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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 6mmol l^{-1} external Ca^{2+}. This paper shows that perfusion of cultured olfactory receptor neurons with GTPγS, 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 μmol 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, 1976*a*,*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.* 1991*c*). They respond to their species-specific sex pheromone blend by opening nonspecific cation channels (Stengl *et al.* 1989, 1992*a*,*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.* 1992*b*). 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.* 1992*b*).

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 (InsP_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 InsP_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 guanylate 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.* 1992*a*).

This study investigates whether cultured ORNs respond to activation by G-proteins, application of InsP_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, 1976*a*; Tolbert *et al.* 1983). They were usually selected for dissection between 08:00 and 10:00 h 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 Ni^{2+} 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 $CaCl_2$, 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 Ca^{2+} dependence and Ca^{2+} permeability of the cation channels, the cells were bathed in $160mmol\,l^{-1}$ CsCl, $10mmol\,l^{-1}$ Hepes, $5\,mmol\,l^{-1}$ glucose, containing $20mmol\,l^{-1}$, $5mmol\,l^{-1}$ or $10^{-7}\,mol\,l^{-1}$ free Ca^{2+} ($11mmol\,l^{-1}$ EGTA and $1mmol\,l^{-1}$ Ca^{2+}). To block Ca^{2+} channels, $6mmol\,l^{-1}$ $NiCl_2$ was added in most experiments. In all experiments, the voltage-dependent Na^+ channels were blocked with $10^{-8}\,mol\,l^{-1}$ external TTX and the delayed rectifier K^+ channels were

blocked with 160mmol l^{-1} internal Cs^+, while the Ca^{2+}-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 $CaCl_2$, 11 EGTA, 1.5 $MgCl_2$, 10 Hepes. For most whole-cell recordings (and all recordings illustrated) KCl and NaCl were replaced by 160 CsCl and $MgCl_2$ was omitted. To examine the Ca^{2+} dependence of the cation channels, intracellular solutions were used with Ca^{2+} 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} $CaCl_2$ (pCa=6); 1mmol l^{-1} BAPTA, 0.5mmol l^{-1} $CaCl_2$ (pCa=7); 1mmol l^{-1} BAPTA, 0.09mmol l^{-1} $CaCl_2$ (pCa=8); or 2mmol l^{-1} BAPTA, 0.09mmol l^{-1} $CaCl_2$ (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 $CaCl_2$, 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 $CaCl_2$, 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 Picospritzer (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} $CaCl_2$ 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 Ca^{2+} 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 Ca^{2+} 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.* 1992*b*) 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

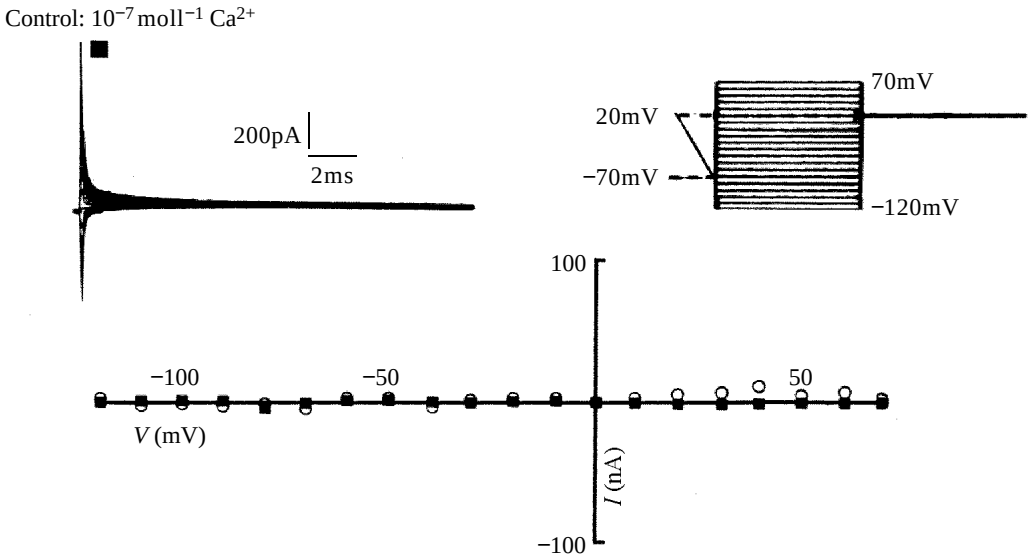

Fig. 1. Responses of cultured ORNs under control conditions. Current traces (upper left, in sodium acetate), voltage protocol (upper right) and current–voltage plot of whole-cell patch-clamp recordings from 3-week cultured ORN from *M. sexta*. Under control conditions, with 160mmol l^{-1} CsCl and 10^{-7} mol l^{-1} Ca^{2+} in the patch pipette and 10^{-8} mol l^{-1} tetrodotoxin (TTX) outside, no currents except leakage currents were elicited. This was observed regardless of the different main cations or anions (156mmol l^{-1} KCl, open circles, 156mmol l^{-1} sodium acetate, filled squares) or of the Ca^{2+} concentrations outside; in this case it was 10^{-7} mol l^{-1}. For all current–voltage plots shown the current was measured at least 5 ms after the start of the voltage steps, during the current plateau. From a holding potential of 20mV a voltage ramp was applied to −70mV (for software reasons only), then the cell was kept at different potentials from −120mV to 70mV in 10mV steps.

inward rectification with 10^{-7} mol l^{-1} Ca^{2+} outside (Stengl *et al.* 1992*b*), but linear *I*/*V* characteristics with 6mmol l^{-1} external Ca^{2+} (Fig. 2); and (d) may be blockable by tetraethylammonium (TEA^+) (Stengl *et al.* 1992*b*). 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 −100mV and +70mV were determined in steps of 10 or 20mV in different extracellular solutions, at various holding potentials. For all recordings shown, 160mmol l^{-1} CsCl and 10^{-8} mol l^{-1} 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

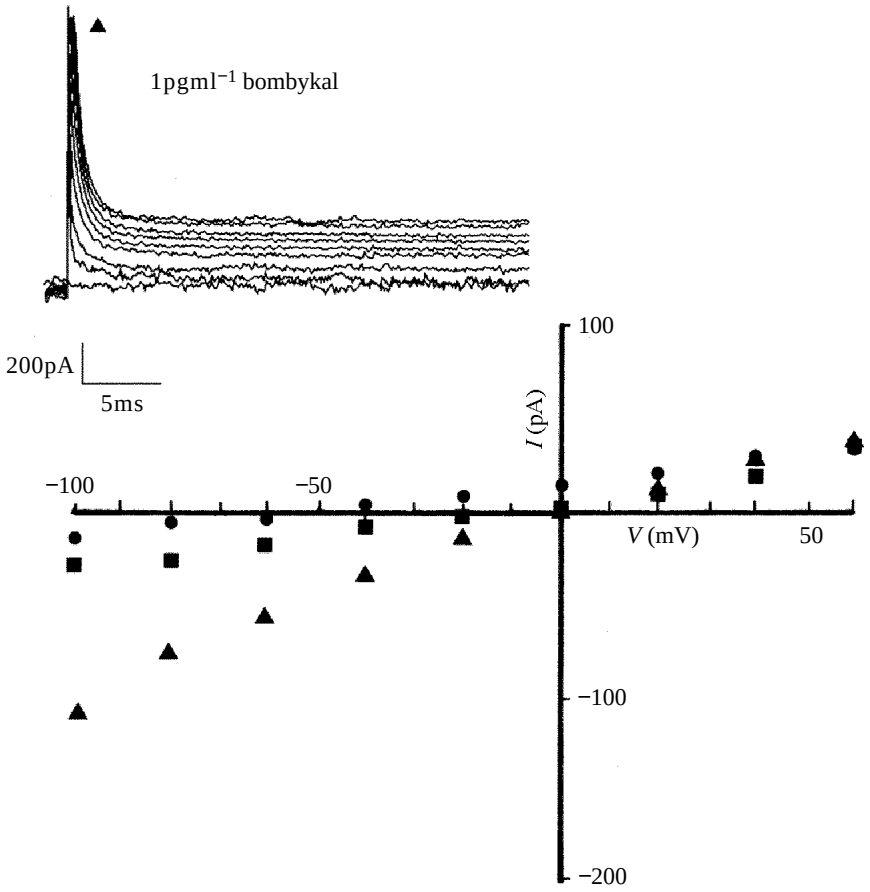

Fig. 2. Responses of cultured ORNs to their species-specific sex pheromone in 6mmol l^{-1} external Ca^{2+}. After application of 1pg ml^{-1} bombykal to 3- to 5-week cultured ORNs, pheromone-dependent currents with a reversal potential around 0mV were elicited. The pheromone-dependent current declined within 3s to a lower, more stable level in extracellular saline containing 6mmol l^{-1} Ca^{2+} and 156mmol l^{-1} NaCl. Although the pheromone-dependent currents are inwardly rectifying in 10^{-7} mol l^{-1} external Ca^{2+} (Stengl *et al.* 1992*b*), they are linear in 6mmol l^{-1} external Ca^{2+}, 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.* 1992*b*), agents that influence G-protein activation were included in the patch pipette during whole-cell recordings.

With $10\,\mu mol\,l^{-1}$ 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 $10\,\mu mol\,l^{-1}$ to $1mmol\,l^{-1}$ GTPγS+ATP ($10\,\mu mol\,l^{-1}$ 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 $6mmol\,l^{-1}$ $NiCl_2$ 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 $10^{-7}\,mol\,l^{-1}$ Ca^{2+} 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 $100\,\mu mol\,l^{-1}$ 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 Ni^{2+} (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 [Ca^{2+}] became obvious (Fig. 4A–C) when the bath solution was changed from $10^{-7}\,mol\,l^{-1}$ Ca^{2+} to $6mmol\,l^{-1}$ Ca^{2+}. 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 Ca^{2+} (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 $6mmol\,l^{-1}$ Ca^{2+} 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 $6\,mmol\,l^{-1}$ Ca^{2+} outside to −86±35pA in $10^{-7}\,mol\,l^{-1}$ Ca^{2+}, both with $10\,\mu mol\,l^{-1}$ GTPγS (*N*=6), and from −36±18pA in high [Ca^{2+}] to −210±96pA in low [Ca^{2+}], 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

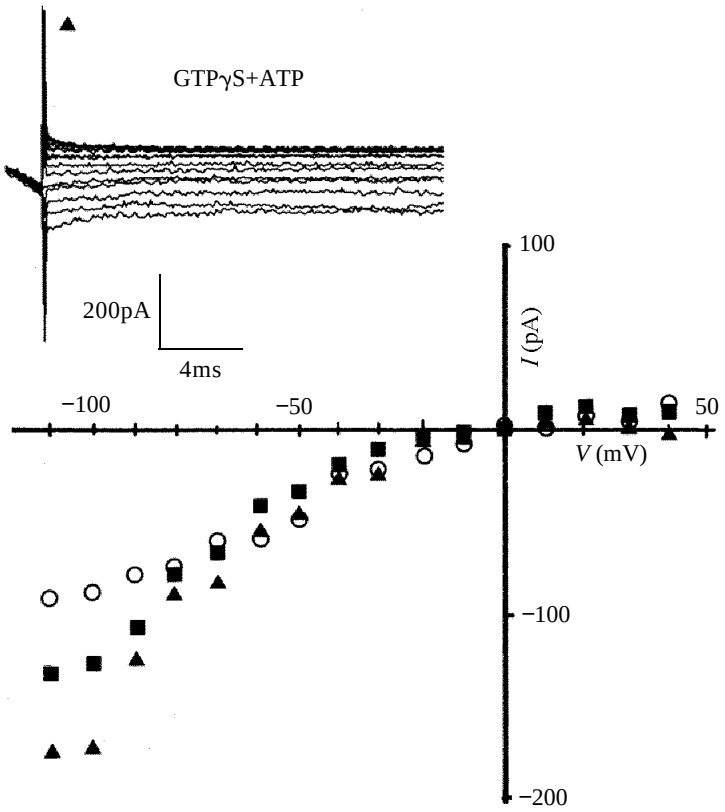

Fig. 3. GTPγS+ATP-dependent cation currents. With 1mmol l^{-1} GTPγS+ATP included in the patch pipette, cation currents are elicited that reverse around 0mV, irrespective of the main extracellular cations. They are inwardly rectifying in 10^{-7} mol l^{-1} extracellular Ca^{2+}. Current traces in 156mmol l^{-1} CsCl are shown in the upper left corner. Current–voltage plots from a 3-week cultured ORN in different extracellular salines containing 10^{-7} mol l^{-1} Ca^{2+} and 156mmol l^{-1} CsCl (filled triangles), KCl (open circles) or sodium acetate (filled squares) are shown. The consecutive recordings in 156mmol l^{-1} NaCl and CsCl are separated by about 3 s (taken from the same cell); the recording in KCl is taken from a different cell. Voltage protocol as described in Fig. 1.

biochemical evidence (Breer *et al.* 1990), was considered. The cultured ORNs were stimulated with inositol 1,4,5-trisphosphate (InsP_3). If InsP_3 (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 −25pA at −110mV in 77% (30/39) of all recordings, even in the presence of external Ni^{2+} (Fig. 5). These cation currents also reversed around 0mV. 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 InsP_3 in the patch pipette, regardless of the extracellular solutions used (N=14).

The amplitude and I/V relationship of the InsP_3-dependent currents were also

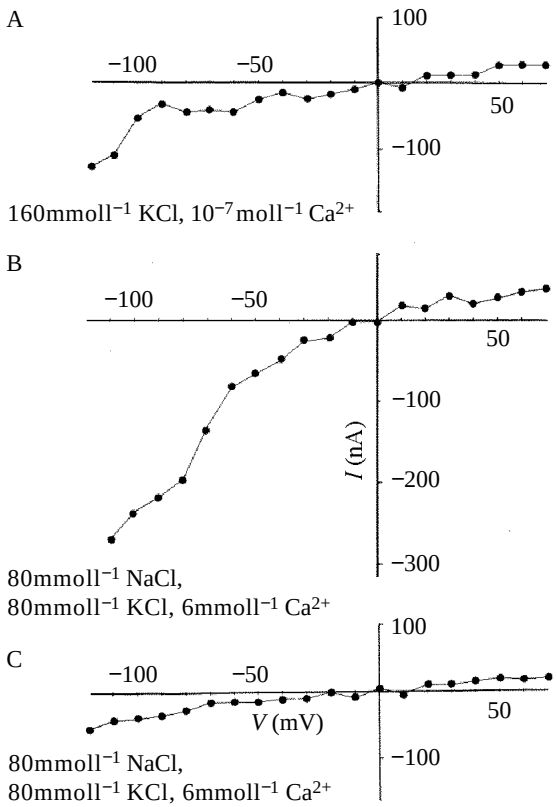

Fig. 4. (A–C). The amplitude of GTPγS+ATP-dependent currents depends on extracellular [Ca^{2+}]. Current–voltage plots of three consecutive measurements (separated by about 3s) from the same cell in different extracellular solutions. After increasing the extracellular Ca^{2+} concentration, the agent-dependent 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 l^{-1} GTPγS, 1mmol l^{-1} ATP, 10^{-7} mol l^{-1} Ca^{2+} and 160mmol l^{-1} CsCl.

dependent on extracellular [Ca^{2+}], in a strikingly similar manner to the GTPγS-dependent currents (Fig. 4). The InsP_3-dependent currents at −110mV ranged from −26±12pA (mean ± S.D.) in 6mmol l^{-1} Ca^{2+} outside to −122±119pA in 10^{-7} mol l^{-1} Ca^{2+} outside, both with 1 μmol l^{-1} InsP_3 (N=5) and from −21±12pA in 6mmol l^{-1} Ca^{2+} to −212±126pA in 10^{-7} mol l^{-1} Ca^{2+}, both with 100 μmol l^{-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} mol l^{-1} (2mmol l^{-1} BAPTA+0.09mmol l^{-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} mol l^{-1} (BAPTA-buffered, without any addition of other intracellular messengers), cation currents with the characteristics of the InsP_3- and

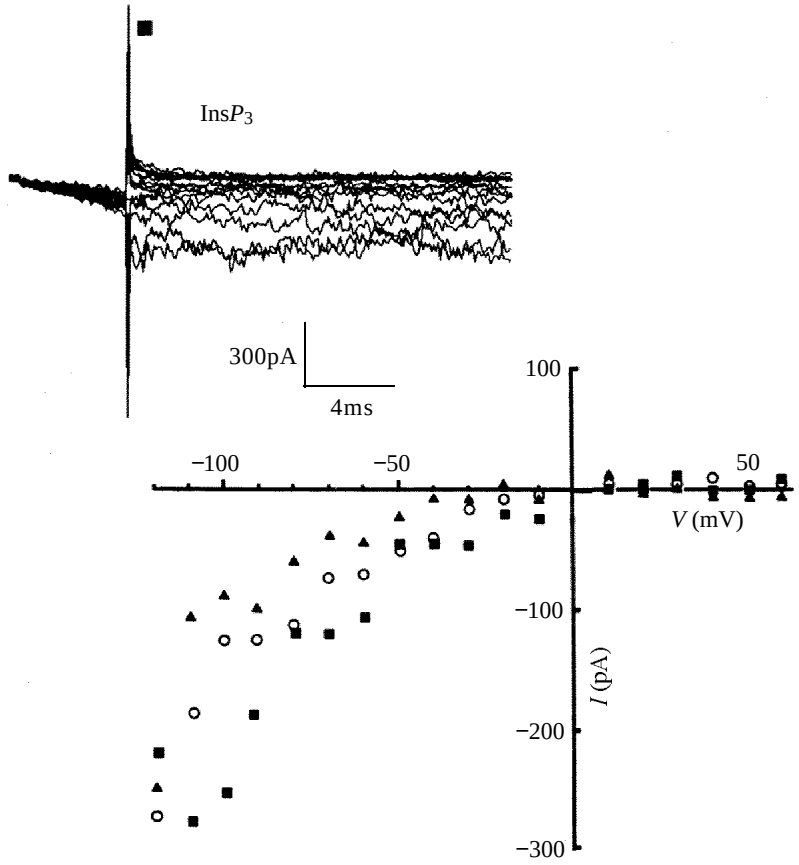

Fig. 5. With 100 μmol l^{-1} InsP_3 included in the shaft of the patch pipette, cation currents are elicited which reverse around 0mV, irrespective of the extracellular monovalent cations present. They are inwardly rectifying with buffered Ca^{2+} outside. Current traces in extracellular solution containing 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.

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).

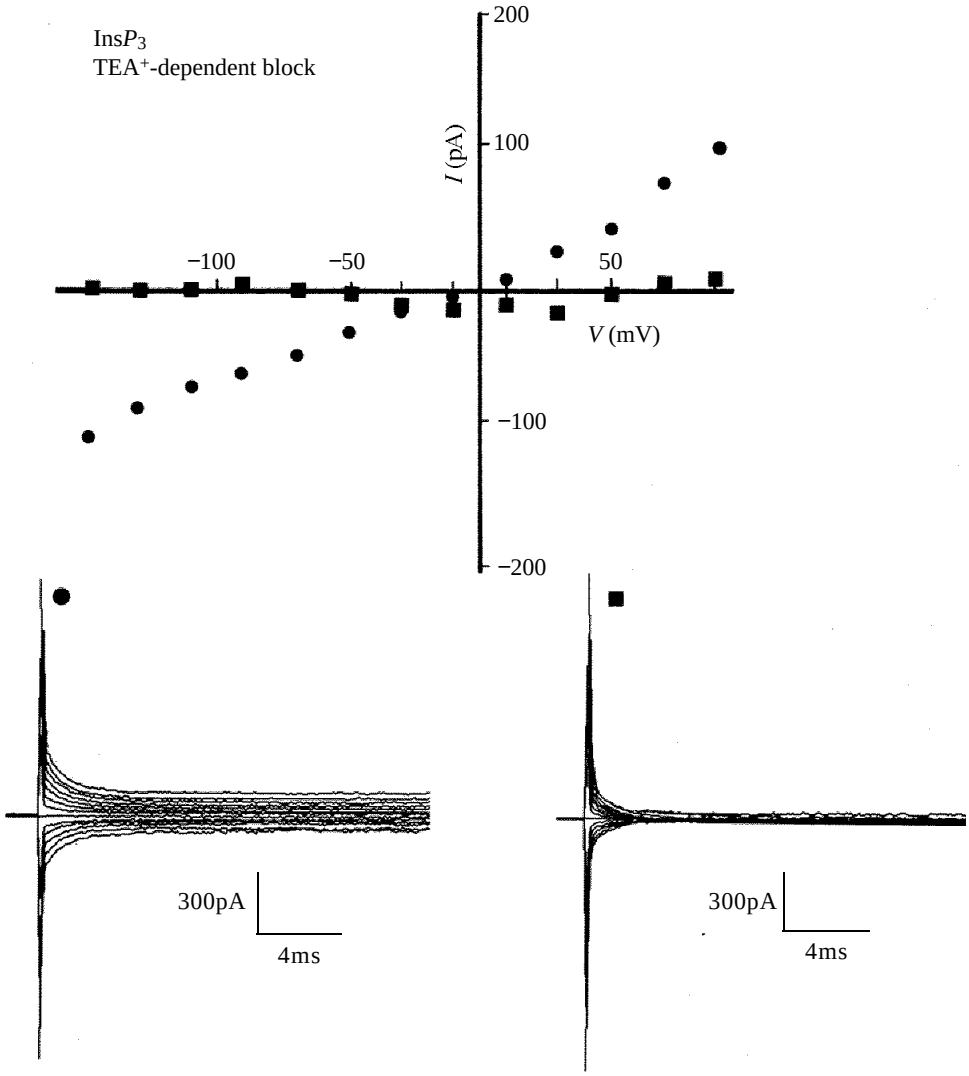

Fig. 6. With 100 μmol l^{-1} InsP_3 in the patch pipette, TEA$^+$-blockable cation currents were elicited. The current–voltage plot shows two consecutive recordings from the same cell (separated by about 6s) in extracellular saline containing 156mmol l^{-1} NaCl, no added Ca^{2+} (which corresponds to at least 2 μmol l^{-1} Ca^{2+}) and 6mmol l^{-1} $NiCl_2$. After the first recording (filled circles), 20mmol l^{-1} TEA$^+$ was applied extracellularly. All cation currents were blocked (filled squares). The current protocols before (filled circles) and after TEA$^+$-application (filled squares) are shown below. Holding potential was −50mV.

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

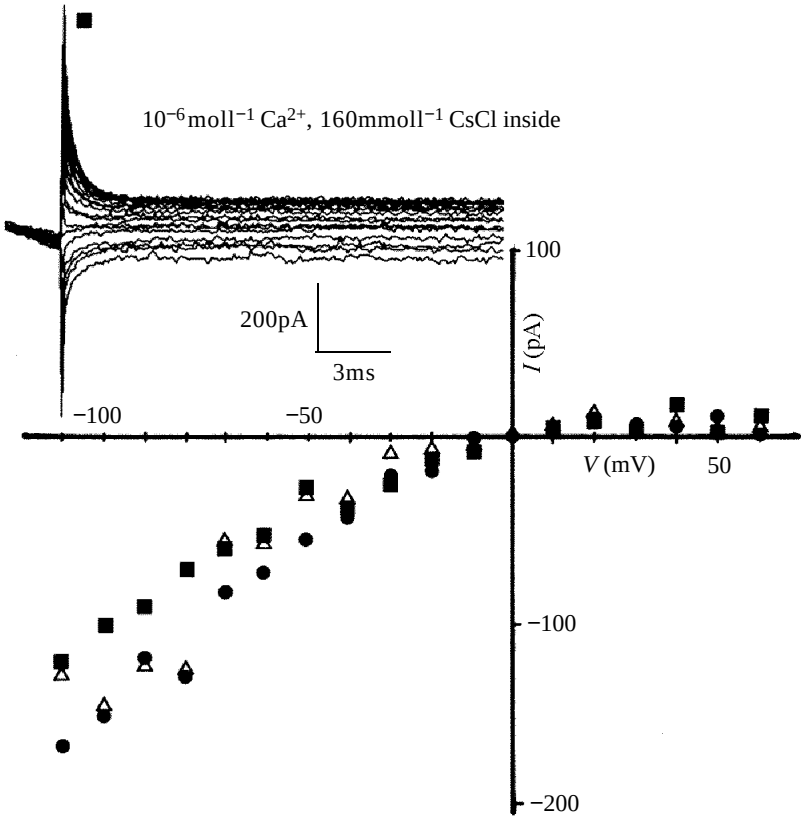

Fig. 7. With 160mmol l^{-1} CsCl and 10^{-6} mol l^{-1} Ca^{2+} in the patch pipette, Ca^{2+}-dependent cation currents are elicited that reverse around 0mV in different extracellular solutions containing 156mmol l^{-1} sodium acetate (filled squares), $SrCl_2$ (open triangles) or potassium acetate (filled circles). The recordings in sodium acetate and $SrCl_2$ are taken from the same ORN (separated by about 6s), while the recording in potassium acetate is from another cell. With 10^{-7} mol l^{-1} Ca^{2+} outside, the currents are inwardly rectifying. Voltage protocol as described in Fig. 1.

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±4.2mV in high [Ca^{2+}] outside (N=12) and −0.3±1.5mV 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-, GTPγS- or Ca^{2+}-dependent currents,

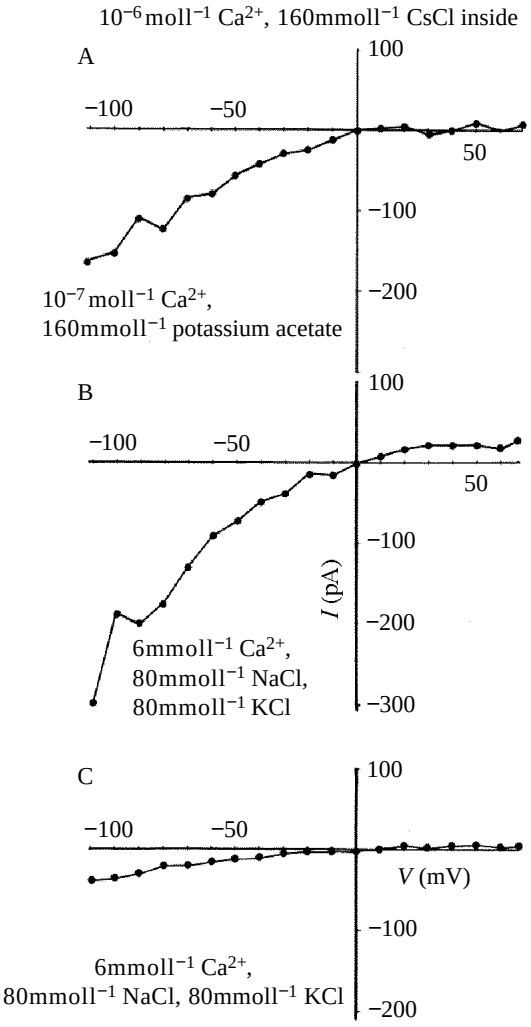

Fig. 8. (A–C). Ca^{2+}-dependent cation currents in 3-week cultured ORNs depend on extracellular [Ca^{2+}]. Current–voltage plots of three consecutive measurements (separated by at least 3s) of the same ORN. After increasing the extracellular Ca^{2+} concentration (irrespective of the monovalent cations present), currents increased transiently (B), before declining within a few seconds to a lower, more stable level (C). Voltage protocol as described in Fig. 1. The patch pipette contained 160mmol l^{-1} CsCl and 10^{-6} mol l^{-1} Ca^{2+} and 10^{-8} mol l^{-1} extracellular TTX was present (as in all other recordings).

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 20mmol l^{-1} extracellular TEA^+ (N=3) (Fig. 10). At −110mV, the PKC-dependent currents ranged from −45±16pA in 6mmol l^{-1} Ca^{2+} (N=6) to −64±34pA in buffered Ca^{2+} (N=3).

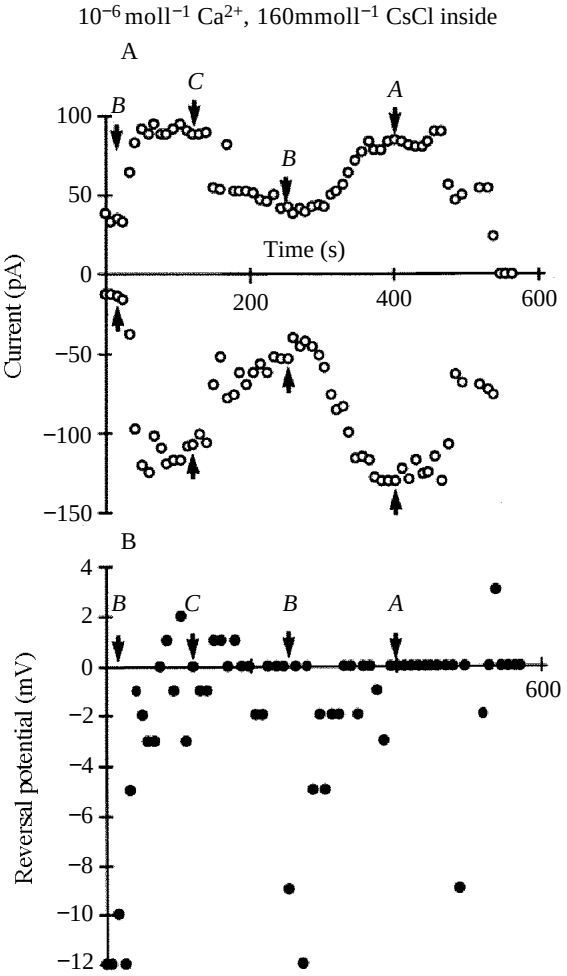

Fig. 9. Calcium-dependent cation currents depend on extracellular Ca^{2+} concentration. With high [Ca^{2+}] outside, they decline within a few seconds to a lower level. (A,B) A 20-day cultured ORN was recorded in symmetrical 160mmol l^{-1} CsCl solutions and 10^{-6} mol l^{-1} $CaCl_2$ in the patch 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).

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 μmol l^{-1} H7 or 2 μmol l^{-1} staurosporine applied with 10 μmol l^{-1} InsP_3, 50% (4/8) of the ORNs tested displayed transient cation currents (Fig. 12). The other 50% did not respond. These

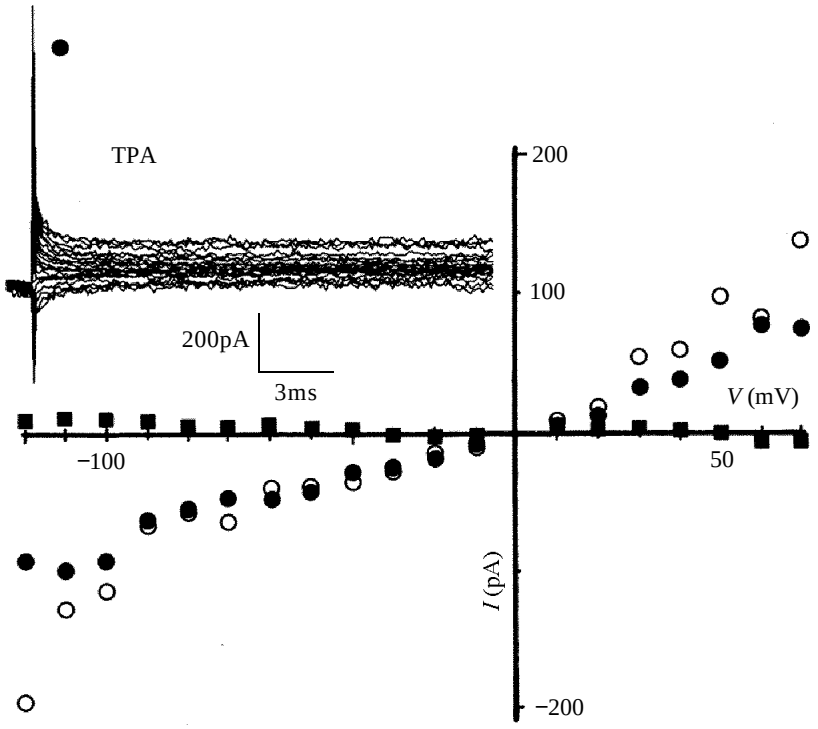

Fig. 10. Phorbol-ester-dependent currents reverse around 0mV and are stable in 6mmol l^{-1} Ca^{2+} outside. In contrast to the Ca^{2+}-dependent currents (Fig. 8B,C), the phorbol-ester-dependent currents do not decline within a few seconds to a lower current plateau in salines with 6mmol l^{-1} Ca^{2+} 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).

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.

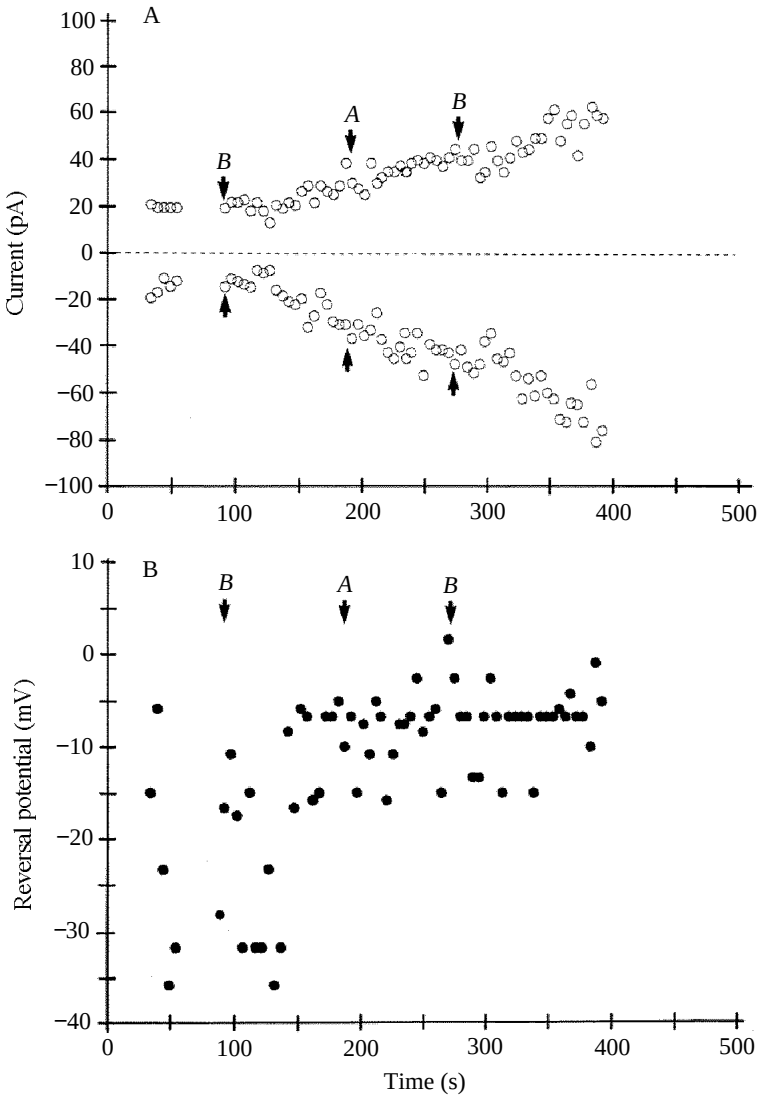

Fig. 11. Protein-kinase-C-dependent cation currents do not depend on extracellular Ca^{2+} concentration. After 21 days, the cultured ORN was recorded in the whole-cell patch-clamp mode, in symmetrical CsCl solutions (160mmol l^{-1} CsCl inside and outside) with 10^{-7} mol l^{-1} Ca^{2+}, 10nmol l^{-1} TPA (phorbol ester) and 250 μmol l^{-1} ATP in the patch pipette. Voltage protocols as described in Fig. 1. (A) Inward currents at −100mV (below) and outward currents at +100mV (above). (B) Reversal potentials. At the arrows, the extracellular Ca^{2+} concentration was changed from 20mmol l^{-1} (*A*) to 10^{-7} mol l^{-1} (*B*).

Whole-cell patch-clamp recordings from the soma of 3-week cultured ORNs showed that GTPγS, ATP, InsP_3, 10^{-6} mol l^{-1} Ca^{2+} and PKC cause inward currents of at least −25pA at −110mV, irrespective of the principal monovalent cation or anion outside the cell. Previous experiments have shown that ORNs from antennae of male *M. sexta*

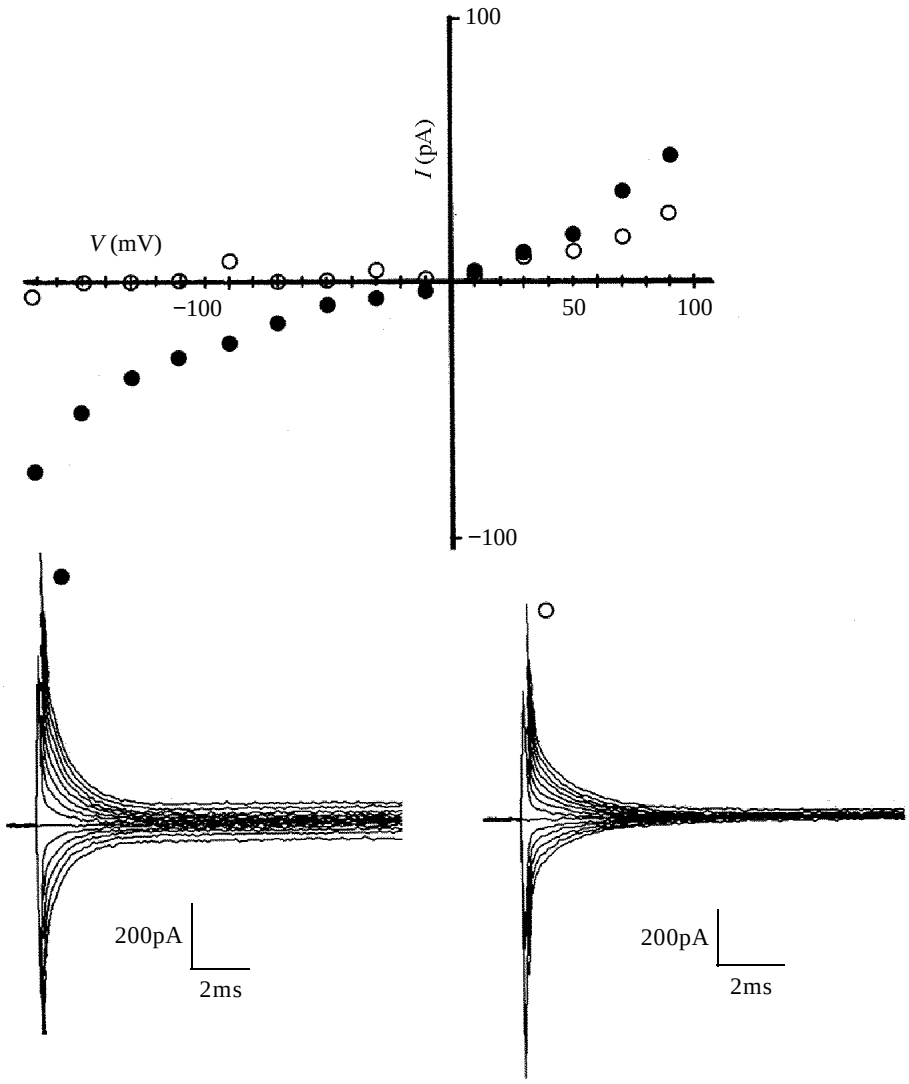

Fig. 12. Application of H7 renders InsP_3-dependent currents more transient. With 10 μmol l^{-1} InsP_3 and 10 μmol l^{-1} H7 in the patch pipette and extracellular saline containing 156mmol l^{-1} NaCl, 6mmol l^{-1} $NiCl_2$ and no added Ca^{2+} (corresponding to at least 2 μmol l^{-1} external Ca^{2+}), InsP_3-dependent currents decline to zero within less than 3s. The current–voltage plots of two consecutive recordings (filled circles show the first recording) from the same cultured ORN are shown. Voltage protocol as described in Fig. 1 with a holding potential of −70mV. Current traces are shown below.

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 Ca^{2+} (Fig. 2) and express inward rectification in 10^{-7} mol l^{-1} external Ca^{2+} (Stengl *et al.* 1992*b*).

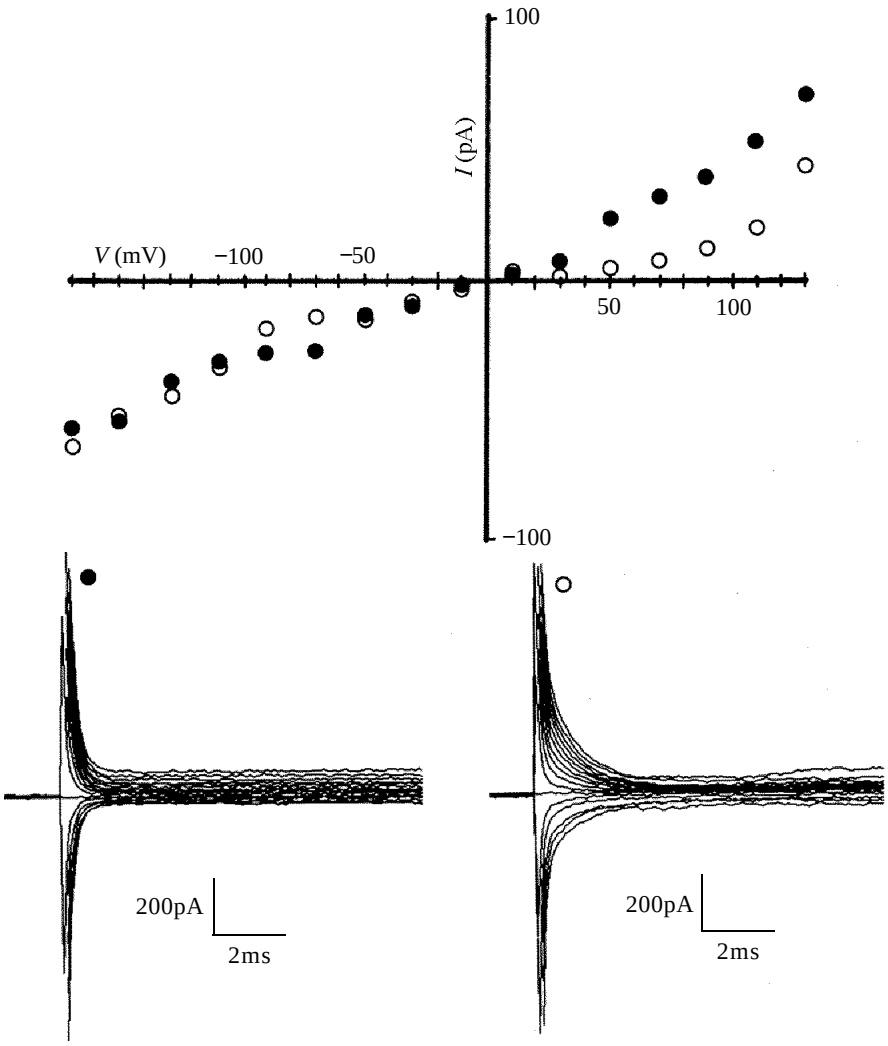

Fig. 13. InsP_3-dependent currents are more stable without the addition of H7. Current–voltage plots of two consecutive recordings (filled circles show the first recording) from the same cultured ORN. With 10 μmol l^{-1} InsP_3 in the patch pipette and extracellular saline containing 156mmol l^{-1} NaCl, 6mmol l^{-1} $NiCl_2$ and no added Ca^{2+}, InsP_3-dependent currents are stable over several seconds. With several micromolar external free Ca^{2+}, the currents are almost linear and develop inward rectification 3s later in the consecutive recording of the same cell. Voltage protocol as described in Fig. 1 with a holding potential of −70mV. Current traces are shown below.

The percentage of pheromone-sensitive cells observed *in vitro* (38%) (Stengl *et al.* 1992*b*) 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

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 GTPγS, 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, GTPγS 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.* 1992*a*). 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.* 1992*b*) 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.* 1991*b*). 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.* 1991*a*), 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 [Ca^{2+}] also activates 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 Ca^{2+} concentration (they remained linear at all Ca^{2+} concentrations tested), they might represent a channel population different from the GTPγS-, ATP-, InsP_3- and Ca^{2+}-dependent cation currents. As an alternative hypothesis, the transient, directly Ca^{2+}-dependent cation channels might change their 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 InsP_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 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.* 1991*b*). Despite the assumption of Zufall *et al.* (1991*b*) that 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.* 1991*b*). Because the *in situ* recordings from extruded dendrites of ORNs in *A. polyphemus* were undertaken with $2\,mmol\,l^{-1}$ 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 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 Ca^{2+}-dependent, it has yet to be shown whether and how internal [Ca^{2+}] increases in the outer dendrite. So far, no intracellular Ca^{2+} stores have been identified in the outer dendrite (Keil and Steinbrecht, 1987; Keil, 1989). In recent experiments, a transient Ni^{2+}-blockable 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 InsP_3-dependent and PKC-dependent Ca^{2+} channels in cultured ORNs.

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