl cyclase and probably a protein kinase C (Boekhoff and Breer, 1992) might be involved in its declining phase and in the adaptation of the receptor potentials (Stengl et al., 1992a).

This study investigates whether cultured ORNs respond to activation by G-proteins, application of Ins\(\text{P}_3\), a rise in internal Ca\(^{2+}\) concentration or activation by a protein kinase C by opening cation channels that might play an important role in pheromone transduction.

**Materials and methods**

**Animals**

The moths *Manduca sexta* (Lepidoptera: Sphingidae), reared from eggs on an artificial diet (modified from that of Bell and Joachim, 1976), were kept on a long-day photoperiod regimen (17h:7h light:dark, with lights on at 07:00h) at 25–26°C and 50–60% relative humidity. Pupae were staged as previously described (Sanes and Hildebrand, 1976a; Tolbert et al. 1983). They were usually selected for dissection between 08:00 and 10:00h and anesthetized by chilling on ice for 10–15min before dissection of the antennal flagellum.

**Cell cultures**

Unless otherwise specified, all culture media were purchased from GIBCO (Grand
Island, NY) and all chemicals and biochemicals, from Sigma Chemical Co. (St Louis, MO).

Details of the culture techniques have been reported previously (Stengl and Hildebrand, 1990). Briefly, antennal flagella from male *M. sexta* pupae (stage 3 of the 18 stages of pupal development) were disrupted by a combination of mechanical and enzymatic treatment. The dissociated cells were plated on concanavalin-A-coated coverslips or uncoated Falcon plastic dishes, in Leibowitz (L15) medium, supplemented with 5% fetal bovine serum (Hyclone) and conditioned medium (supernatant fluid from cultures of a non-neuronal *M. sexta* cell line, generously provided by Drs J. Hayashi and L. Oland). The cultures were maintained in an incubator for 2–4 weeks at about 20°C and high humidity.

**Patch-clamp technique and data analysis**

Patch-clamp experiments followed the method described by Hamill *et al.* (1981). Patch pipettes were made from borosilicate glass capillaries (Clark Electromedical Instruments, Reading, UK) with a Sutter Instruments micropipette puller (model P80/PC). The pipettes were coated with Sylgard (Dow Corning, Midland, MI). The tip resistance was 5–20 MΩ when the electrodes were filled with physiological saline. The cells were viewed at 400× magnification with an Olympus inverted microscope equipped with phase contrast or Hoffmann modulation contrast optics. After formation of a seal between the pipette and the cell membrane, the electrode capacitance was compensated.

Whole-cell currents were measured at room temperature with an Axopatch 1C patch-clamp amplifier (Axon Instruments Co., Burlingame, CA). The currents were acquired on-line with an 80386-based microcomputer (Dell Computer Corp., Austin, TX) using pClamp software (Axon Instruments Co.), which was also used for data analysis. Leakage currents were generally not subtracted since they remained negligible. Only in a few cells (as indicated in the figure legends), where Cs⁺, TTX and Ni²⁺ did not block all the voltage-dependent currents, were leakage currents subtracted. Junction potential drifts (usually around 10pA, as determined by the amplifier) that occurred during some recordings were subtracted with pClamp software. For current–voltage plots, currents were generally measured at least 5ms after the start of the voltage pulse and during the current plateau.

**Solutions and stimulus application**

During whole-cell recordings, cultured ORNs were kept in ‘extracellular saline solution’ containing (in mmol l⁻¹): 156 NaCl, 4 KCl, 6 CaCl₂, 5 glucose and 10 Hepes (adjusted to pH7.1 with NaOH). For determination of the ion-specificity of the cation channels, Na⁺ was replaced by 156mmol l⁻¹ KCl or CsCl; Cl⁻ was replaced by acetate or aspartate. During examination of the Ca²⁺ dependence and Ca²⁺ permeability of the cation channels, the cells were bathed in 160mmol l⁻¹ CsCl, 10mmol l⁻¹ Heps, 5mmol l⁻¹ glucose, containing 20mmol l⁻¹, 5mmol l⁻¹ or 10⁻⁷ mol l⁻¹ free Ca²⁺ (11mmol l⁻¹ EGTA and 1mmol l⁻¹ Ca²⁺). To block Ca²⁺ channels, 6mmol l⁻¹ NiCl₂ was added in most experiments. In all experiments, the voltage-dependent Na⁺ channels were blocked with 10⁻⁵ mol l⁻¹ external TTX and the delayed rectifier K⁺ channels were
blocked with 160mmol l⁻¹ internal Cs⁺, while the Ca²⁺-dependent K⁺ channels could not be blocked by Cs⁺ or any other blocker tested.

The ‘intracellular saline solution’ used to fill pipettes was (in mmol l⁻¹): 150 KCl, 5 NaCl, 1 CaCl₂, 11 EGTA, 1.5 MgCl₂, 10 Hepes. For most whole-cell recordings (and all recordings illustrated) KCl and NaCl were replaced by 160 CsCl and MgCl₂ was omitted. To examine the Ca²⁺ dependence of the cation channels, intracellular solutions were used with Ca²⁺ concentrations of 10⁻⁶, 10⁻⁷, 10⁻⁸ mol l⁻¹ or of less than 10⁻⁸ mol l⁻¹ buffered with BAPTA (Calbiochem, La Jolla CA): 1mmol l⁻¹ BAPTA, 0.9mmol l⁻¹ CaCl₂ (pCa=6); 1mmol l⁻¹ BAPTA, 0.5mmol l⁻¹ CaCl₂ (pCa=7); 1mmol l⁻¹ BAPTA, 0.09mmol l⁻¹ CaCl₂ (pCa=8); or 2mmol l⁻¹ BAPTA, 0.09mmol l⁻¹ CaCl₂ (pCa<8).

‘Normal’ ionic gradients were defined as high [NaCl] externally and high [KCl] internally. The bath solution contained (in mmol l⁻¹): 156 NaCl, 4 KCl, 6 CaCl₂, 5 glucose and 10 Hepes (adjusted to pH7.1 with NaOH) outside. The solutions in the pipette contained (in mmol l⁻¹): 156 KCl, 5 NaCl, 1 CaCl₂, 11 EGTA (pCa=7), 10 Hepes. For almost all recordings (and for all recordings illustrated), KCl and NaCl were replaced by 160mmol l⁻¹ CsCl.

Before breaking into the whole-cell configuration, the patches were kept at 0mV pipette potential while phorbol esters (TPA, phorbol 12-myristate 13-acetate), or protein kinase C inhibitors such as staurosporine (Boehringer Mannheim, Indianapolis, IN) or H7, were applied to the cell via a glass pipette (tip opening about 10 μm) which was driven by a Picospripter (General Valve Corp., Fairfield, NJ). All other agents were included in the patch pipette filling solutions, but only in the shaft; the tip of the electrode was filled with about equal parts of the intracellular control solution. Therefore, the concentrations of the agents used are more dilute (to a maximum of 50%) than indicated.

Results

After 2–3 weeks in vitro, ORNs originating from pupal antennae of 3-day-old M. sexta males were examined for the presence of intracellular-messenger-gated cation currents. In whole-cell patch-clamp recordings with 160mmol l⁻¹ CsCl, 10⁻⁷ mol l⁻¹ CaCl₂ in the patch pipette, and 10⁻⁸ mol l⁻¹ tetrodotoxin (TTX) outside (to block all the voltage-dependent Na⁺ and K⁺ channels), no cation currents were elicited (N=21) at potentials between −120mV and 70mV (Fig. 1). This was independent of the principal cation (Na⁺, K⁺ or Cs⁺), the principal anion (Cl⁻, acetate or aspartate) and the external Ca²⁺ concentration. The mean ± s.d. of inward currents elicited at −120mV was −4.8±12.2pA (N=21). Extracellular Ni⁺ was added in most recordings to block presumptive Ca²⁺ channels.

Experiments designed to identify intracellular-messenger-dependent cation channels with properties similar to the pheromone-dependent cation channels (Fig. 2, and Stengl et al. 1992b) sought channels that (a) carry inward currents at potentials more negative than the resting potential, with currents less than −25pA at −110mV (exceeding the mean leak currents by about five times) under ‘normal’ ionic gradients; (b) show approximately the same reversal potentials (around 0mV) in various extracellular solutions with different principal cations, irrespective of the main anion outside; (c) show
inward rectification with $10^{-7}\text{mol}\cdot\text{l}^{-1}\text{Ca}^{2+}$ outside (Stengl et al. 1992b), but linear I/V characteristics with $6\text{mmol}\cdot\text{l}^{-1}\text{Ca}^{2+}$ outside (Stengl et al. 1992b); and (d) may be blockable by tetraethylammonium (TEA$^+$) (Stengl et al. 1992b). Criterion b was not always a reliable indication of the presence of intracellular-messenger-dependent cation currents since, in some cells, Ca$^{2+}$-dependent K$^+$ currents, which were unaffected by any blocker tested, were superimposed on TEA$^+$-blockable cation currents. For all cells tested, currents at potentials between at least $-100\text{mV}$ and $+70\text{mV}$ were determined in steps of 10 or 20 mV in different extracellular solutions, at various holding potentials. For all recordings shown, $160\text{mmol}\cdot\text{l}^{-1}\text{CsCl}$ and $10^{-8}\text{mol}\cdot\text{l}^{-1}\text{TTX}$ were present to block the voltage-dependent K$^+$ and Na$^+$ channels. The ORNs were recorded on-line with a microcomputer, which also generated the voltage protocols (see Fig. 1).

This procedure allowed quick determination of the reversal potentials and the rectification properties of the currents in different extracellular salines. It did not allow measurement of the exact time course or the exact maximal amplitude of transient currents, because the data were usually not collected as a continuous time record, but were recorded at different times after obtaining the whole-cell configuration. Leakage
Fig. 2. Responses of cultured ORNs to their species-specific sex pheromone in 6mmol l⁻¹ external Ca²⁺. After application of 1pgml⁻¹ bombykal to 3- to 5-week cultured ORNs, pheromone-dependent currents with a reversal potential around 0mV were elicited. The pheromone-dependent current declined within 3s to a lower, more stable level in extracellular saline containing 6mmol l⁻¹ Ca²⁺ and 156mmol l⁻¹ NaCl. Although the pheromone-dependent currents are inwardly rectifying in 10⁻² mol l⁻¹ external Ca²⁺ (Stengl et al. 1992b), they are linear in 6mmol l⁻¹ external Ca²⁺; mainly because of a decrease in inward current at negative potentials. The holding potential was −80mV. After a step from −80mV to −100mV, the cell potential was changed in 20-mV steps from −100 to 60mV. Filled circles mark the current–voltage plot of a whole-cell patch-clamp recording 30s before application of the pheromone. The filled triangles mark the current–voltage plot during bombykal application, after subtraction of the previous control recording (filled circles) taken 3s before the pheromone response. The current recording during pheromone application without leakage-subtraction is marked with a filled triangle in the upper left corner. Filled squares indicate the leakage-subtracted current–voltage plot about 4s after the pheromone application. In this recording, as well as in all following recordings shown, 160mmol l⁻¹ CsCl and 10⁻⁸ mol l⁻¹ TTX are present to block voltage-dependent K⁺ and Na⁺ channels.
currents were generally not subtracted in the following experiments, since they remained negligible in control recordings (Fig. 1).

To test whether activation of G-proteins elicits cation currents with the same properties as the pheromone-dependent cation currents (Fig. 2, and Stengl et al. 1992b), agents that influence G-protein activation were included in the patch pipette during whole-cell recordings.

With 10 μmol l⁻¹ to 1mmol l⁻¹ GTPγS (a non-hydrolysable GTP-analog; Dunlap et al. 1987) in the shaft of the patch pipette (without ATP present), 25% (2/8) of the analyzed ORNs displayed currents greater than −25pA at −110mV. With 10 μmol l⁻¹ to 1mmol l⁻¹ GTPγS+ATP (10 μmol l⁻¹ to 5mmol l⁻¹), the number of responding cells increased, so that 75% (9/12) of all ORNs tested displayed currents greater than −25pA at −110mV (Figs 3, 4A–C), even with 6mmol l⁻¹ NiCl₂ outside (N=3). The reversal potential of the GTPγS and the GTPγS+ATP-dependent currents remained around 0mV if the main cation outside was Na⁺, K⁺ or Cs⁺, regardless of whether Cl⁻ or acetate was the anion (Fig. 3). The currents showed inward rectification with 10⁻⁷ mol l⁻¹ Ca²⁺ outside (Fig. 3). GTPγS-dependent cation currents (with or without ATP) were blocked with 20mmol l⁻¹ TEA⁺ in the extracellular solution (N=4). Perfusion with 1mmol l⁻¹ GDPβS (a non-hydrolysable GDP analog) did not invoke any currents that differed from leakage currents (N=4).

With 100 μmol l⁻¹ to 5mmol l⁻¹ ATP added to the pipette solution, 44% of the ORNs tested displayed nonspecific cation currents (N=22) in the absence of external Ni²⁺ (not shown). Thus, ATP alone can elicit cation currents with the same properties as the GTPγS-dependent currents, either directly, or indirectly, in cultured ORNs.

When GTPγS-dependent currents, as well as ATP-dependent currents or GTPγS+ATP-dependent currents, were recorded consecutively in the same cell, a significant dependence of the current amplitude on external [Ca²⁺] became obvious (Fig. 4A–C) when the bath solution was changed from 10⁻⁷ mol l⁻¹ Ca²⁺ to 6mmol l⁻¹ Ca²⁺. Irrespective of the monovalent cations present (see Fig. 3), the inward current at a constant negative potential increased transiently and reached a stationary lower current level within less than 2s (Fig. 4B,C). In 10⁻⁷ mol l⁻¹ extracellular Ca²⁺ (Figs 3, 4A), the elicited cation currents appeared to be more stable and showed inward rectification. The non-linear I/V curve became linear after several seconds in 6mmol l⁻¹ Ca²⁺ outside (Fig. 4C).

Quantification of the amplitudes of the GTPγS-dependent currents was difficult and dose–response curves could not be obtained reliably since the currents contained a transient component that was not always detected on the non-continuous time record used. A detailed analysis of the kinetics of the currents will be provided elsewhere. The current amplitudes observed at −110mV (measured at least 5ms after the start of the voltage pulse, during the current plateau) ranged from −37±17pA (mean ± s.d.) in 6mmol l⁻¹ Ca²⁺ outside to −86±35pA in 10⁻⁷ mol l⁻¹ Ca²⁺, both with 10 μmol l⁻¹ GTPγS (N=6), and from −36±18pA in high [Ca²⁺] to −210±96pA in low [Ca²⁺], both with 1mmol l⁻¹ GTPγS (N=5).

The possibility that a G-protein-dependent activation of phosphoinositidase C might have caused opening of these GTPγS-dependent cation channels, as suggested by
biochemical evidence (Breer et al. 1990), was considered. The cultured ORNs were stimulated with inositol 1,4,5-trisphosphate (InsP3). If InsP3 (0.01–100 μmol l\(^{-1}\) and no ATP) was included in the patch pipette during the whole-cell recordings, cation currents were larger than −25 pA at −110 mV in 77% (30/39) of all recordings, even in the presence of external Ni\(^{2+}\) (Fig. 5). These cation currents also reversed around 0 mV. The reversal potential was independent of the principal external cation, and the external anions (Fig. 5). If TEA\(^+\) was added extracellularly (Fig. 6), no currents were elicited in ORNs with InsP3 in the patch pipette, regardless of the extracellular solutions used (N=14).

The amplitude and \(I/V\) relationship of the InsP3-dependent currents were also
dependent on extracellular $[\text{Ca}^{2+}]$, in a strikingly similar manner to the GTP$\gamma$S-dependent currents (Fig. 4). The InsP$_3$-dependent currents at $-110\text{mV}$ ranged from $-26\pm12\text{pA}$ (mean ± s.d.) in 6mmol$^{-1}$ Ca$^{2+}$ outside to $-122\pm119\text{pA}$ in $10^{-7}\text{mol}^{-1}$ Ca$^{2+}$ outside, both with 1$\mu$mol$^{-1}$ InsP$_3$ ($N=5$) and from $-21\pm12\text{pA}$ in 6mmol$^{-1}$ Ca$^{2+}$ to $-212\pm126\text{pA}$ in $10^{-7}\text{mol}^{-1}$ Ca$^{2+}$, both with 100$\mu$mol$^{-1}$ InsP$_3$ ($N=6$).

To determine whether InsP$_3$ opens cation channels via an increase in internal Ca$^{2+}$ concentration, agents known to change the Ca$^{2+}$ levels were included in the patch pipette solution. With Ca$^{2+}$ inside buffered to less than $10^{-8}\text{mol}^{-1}$ (2mmol$^{-1}$ BAPTA+0.09mmol$^{-1}$ CaCl$_2$) during perfusion with InsP$_3$, only 22% (2/9) of the recorded ORNs displayed cation currents. After increasing the Ca$^{2+}$ concentration in the patch pipette solution to $10^{-6}\text{mol}^{-1}$ (BAPTA-buffered, without any addition of other intracellular messengers), cation currents with the characteristics of the InsP$_3$- and

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**Fig. 4.** (A–C). The amplitude of GTP$\gamma$S+ATP-dependent currents depends on extracellular $[\text{Ca}^{2+}]$. Current–voltage plots of three consecutive measurements (separated by about 3s) from the same cell in different extracellular solutions. After increasing the extracellular Ca$^{2+}$ concentration, the agent-dependent currents increase transiently (B), before declining to a lower, more stable level (C) (irrespective of the monovalent cations present, as shown in Fig. 3). Voltage protocol as described in Fig. 1. The patch pipette contained 1mmol$^{-1}$ GTP$\gamma$S, 1mmol$^{-1}$ ATP, $10^{-7}\text{mol}^{-1}$ Ca$^{2+}$ and 160mmol$^{-1}$ CsCl.
GTPγS-dependent currents appeared in 85% (11/13) of the cells tested (Figs 7–9). The Ca^{2+}-dependent currents at −110mV ranged from −148±120pA (mean ± s.d.) in 6 mmol l^{-1} Ca^{2+} outside (N=9) to −141±136pA in 10^{-7} mol l^{-1} Ca^{2+} outside (N=13). Again, these Ca^{2+}-dependent cation currents reversed around 0mV, irrespective of the main anion or cation outside (Fig. 7), were TEA^{+}-blockable and depended on the extracellular Ca^{2+} concentration (Figs 7–9). Switching from low [Ca^{2+}] outside to 6 mmol l^{-1} Ca^{2+} transiently increased the Ca^{2+}-activated current (Fig. 8B,C), which then reached a more stable lower current level with a linear I/V relationship (Figs 8C, 9A). The transient current increase was not observed in all recordings (compare Figs 8A–C, 9A).

Fig. 5. With 100 μmol l^{-1} InsP_{3} included in the shaft of the patch pipette, cation currents are elicited which reverse around 0mV, irrespective of the extracellular monovalent cations present. They are inwardly rectifying with buffered Ca^{2+} outside. Current traces in extracellular solution containing sodium acetate as the main cation are shown in the upper left corner. The current–voltage plot shows three consecutive recordings (separated by at least 3s) from the same cell in different extracellular solutions: 156mmol l^{-1} CsCl, filled triangles; 156mmol l^{-1} KCl, open circles; and 156mmol l^{-1} sodium acetate, filled squares. Voltage protocol as described in Fig. 1.
After switching from 6mmol l\(^{-1}\) Ca\(^{2+}\) outside to 10\(^{-7}\) mol l\(^{-1}\) Ca\(^{2+}\), the inward currents increased and became inwardly rectifying (Figs 7, 9A). Switching back to 6mmol l\(^{-1}\) external Ca\(^{2+}\), the inward currents increased temporarily to a higher current amplitude (Fig. 8B), before declining again and becoming linear (Figs 8C, 9A). Maximal shifts of
about 12mV to more positive values in the reversal potential of the Ca\(^{2+}\)-dependent cation currents were observed when the extracellular Ca\(^{2+}\) concentrations were changed from 6mmol l\(^{-1}\) to 10\(^{-7}\)mol l\(^{-1}\) (=buffered Ca\(^{2+}\)) in symmetrical CsCl solutions (Fig. 9B). The mean ± s.d. of the reversal potential shortly after changing the extracellular Ca\(^{2+}\) concentration was \(-7.7±4.2\) mV in high [Ca\(^{2+}\)] outside (N=12) and \(-0.3±1.5\) mV in buffered Ca\(^{2+}\) outside (N=18).

Finally, the possibility that Ca\(^{2+}\) also opens cation channels in cultured ORNs indirectly via activation of a protein kinase C (PKC) was investigated. In 52% of the ORNs tested (N=44), cation currents with a reversal potential around 0mV were elicited when 10pgml\(^{-1}\) phorbol ester (TPA=PKC activator) was applied to the cell via a micropipette before obtaining the whole-cell configuration or with TPA in the patch pipette (Figs 10, 11A,B). In contrast to the InsP\(_3\)-, GTP\(\gamma\)S- or Ca\(^{2+}\)-dependent currents,
the TPA-evoked currents were not dependent on extracellular [Ca$^{2+}$] (Fig. 11A,B), since they did not show inward rectification in $10^{-7}$ mol l$^{-1}$ external Ca$^{2+}$. The PKC-dependent cation currents were blocked with 20 mmol l$^{-1}$ extracellular TEA$^+$ ($N=3$) (Fig. 10). At $-110$ mV, the PKC-dependent currents ranged from $-45\pm16$ pA in 6 mmol l$^{-1}$ Ca$^{2+}$ ($N=6$) to $-64\pm34$ pA in buffered Ca$^{2+}$ ($N=3$).
To test whether the InsP$_3$-dependent currents were also elicited from a Ca$^{2+}$-dependent phosphorylation via a PKC, PKC inhibitors were applied (Figs 12, 13). With 10$\mu$mol l$^{-1}$ H7 or 2$\mu$mol l$^{-1}$ staurosporine applied with 10$\mu$mol l$^{-1}$ InsP$_3$, 50% (4/8) of the ORNs tested displayed transient cation currents (Fig. 12). The other 50% did not respond. These

Fig. 9. Calcium-dependent cation currents depend on extracellular Ca$^{2+}$ concentration. With high [Ca$^{2+}$] outside, they decline within a few seconds to a lower level. (A,B) A 20-day cultured ORN was recorded in symmetrical 160mmol l$^{-1}$ CsCl solutions and 10$^{-6}$mol l$^{-1}$ CaCl$_2$ in the patch pipette. After keeping the cells in 6mmol l$^{-1}$ external Ca$^{2+}$, the Ca$^{2+}$ concentrations in the extracellular solutions were exchanged (arrows) from 6mmol l$^{-1}$ CaCl$_2$ (solution A) to 10$^{-7}$mol l$^{-1}$ CaCl$_2$ (solution B), and to 20mmol l$^{-1}$ CaCl$_2$ (solution C). (A) The inward currents at −100mV (below), as well as the outward currents at +100mV (above) are shown over time (A). The first current–voltage protocol taken after the break from the cell-attached configuration to the whole-cell configuration (in solution A) was recorded at time zero. Shifts in the reversal potential occur during changes in extracellular Ca$^{2+}$ concentrations (B).
Transient cation currents were elicited only during the first voltage protocol (Fig. 12). In comparison, 77% (30/39) of the ORNs tested expressed more-stable currents without the addition of PKC inhibitors (Fig. 13), as did the TPA-dependent currents (Fig. 10).

**Discussion**

The development of a primary cell culture system of differentiating, identifiable ORNs from *M. sexta* antennae has greatly facilitated studies of the physiological properties of olfactory neurons (Stengl and Hildebrand, 1990). Biochemical evidence indicates the involvement of second messengers in pheromone transduction in these olfactory neurons (Ziegelberger *et al.* 1990; Breer *et al.* 1990). Furthermore, cultured ORNs respond to pheromonal stimulation by opening of nonspecific cation channels that appear not to be directly gated by the pheromone. Therefore, the possible occurrence of cation channels mediated by an intracellular messenger was sought.

Fig. 10. Phorbol-ester-dependent currents reverse around 0mV and are stable in 6mmol l⁻¹ Ca²⁺ outside. In contrast to the Ca²⁺-dependent currents (Fig. 8B,C), the phorbol-ester-dependent currents do not decline within a few seconds to a lower current plateau in salines with 6mmol l⁻¹ Ca²⁺ outside. Two consecutive recordings (filled circles show the first recording) separated by about 3s were taken from the same cell, after application of 10ng ml⁻¹ TPA via a picospritzer-driven glass capillary. After application of 20mmol l⁻¹ TEA⁺, TPA-dependent currents are blocked (filled squares).
Whole-cell patch-clamp recordings from the soma of 3-week cultured ORNs showed that GTPγS, ATP, InsP₃, $10^{-6}$ mol l⁻¹ Ca²⁺ and PKC cause inward currents of at least $-25$ pA at $-110$ mV, irrespective of the principal monovalent cation or anion outside the cell. Previous experiments have shown that ORNs from antennae of male *M. sexta*
respond to their sex pheromone in vitro with the opening of cation channels that are distinguished from other channels by inward currents at potentials more negative than the resting potential. They have a reversal potential around 0mV, irrespective of the principal external cation or anions, promote linear currents in 6mmol l⁻¹ external Ca²⁺ (Fig. 2) and express inward rectification in 10⁻⁷ mol l⁻¹ external Ca²⁺ (Stengl et al. 1992b).
The percentage of pheromone-sensitive cells observed in vitro (38%) (Stengl et al. 1992b) correlates well with the percentage of pheromone-sensitive cells in the antenna, which is about 32% (Lee and Strausfeld, 1990). Because more than 70% of the cultured ORNs responded to intracellular messenger application, it is assumed that ORNs sensitive to plant odors as well as those sensitive to pheromone contain cation channels.

Fig. 13. InsP3-dependent currents are more stable without the addition of H7. Current–voltage plots of two consecutive recordings (filled circles show the first recording) from the same cultured ORN. With 10 μmol l⁻¹ InsP3 in the patch pipette and extracellular saline containing 156 mmol l⁻¹ NaCl, 6 mmol l⁻¹ NiCl₂ and no added Ca²⁺, InsP3-dependent currents are stable over several seconds. With several micromolar external free Ca²⁺, the currents are almost linear and develop inward rectification 3 s later in the consecutive recording of the same cell. Voltage protocol as described in Fig. 1 with a holding potential of −70 mV. Current traces are shown below.
that are mediated by intracellular messengers. Considering the transient nature of at least some of these channels, they may be present in most, or perhaps all, ORNs. But since at least 2s is required to generate the first computer-driven voltage protocol after breaking into the whole-cell configuration, they might have been overlooked. Furthermore, since these agent-dependent currents possibly depend on intracellular organelles and molecules (such as intracellular Ca$^{2+}$ stores), fast washout of the intracellular medium could obliterate the intracellular-messenger-dependent currents. Finally, the presence of intracellular Ca$^{2+}$ buffers could decrease the agent-dependent cation currents in some of the cells to below the levels of leakage currents.

Currents elicited by GTP$\gamma$S, ATP, InsP$_3$ and $10^{-6}$ mol l$^{-1}$ Ca$^{2+}$ had similar properties and could be blocked by reducing the intracellular Ca$^{2+}$ concentration to less than $10^{-8}$ mol l$^{-1}$ with BAPTA. Therefore, it is likely that InsP$_3$, GTP$\gamma$S and ATP might act via an increase in internal Ca$^{2+}$ concentration, possibly via activation of a G-protein-dependent phosphoinositidase C (Berridge, 1987; Ferris and Snyder, 1992; Gilman, 1987; Stengl et al. 1992a). This has been suggested by biochemical experiments with antennal extracts (Boeckhoff et al. 1990; Breer et al. 1990). It is assumed that InsP$_3$ might produce a rise in internal Ca$^{2+}$ concentration by promoting Ca$^{2+}$ influx from outside (Restrepo et al. 1990) and/or via release from internal stores, as has been reported in many other systems (Berridge and Irvine, 1989). A release of Ca$^{2+}$ from internal stores in the soma (Berridge and Irvine, 1989) seems likely, since cation channels opened after perfusion with InsP$_3$ in the presence of external Ca$^{2+}$ channel blockers.

Direct activation of at least a subpopulation of the intracellular-messenger-dependent cation channels by a rise in internal [Ca$^{2+}$] seems probable, since $10^{-6}$ mol l$^{-1}$ Ca$^{2+}$ elicits cation currents, even in the presence of PKC blockers. Similarly, the ‘spontaneous’ opening of cation channels after patch excision (McClintock and Ache, 1990; Stengl et al. 1992b) in solutions containing a high Ca$^{2+}$ concentration could be explained by Ca$^{2+}$-dependent activation of cation channels. The theory of cation channels directly dependent on [Ca$^{2+}$] is supported by in situ recordings from extruded dendrites of ORNs of the moth Antheraea polyphemus (Zufall et al. 1991b). Calcium-dependent cation channels have also been reported in vertebrate ORNs (Schild and Bischofberger, 1991) and in cultured lobster ORNs sustained inward currents were reported after InsP$_3$ application (Fadool et al. 1991).

The intracellular-messenger-dependent cation channels of cultured ORNs appear to be permeable to Ca$^{2+}$, since a switch to extracellular solutions containing a high (6–20mmol l$^{-1}$) Ca$^{2+}$ concentration moved the reversal potentials of the cation currents to more positive values and transiently increased the amplitude of the currents. It is assumed that the influx of extracellular Ca$^{2+}$ through the cation channels causes activation of more Ca$^{2+}$-dependent cation channels, which are later closed (in a Ca$^{2+}$-dependent manner) via an unknown mechanism. The Ca$^{2+}$-dependent rapid inactivation of cation channels, also found in vertebrate ORNs (Zufall et al. 1991a), could constitute a mechanism for rapid termination of the physiological response to odor stimulation. This rapid inactivation has been postulated for pheromone transduction by Kaissling (1972). The transient opening of channels might also underlie the on-responses of ORNs that can follow odor pulses.
It is likely that the rise in internal \([\text{Ca}^{2+}]\) also activates \(\text{Ca}^{2+}\)-dependent kinases (Nishizuka, 1984). This assumption is supported by the finding that the addition of kinase blockers decreases the number of cells in which intracellular-messenger-dependent cation currents are observed. Furthermore, cation channels in cultured ORNs were opened via stimulation by a protein kinase C. Since the PKC-dependent currents were more stable and were not dependent on extracellular \(\text{Ca}^{2+}\) concentration (they remained linear at all \(\text{Ca}^{2+}\) concentrations tested), they might represent a channel population different from the GTP\(\gamma\)S-, ATP-, Ins\(P_3\) and \(\text{Ca}^{2+}\)-dependent cation currents. As an alternative hypothesis, the transient, directly \(\text{Ca}^{2+}\)-dependent cation channels might change their \(\text{Ca}^{2+}\)-dependence (activation as well as blockage) after PKC-dependent phosphorylation (Ewald et al. 1985). This assumption is supported by the observation that the addition of PKC blockers appeared to render Ins\(P_3\)-dependent currents more transient and that all agent-dependent currents could be blocked by TEA\(^+\). Single-channel experiments will distinguish between the two hypotheses.

Since PKC-dependent and \(\text{Ca}^{2+}\)-dependent cation channels have been observed in excised patches from extruded dendrites of ORNs of the moth \(A.\) polyphemus, the cation currents probably play an important role in olfactory transduction (Zufall and Hatt, 1991; Zufall et al. 1991b). Despite the assumption of Zufall et al. (1991b) that \(\text{Ca}^{2+}\)-dependent cation channels occur only in inner dendrites, it appears more likely that both channel types occur in outer dendrites. The inner dendritic segment and the soma of the ORNs are tightly wrapped by an auxiliary cell, forming a compartment separate from the outer dendritic segment, which extends uncovered beyond the cuticle into the hairshaft (Keil and Steinbrecht, 1987). The inner dendritic membranes are, therefore, not easily accessible, and extruded dendritic vesicles probably consist of outer dendritic membranes, even when the antennal shank is completely shaved off (Keil and Steinbrecht, 1987; Zufall and Hatt, 1991; Zufall et al. 1991b). Because the \textit{in situ} recordings from extruded dendrites of ORNs in \(A.\) polyphemus were undertaken with 2 mmol l\(^{-1}\) \(\text{Ca}^{2+}\) outside, and because the dendrites were preincubated with the pheromone up to an hour before recording (Zufall and Hatt, 1991), transient ion channels would not be detected. Therefore, whether transiently activated cation channels and directly \(\text{Ca}^{2+}\)-dependent cation channels also occur on outer dendrites has yet to be determined. Since all the channels found so far on dendrites of ORNs are either directly or indirectly \(\text{Ca}^{2+}\)-dependent, it has yet to be shown whether and how internal \([\text{Ca}^{2+}]\) increases in the outer dendrite. So far, no intracellular \(\text{Ca}^{2+}\) stores have been identified in the outer dendrite (Keil and Steinbrecht, 1987; Keil, 1989). In recent experiments, a transient Ni\(^{2+}\)-blockable \(\text{Ca}^{2+}\) current, followed by cation currents, was observed after intracellular messenger application (Stengl et al. 1991). We are therefore investigating whether pheromone triggers the transient opening of both Ins\(P_3\)-dependent and PKC-dependent \(\text{Ca}^{2+}\) channels in cultured ORNs.

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