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A CALCIUM-ACTIVATED NONSPECIFIC CATION CHANNEL FROM OLFACTORY RECEPTOR NEURONES OF THE SILKMOTH *ANTHERAEA POLYPHEMUS*

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Summary

Single-channel patch-clamp techniques were used to identify and characterize a Ca^{2+}-activated nonspecific cation channel (CAN channel) on insect olfactory receptor neurones (ORNs) from antennae of male *Antheraea polyphemus*. The CAN channel was found both in acutely isolated ORNs from developing pupae and in membrane vesicles from mature ORNs that presumably originated from inner dendritic segments. Amplitude histograms of the CAN single-channel currents presented well-defined peaks corresponding to at least four channel substates each having a conductance of about 16 pS. Simultaneous gating of the substates was achieved by intracellular Ca^{2+} with an EC_{50} value of about 80 nmol l^{-1}. Activity of the CAN channel could be blocked by application of amiloride (IC_{50}<100 nmol l^{-1}). Moreover, in the presence of 1 μmol l^{-1} Ca^{2+}, opening of the CAN channel was totally suppressed by 10 μmol l^{-1} cyclic GMP, whereas ATP (1 mmol l^{-1}) was without effect. We suggest that the CAN channel plays a specific role in modulation of cell excitability and in shaping the voltage response of ORNs.

Introduction

Male moths have long been known for their remarkable ability to detect extremely low concentrations of female sex pheromones (Kaissling and Priesner, 1970; Kaissling, 1987). The molecular mechanism underlying chemo-electrical transduction, however, remained elusive.

The male silkmoth *Antheraea polyphemus* detects female sex pheromone components *via* specialized olfactory receptor neurones (ORNs) innervating long, trichoid sensilla located on its antennae (Kaissling, 1987). Each sensillum consists of a hollow cuticular hair innervated by the dendrites of two to three bipolar ORNs

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and three auxiliary cells (thecogen, tormogen and trichogen cells) enveloping the sensory neurones (Keil and Steinbrecht, 1984). In the lumen of the hair, the outer dendritic segments are bathed by a solution containing K^+ as the main extracellular cation (Kaissling, 1987).

Owing to small size of ORNs and the obstacle posed by the closely apposed supporting cells, no intracellular membrane potential recordings of insect ORNs have been obtained. Extracellular recordings revealed that pheromone components elicit a graded depolarizing receptor potential, which leads to the generation of action potentials (reviewed by Kaissling, 1986; Meng *et al.* 1989).

To investigate the ion channels involved in chemo-electrical transduction and in the shaping of the graded receptor potential and the phasic action potential response, three alternative strategies were used. A long-term cell-culture system was developed (Stengl and Hildebrand, 1990) and several types of ion channels were shown to be expressed in these ORNs after 2–3 weeks *in vitro* (Zufall *et al.* 1991). These experiments were carried out in the sphinx moth *Manduca sexta*. In another study we showed that the outer dendritic plasma membrane of silkmoth ORNs *in situ* seems to be exclusively equipped with a specific sex-pheromone-sensitive ion channel (the AC_1 channel) not previously described in cultured ORNs (Zufall and Hatt, 1991). This study suggested that channel phosphorylation *via* protein kinase C was the crucial step in channel activation, although the AC_1 channel was also sensitive to intracellular cyclic GMP.

Here we describe some of the electrical properties of silkmoth ORNs following acute isolation. We characterize a Ca^{2+}-activated nonspecific cation channel (CAN channel). This channel is of particular significance because a similar cation channel has been shown to be active in the soma membrane of cultured ORNs after pheromone application (Zufall, 1989; Stengl *et al.* 1989). The mechanism of activation of this channel, however, has not been explained. A preliminary report of some of this work has already been presented (Zufall *et al.* 1988).

Materials and methods

Preparation

For acute isolation of silkmoth ORNs we slightly changed the method described by Keil (1987). Diapausing pupae of male *Antheraea polyphemus* (Cramer) were kept at 4°C in a light/dark rhythm of 11 h/13 h. To initiate adult development, pupae were placed in an incubator at 22°C at a humidity of 75 %. Antennae were dissected out of the pupae after 5–8 days just before the beginning of cuticle formation and immersed in physiological saline at room temperature. Dissociation of the epithelium was obtained by gentle trituration of the antennal side branches in 'Ca^{2+}-free' saline (buffered to 100 nmol l^{-1} free Ca^{2+} with EGTA). No digesting enzymes were needed for this treatment. The resulting suspension was diluted 1:100 in physiological saline and antennal cells were plated on uncoated Nunc Petri dishes, where they adhered after 10–15 min. Cells obtained in this way were used for about 4 h.

In a different approach, ORN membranes were isolated *in situ* from adult moths by using a similar technique to that described by Williams (1988) and Zufall and Hatt (1991). In contrast to our earlier study, in which special care was taken only to protrude outer dendritic segments, here single olfactory trichoid hairs were almost completely severed from the antenna. Application of considerable positive pressure to the stem of the antenna seemed to break tight contacts within the sensillum and normally resulted in extrusion of a number of membrane vesicles (so-called blebs) up to a few micrometres in diameter.

Patch-clamp technique and data analysis

Patch-clamp recording closely followed the methods described by Zufall *et al.* (1991) and Zufall and Hatt (1991). Briefly, electrodes were made from borosilicate glass capillaries, coated with Sylgard and then fire-polished. The neurones and blebs were viewed at 320× magnification with a Zeiss Axiovert 10 inverted microscope equipped with phase-contrast and Nomarski optics. Whole-cell and single-channel currents were measured at room temperature with an EPC-7 amplifier (List Electronic, Darmstadt, FRG) and stored on video tape. Single-channel currents were then low-pass filtered at 2–5 kHz with an eight-pole Bessel filter and digitally sampled at 20 kHz using a Hewlett-Packard HP 9802 computer. The event detection program described by Dudel and Franke (1987) was used to evaluate single-channel currents. Only those patches that contained no superpositions of openings, indicating only one active channel in the patch, were evaluated for this study.

Solutions and drug application

Cells and blebs were continuously superfused with saline containing (in $mmol\,l^{-1}$): 150 NaCl; 4 KCl; 2 $CaCl_2$; 5 glucose; 10 Hepes; pH 7.1. In one experiment all Na^+ and K^+ in this solution was replaced by choline. The 'intracellular' pipette-filling solution for whole-cell and outside-out recordings contained (in $mmol\,l^{-1}$): 1 NaCl; 150 KCl; 1 $CaCl_2$; 10 EGTA; 2 $MgCl_2$; pH 7.1. For desired levels of free Ca^{2+} in the inside-out experiments the Ca^{2+}/EGTA ratio was calculated according to Hagiwara (1983). All chemicals used in this study were obtained from Sigma except amiloride hydrochloride, which was kindly donated by Merck, Sharp & Dohme (FRG).

Solution changes were made by moving the excised patches to a microchamber equipped with a liquid filament switch, allowing a change of test solutions within 200 μs (Dudel *et al.* 1990).

Results

Following acute isolation, silkmoth ORNs can easily be identified from other antennal cells by their typical morphology (Keil, 1987). Fig. 1 shows a bundle of three receptor neurones. The perikarya and olfactory dendrites can be clearly distinguished. Only those neurones with a soma membrane completely unwrapped

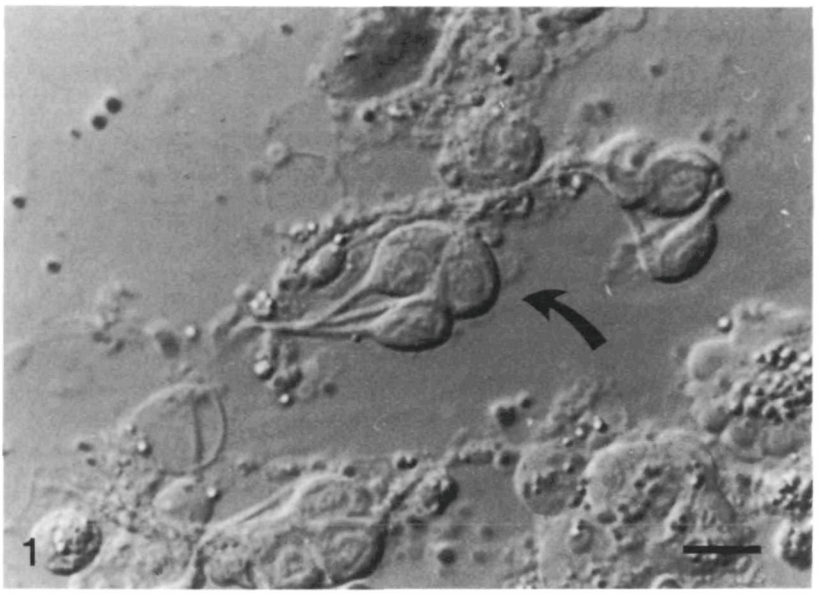

Fig. 1. A group of acutely isolated olfactory receptor neurones (arrow) from antennae of *Antheraea polyphemus* pupae. The cell bodies of the three neurones seem to be completely free of their auxiliary cells. Cell bodies and dendrites still adhere to each other. Scale bar, 10 μm.

from their auxiliary cells were chosen for electrophysiological analysis and this was a prerequisite for successful whole-cell recordings.

In whole-cell recordings, ORNs had a resting potential V_0 of -41 ± 8 mV (mean±S.D.; $N=9$). The input resistance was determined from the slope of the current voltage (I–V) curve between -70 and -150 mV, where no voltage-gated currents were observed. The input resistance was 5.2 ± 0.8 GΩ ($N=6$). The membrane time constant was typically about 30 ms, giving a membrane capacitance of about 5.8 pF. Using the membrane time constant and a specific membrane capacitance of 1 μF cm^{-2}, a specific membrane resistance of 30 kΩ cm^2 could be calculated.

Fig. 2A shows a family of whole-cell currents from an acutely isolated ORN in response to depolarizing voltage steps from a holding potential of -85 mV to the indicated values. Only one current component is obvious: a fast activating and then inactivating outward current. The transient outward current appears at -40 mV and becomes larger and faster with increasing depolarization. The time constant of inactivation is about 14 ms and does not seem to be voltage dependent. The transient outward current was absent when the cell was depolarized from a holding potential more positive than -50 mV and could be inhibited by the external application of 5 mmol l^{-1} 4-aminopyridine (not shown). Thus, it appears that the first voltage-activated current expressed during pupal development of silkmoth ORNs is a current with characteristics very similar to the A-current (I_A)

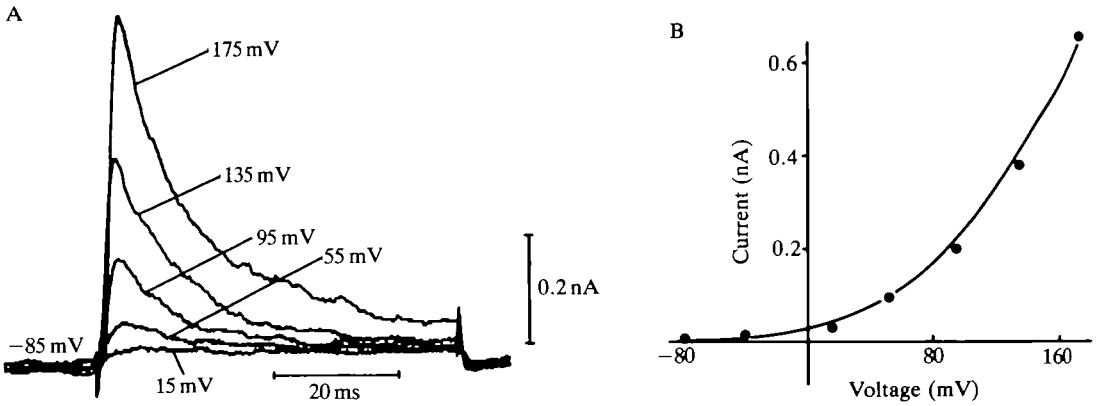

Fig. 2. (A) A family of whole-cell currents from an acutely isolated olfactory receptor neurone (ORN). Currents were activated by depolarizing voltage steps from a holding potential of −85 mV to the indicated values. Leakage currents and capacity transients are not subtracted. (B) Current–voltage relationship for the peak value of the transient outward current shown in A.

(Neher, 1971; Connor and Stevens, 1971), a result that is supported by analysis of ionic currents in developing *Drosophila* muscle (Salkoff and Wyman, 1981).

Further information on membrane properties of acutely isolated ORNs was obtained from single-channel measurements. In agreement with the result shown in Fig. 2, no active ion channels were found after giga-seal formation (on-cell patch) at the cell's resting potential. Surprisingly, in some of these 'silent' patches the opening of a specific ion channel could be observed after the formation of outside-out patches (at a holding potential of −60 mV, near the reversal potential for K^+). This ion channel seemed to be activated by patch excision. Fig. 3 shows a continuous recording at low time resolution over 44 s. Channel openings are grouped in bursts that can last from tens of milliseconds to several seconds. Bursts are interrupted by closed periods of up to several hundred milliseconds. The channel was active as long as the patch was stable (up to 20 min) with no obvious loss of activity.

A more detailed analysis of these channel openings is given in Fig. 4. Some single-channel events are shown in Fig. 4A on an expanded time scale. The characteristic fluctuation behaviour during the bursts of openings is obvious. Most transitions do not occur between the closed and open states but between different current sublevels. The complicated kinetics of these single-channel events is reflected in the amplitude distribution (Fig. 4C). Current amplitudes are broadly distributed with a peak at −2.8 pA (at −60 mV). It appears that discrete amplitude sublevels occur in the distribution, although they cannot be fully resolved (see also Fig. 6C). The peak values of the amplitude distributions at different holding potentials were used to generate the *I–V* curve (Fig. 4B). A slight inward rectification under the ionic conditions employed is obvious. This rectification may be explained by a Mg^{2+} block, as has been shown for a very similar ion channel

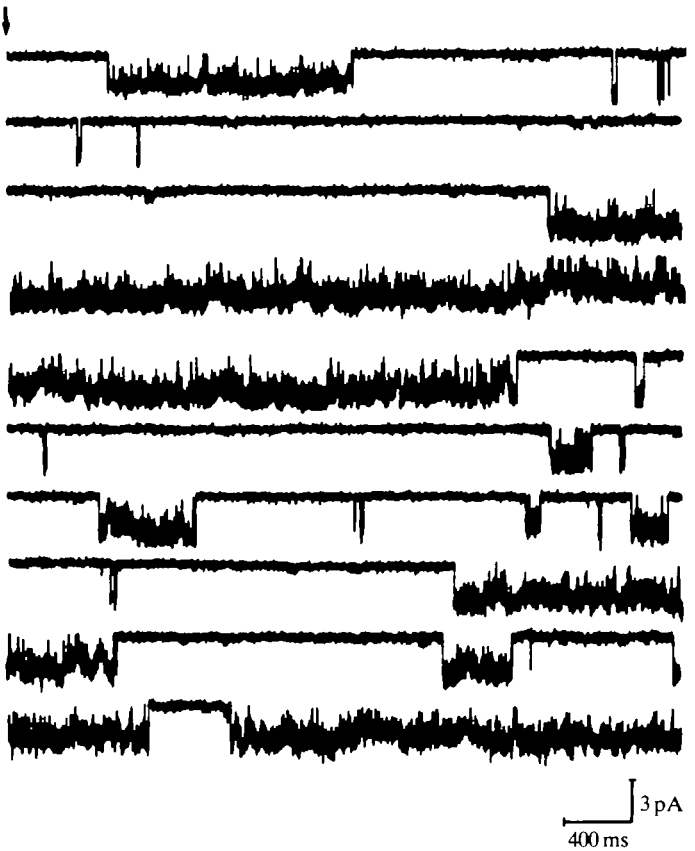

Fig. 3. Continuous recording at low time resolution over 44 s of single-channel events activated after patch excision (arrow) from an acutely isolated ORN. Outside-out patch, holding potential −60 mV. Bandwidth 0–2 kHz.

from lobster ORNs (McClintock and Ache, 1990). The presumed Mg^{2+} block may also account for open-channel current noise. From a reversal potential near 0 mV and the observation that substitution of all monovalent extracellular cations by choline resulted in a complete loss of current at negative potentials, we considered this ion channel to be nonspecifically permeable for cations. A single-channel conductance of 48±1.5 pS (N=6) was calculated from the slope of the I–V curve at negative holding potentials. Except for the inward rectification, no other obvious voltage dependence of the channel was observed. Fig. 4D shows the distribution of open times in a semi-logarithmic plot. The distribution can be fitted by two exponentials with time constants of 1 ms and 4.6 ms. No attempts were made in this study to obtain the distributions of closed times or burst lengths.

Since excised patches from acutely isolated silkmoth ORNs were extremely difficult to achieve, a different approach was used in the following experiments. We have shown in a previous study that single-channel recordings from mem-

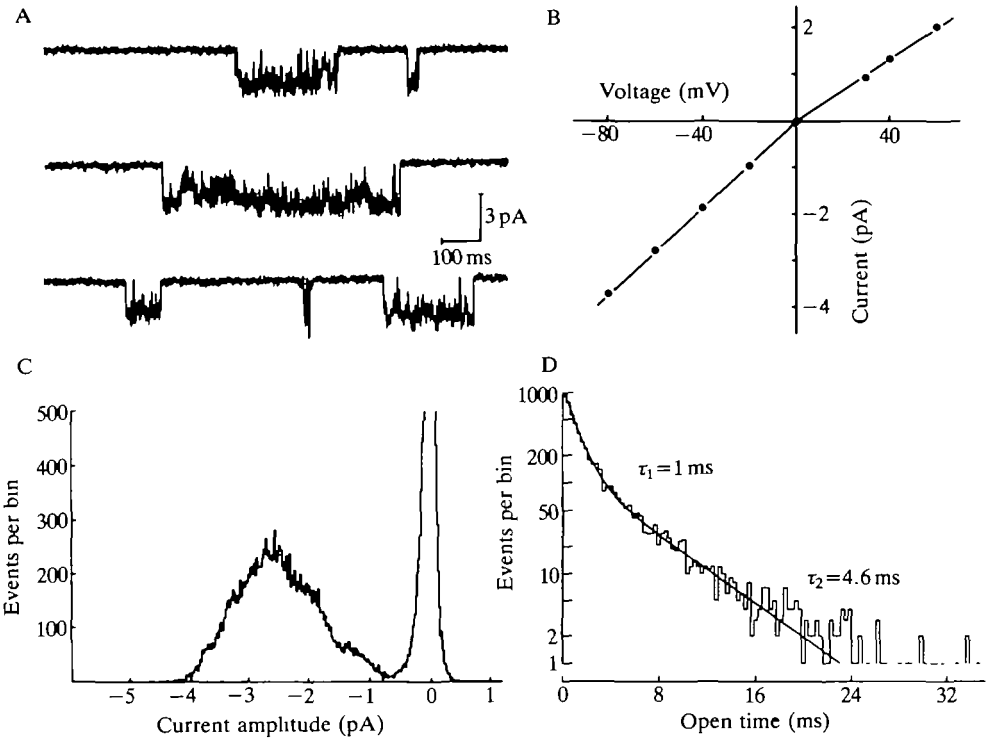

Fig. 4. Evaluation of single-channel currents activated after patch excision. Same patch as in Fig. 3. Holding potential −60 mV. (A) Examples of bursts of openings on an expanded time scale. Bandwidth 0–5 kHz. (B) *I–V* relationship of single-channel events from A. Each point on the curve represents the maximum of amplitude distributions like that in C. The curve shows distinct inward rectification. A single-channel conductance of 48 pS is derived from the slope of the *I–V* curve at negative potentials. (C) Amplitude distribution of single-channel openings, binwidth 0.02 pA. (D) The distribution of open times can be fitted by two exponentials with time constants τ of 1 ms and 4.6 ms. Binwidth 300 μs.

branes of the outer dendritic segment of mature silkmoth ORNs can be obtained consistently by using a new *in situ* preparation (Zufall and Hatt, 1991). These outer dendritic membranes did not exhibit the 48 pS cation channel described here. In the *in situ* preparation it was also possible to extrude membrane vesicles (so-called blebs) after first cutting off most of the olfactory hairs. These membrane fragments probably did not contain outer dendritic membranes because they never incorporated the pheromone-sensitive AC_1 channel (F. Zufall, unpublished observation). Likewise, the typical channel distribution of auxiliary cells (Zufall, 1989) was not found in these membranes. Instead, these vesicles contained the 48 pS cation channel described above, suggesting that they probably originated either from inner dendritic or from somal ORN membrane. The latter possibility, however, seems unlikely, since we never saw on these membranes openings of the

delayed rectifier K^+ channel that is distributed in the somal membrane at a high density (Zufall *et al.* 1991).

To gain further insight into the gating mechanism of the 48 pS cation channel, inside-out patches were prepared from vesicle membranes. Surprisingly, these patches were particularly stable so that channels could be recorded at more hyperpolarized potentials, which greatly improved the resolution of current sublevels. Because these channels were activated by patch excision (see above), we tested the effect of Ca^{2+}. Fig. 5A shows an experiment in which the intracellular Ca^{2+} concentration was buffered to 10 nmol l^{-1}. No channel openings occurred at this Ca^{2+} concentration. Rapid switching (by means of the liquid-filament switch) to an intracellular solution containing 100 nmol l^{-1} free Ca^{2+} induced the opening of a specific ion channel. Distinct current sublevels during

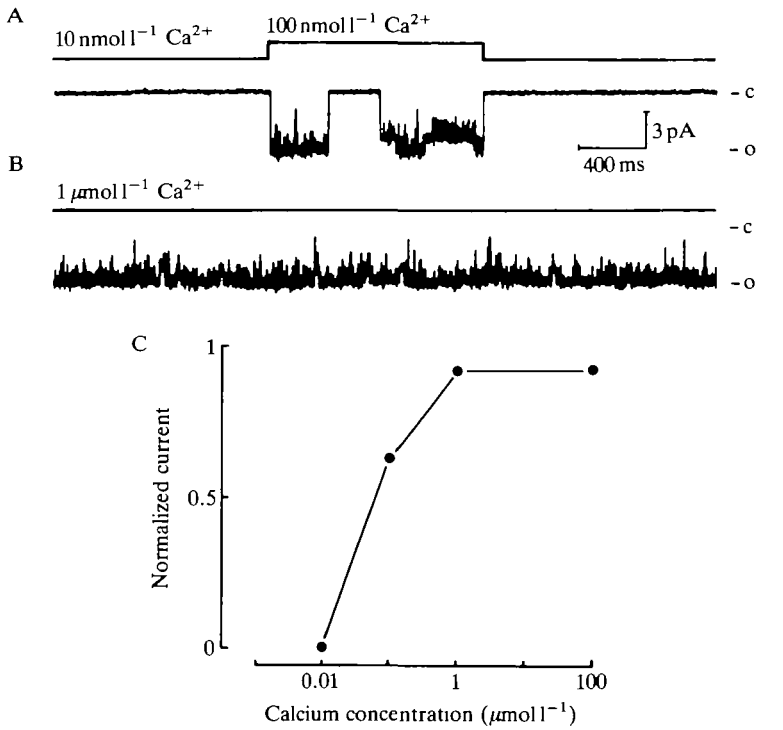

Fig. 5. Gating of the 48 pS cation channel by intracellular Ca^{2+}. Inside-out patch, holding potential −100 mV. (A) Fast application of a solution containing 100 nmol l^{-1} free Ca^{2+} leads to opening of the cation channel, which is totally closed at a free Ca^{2+} concentration of 10 nmol l^{-1}. Maximal open (o) and closed (c) states of the channel are indicated. (B) The channel is almost always in one of the open states during continuous superfusion with intracellular solution containing 1 μmol l^{-1} free Ca^{2+}. (C) Ca^{2+} sensitivity of the CAN channel. Each point on the curve represents the normalized mean current at the indicated Ca^{2+} concentration. Mean current was calculated by integrating current flow during channel opening and dividing the integral by the total sample time. Sample time for each value was 50 s.

opening are obvious at the holding potential of −100 mV. The Ca^{2+} effect could easily be reversed. In Fig. 5B, during a continuous application of 1 μmol l^{-1} Ca^{2+} to the same patch, the channel was almost always in the open state. This ion channel had characteristics similar in terms of ion selectivity, I–V relationship and kinetics (not shown) to those of the 48 pS cation channel from freshly isolated ORNs (see above). Thus, it seemed justified to conclude that both ion channels belong to the same type. Further, our results characterize the cation channel described here as a member of the family of Ca^{2+}-activated nonspecific cation channels (CAN channel) (Partridge and Swandulla, 1988).

Fig. 5C shows the steep Ca^{2+} dependence of the CAN channel. The normalized mean current was plotted *versus* the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. At a $[Ca^{2+}]_i$ of 10 nmol l^{-1}, no channel openings were detected. As $[Ca^{2+}]_i$ was raised to 100 nmol l^{-1}, the mean normalized current increased dramatically to 0.63 and reached a plateau of 0.92 at a $[Ca^{2+}]_i$ of 1 μmol l^{-1}. The slope of the double-logarithmic dose–response curve in the steep range (from the data of Fig. 5C) is greater than 1, indicating more than one binding step for Ca^{2+}. The EC_{50} value for Ca^{2+} activation was about 80 nmol l^{-1} (N=4). In view of this Ca^{2+} activation curve, the above result of channel activation by patch excision can be explained. In the outside-out recordings of Figs 3 and 4 a patch pipette solution with a free Ca^{2+} concentration of 100 nmol l^{-1} was used, which is enough to activate the CAN channel to a high degree.

Next we tried to inhibit the activity of the CAN channel by application of amiloride to the cytoplasmic side. Amiloride has been well characterized as a channel blocker of epithelial Na^+ channels (Palmer and Frindt, 1986). Moreover, it has been shown to inhibit odour responses of isolated frog olfactory receptor cells (Frings and Lindemann, 1988). Fig. 6A shows a continuous recording in which the effect of 100 nmol l^{-1} amiloride was tested. As can be seen, the activity of the CAN channel steadily decreased over several hundred milliseconds after a fast application of the drug. In Fig. 6B the continuous application of a higher concentration of amiloride (500 nmol l^{-1}) resulted in a stronger inhibition of the activity of the CAN channel. Clusters of openings disappeared and the open time was drastically reduced (to 0.2 ms in this experiment from a single-exponential fit of the open-time distribution). In Fig. 6C we evaluated the distribution of current amplitudes. Under control conditions the histogram clearly showed four different conductance levels. The peaks appeared to occur at multiples of about −1.6 pA (corresponding to a conductance of 16 pS). After addition of amiloride to the bath solution, only openings of the lowest conductance state were detected in the amplitude distribution. Very similar results have been obtained in five other patches.

In another series of experiments we tested whether the activity of the CAN channel could be controlled by intracellular nucleotides. In this respect, the effect of cyclic GMP was of special interest. An increase in cyclic GMP concentration in silkmoth ORNs has previously been shown after pheromonal stimulation (Ziegelberger *et al.* 1990) as has a potential modulatory role of cyclic GMP for at least two

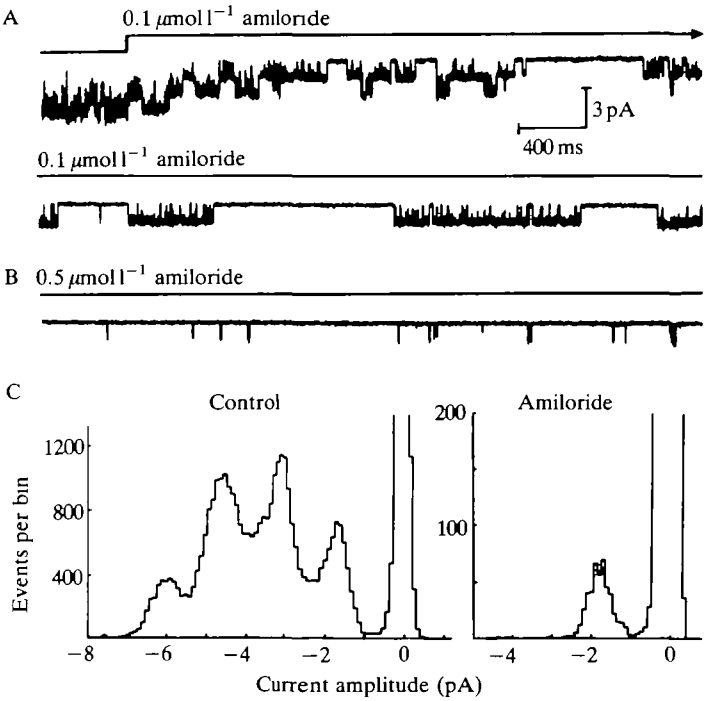

Fig. 6. The effect of amiloride on the activity of the CAN channel. Inside-out patch, holding potential −100 mV. (A) After fast application of amiloride hydrochloride (0.1 μmol l^{-1}) the activity of the CAN channel steadily declines. The IC_{50} value for the amiloride effect is less than 0.1 μmol l^{-1}. Upper and lower traces are a continuous recording. (B) Effect of a higher concentration of amiloride (0.5 μmol l^{-1}) during continuous application. (C) Amplitude histograms obtained under control conditions and after application of amiloride (0.1 μmol l^{-1}). Binwidth 0.05 pA.

different ion channels expressed in ORNs, including the AC_1 channel (Zufall *et al.* 1991; Zufall and Hatt, 1991). In Fig. 7A, the CAN channel was activated by the continuous application of intracellular solution containing 1 μmol l^{-1} free Ca^{2+}. Rapid switching to a solution to which 10 μmol l^{-1} cyclic GMP (guanosine 3′5′-cyclic monophosphate) was added resulted in a fast (<10 ms) and complete suppression of the activity of the CAN channel. This effect was reversible. The cyclic-GMP-mediated inhibition was repeated in the same patch up to 20 times without any obvious change. No ATP was needed for this effect, suggesting a direct action of cyclic GMP rather than *via* a cyclic-GMP-dependent protein kinase. In Fig. 7B we tested the effect of ATP (1 mmol l^{-1}), which is known for its Ca^{2+}-chelating action. ATP, however, was not able to inhibit the activity of the CAN channel. Thus, the cyclic GMP effect seemed to be specific.

Discussion

We have characterized a Ca^{2+}-activated nonselective cation channel (CAN

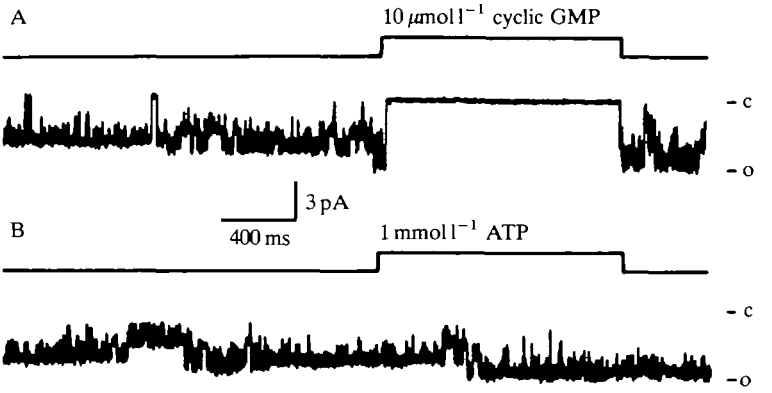

Fig. 7. (A) The effect of cyclic GMP. The activity of the CAN channel is totally suppressed by a fast application of cyclic GMP ($10\,\mu mol\,l^{-1}$), whereas ATP ($1\,mmol\,l^{-1}$) is without effect (B).

channel) from olfactory receptor neurones of the silkmoth *Antheraea polyphemus*. This channel is expressed in the soma membrane during the early phase of pupal development, at a time when only one type of voltage-activated current, the A-current, can be found in these neurones. Most probably, at this developmental stage insect ORNs are not able to respond to pheromones (Schweitzer *et al.* 1976). The CAN channel can easily be distinguished from the previously described AC_1 channel (Zufall and Hatt, 1991), which probably mediates primary olfactory transduction. The CAN channel is never found in outer dendritic membranes, has slightly inwardly rectifying properties, is activated by patch excision, is gated by intracellular Ca^{2+} and is inhibited by cyclic GMP. In contrast, the AC_1 channel seems to be exclusively expressed in the outer dendritic membrane, has a linear *I–V* curve, is not gated by Ca^{2+}, is not activated by patch excision and is stimulated by cyclic GMP. Although the CAN channel does not seem to mediate primary olfactory transduction, it is of particular significance, since we have previously described a very similar cation channel that is active after pheromone stimulation in somatic membranes of cultured ORNs from *Manduca sexta* (Zufall, 1989; Stengl *et al.* 1989). These cation channels resemble each other in their conductance, inward rectifying *I–V* relationship, the occurrence of characteristic current sublevels and their activation by patch excision. It seems safe, therefore, to conclude that the *Manduca* cation channel also belongs to the family of Ca^{2+}-activated nonselective cation channels.

The CAN channel obviously has more complex kinetics than would be expected from a simple two-state model. It displays a double-exponential opening probability distribution, clusters of openings with long-lived closed states, and rapid fluctuations from the closed state to transient open states. Some of these observations may be additionally confused by the variability in conductance. Since this variability occurred in quantal steps, we attribute it to the synchronized opening of variable numbers of elementary conductance pathways or ion channel

subunits. Similar behaviour has been described in a number of channels, including chloride and potassium channels (Krouse *et al.* 1986; Hunter and Giebisch, 1987; Premkumar *et al.* 1990).

CAN channels have been described in a number of cells, including neurones, muscle cells, secretory cells, glial cells and epithelial cells (Partridge and Swandulla, 1988). Their role, however, is not well understood. The best evidence comes from *Helix* burster neurones, where CAN channels can provide a maintained depolarizing current (Partridge and Swandulla, 1988). What could be the functional role of the CAN channel in ORNs? As in other sensory neurones, like photoreceptors (for a review, see Attwell, 1986), the voltage response of ORNs to the incoming stimulus may not be determined simply by the opening of the transducer channels in the outer segments, but may be further shaped by distinct voltage- and time-dependent currents in the inner segment and the soma membrane. For example, in silkmoth ORNs, application of the odour stimulus leads to a graded receptor potential (with unknown exact time course), whereas the output of the neurone is a phasic action potential sequence (Meng *et al.* 1989). The CAN channel described here is highly sensitive to slight changes in the intracellular Ca^{2+} concentration. If Ca^{2+} concentration were elevated, it would open and further depolarize the cell. Thus, it would 'boost' the incoming depolarisation from the outer dendritic segment. This function could be coupled to the reported transient increase in inositol 3-phosphate following pheromone stimulation (Breer *et al.* 1990), which would result in a transient depolarization of the soma. In contrast, current spread within the cell would hardly be a problem at the high input resistance of 5 GΩ. The simultaneous opening of just a few CAN channels would depolarize the cell by tens of millivolts on top of the already large depolarization generated by activation of enough of the AC_1 channels, making a 'boosting' function of the CAN channel questionable. Further speculation on the role of the CAN channel is complicated since we know neither the input resistance of mature ORNs within their functional cytoarchitecture (e.g. cultured ORNs after 2–3 weeks of differentiation had a 10 times lower input resistance, Zufall *et al.* 1991, than that described here) nor the local concentrations of cyclic GMP within the cell. Thus, a functional role for the CAN channel in mature ORNs remains to be demonstrated.

In conclusion, the CAN channel seems to offer an additional tool for modulating cell excitability and for shaping the output of ORNs.

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