Ionic Currents of Kenyon Cells from the Mushroom Body of the Honeybee

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The mushroom bodies have been suggested to be essentially involved in learning and memory in insects. In the honeybee *Apis mellifera* they are composed of about 340,000 intrinsic elements, called Kenyon cells, which can be easily separated from all other neurons of the brain. Here we describe a preparation in which we studied ionic currents in the isolated Kenyon cell somata, using tight-seal whole-cell recording. Several outward and inward currents were identified and investigated by the use of pharmacological agents and in ion substitution experiments: a rapidly inactivating A-type potassium current that is completely blocked with 5 mM 4-aminopyridine; a calcium-activated potassium current that is blocked by 1–100 nM charybdotoxin; a delayed rectifier-type potassium current that is only weakly sensitive to tetraethylammonium but is blocked by 100 μ M quinidine; a rapidly activating and inactivating, TTX-sensitive sodium current; a persistent sodium current that is both TTX and cadmium sensitive; and a calcium current that is completely blocked at 50 μ M cadmium and is affected by verapamil and nifedipine only at high concentrations (100 μ M). The currents described here are very similar to currents found in other insect neurons or muscle cells.

This preparation will not only facilitate studies concerning the action of transmitters and neuromodulators that are contained within neurons converging onto the Kenyon cells, but will also allow a study of the role of the adenylyl cyclase pathway, elements of which are expressed in Kenyon cells, and are known to be essential for learning in invertebrates.

[Key words: insect, whole-cell patch clamp, potassium currents, sodium currents, calcium currents]

The mushroom bodies are probably the most peculiar structures in the insect brain, which has encouraged wide speculations about their possible function. Evidence for an involvement in learning and memory was initially derived from studies in the honeybee, which showed that selectively disturbing mushroom body function by cooling causes retrograde amnesia after olfactory training (Menzel et al., 1974; Masuhr, 1976; Erber et al., 1980). Behavioral analysis of *Drosophila* mutants defective in

mushroom body structure revealed that the mutants behave poorly in olfactory conditioning tasks compared to wild-type animals (Heisenberg et al., 1985). The gene products of *Drosophila* learning mutants *dunce* (*dnc*) and *rutabaga* (*rut*) have been shown to be preferentially expressed in the intrinsic elements of the mushroom bodies, the Kenyon cells (Nighorn et al., 1991; Han et al., 1992; see Davis, 1993, for review). Both mutations interfere with the cAMP pathway by affecting a cAMP-specific phosphodiesterase and a calcium/calmodulin-responsive adenylyl cyclase, respectively (Byers et al., 1981; Livingstone et al., 1984). Behavioral pharmacological experiments with honeybees revealed that octopamine injected into the mushroom bodies facilitates olfactory learning and memory retrieval (Bicker and Menzel, 1989; Menzel et al., 1990), and biochemical studies indicate that the cAMP cascade may be involved in mediating this effect (Menzel et al., 1991). In an electrophysiological study, a presumably octopaminergic neuron has been identified, which projects into the main input region of the bee mushroom bodies, the calyces, and mediates the reward in olfactory learning (Hammer, 1993). Furthermore, Mauerlshagen (1993) demonstrated changes in the response of an identified mushroom body extrinsic neuron to an odorant after applying a classical olfactory conditioning paradigm. Taken together, these results provide evidence for an important role of the mushroom body intrinsic elements, the Kenyon cells, in olfactory learning and memory.

Unfortunately, due to the small size of cell bodies and processes in flies and bees, electrophysiological investigations on the Kenyon cells cannot be performed *in vivo*. We therefore decided to study the isolated Kenyon cell somata of honeybee pupae using tight-seal whole-cell recording (Hamill et al., 1981). This technique is particularly suitable for small cells and has already been successfully applied to a variety of insect neuronal preparations (Byerly and Leung, 1988; O'Dowd and Aldrich, 1988; Solc and Aldrich, 1988; Hardie and Weckström, 1990; Hardie, 1991; Saito and Wu, 1991; Laurent et al., 1993; Pearson et al., 1993). Anatomical studies in the honeybee revealed that each mushroom body contains about 170,000 Kenyon cells as the only intrinsic neuronal type (Witthöft, 1967; Mobbs, 1982). Because of their morphology, the mushroom bodies in this insect can easily be dissected out of the brain (Fig. 1) and their subsequent dissociation yields a pure preparation of Kenyon cells.

With this article we give a description of the variety of voltage-gated and calcium-activated currents that are present in the isolated somata of pupal Kenyon cells. The currents are compared to those found in other insect neuronal and muscle preparations. Since we were the first to do voltage-clamp studies with these neurons, we started to characterize their whole-cell

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Figure 1. The mushroom body and its intrinsic neurons. *A*, Pupal brain with the left mushroom body dissected out of the brain and the right one still in place within the dorsal protocerebrum. *B*, Bodian-stained frontal section through the mushroom body, showing the somata of Kenyon cells located within two cup-shaped structures, the calyces. This picture illustrates how well the cell bodies of the Kenyon cells are separated from the somata of other neurons of the protocerebrum. *C*, Isolated somata of Kenyon cells viewed under phase contrast. Scale bars: *a*, 500 μm ; *b*, 100 μm ; *c*, 20 μm .

currents. The results provide the basis for future investigations on the action of transmitter substances and neuromodulators that, with immunocytochemical techniques, have been shown to be contained within neurons converging onto the Kenyon cells. Since the *dnc* and *rut* gene products have been shown to be present in Kenyon cells, we are particularly interested in a possible involvement of the adenylyl cyclase pathway in modulating ionic currents of these cells.

A preliminary report of some of the work presented here has appeared in abstract form (Schäfer et al., 1993).

Materials and Methods

Animals. Honeybee (*Apis mellifera*) pupae were collected from the comb between days 2 and 6 of the pupal development, which lasts 9 d under natural conditions. They were reared at 28°C and 80% relative humidity in an incubator until they had reached 70–80% of adult development (i.e., pupal day 7) judged on the basis of the body pigmentation.

Cell preparation. Kenyon cells of 7-d-old pupae were dissected and cultured following the protocol of Kreissl (1992; Kreissl and Bicker, 1992) with only minor modifications. In brief, brains were removed from the head capsule in a Leibovitz L15 medium (GIBCO-Bethesda Research Laboratory) supplemented with sucrose, glucose, fructose, and proline (42.0, 4.0, 2.5, and 3.3 gm/500 ml, respectively) to reach a final osmolarity of 500 mOsm (L15-500). After the brains had been exposed to a hyperosmolar medium (containing 59 gm instead of 42 gm sucrose/500 ml; 600 mOsm), the glial sheath could be removed readily. This was followed by another step in L15-500 medium in which mushroom bodies were dissected out of the brains. After a 10 min incubation in a calcium-free Ringer solution (in mM: 130 NaCl, 5 KCl, 10 MgCl₂, 25 glucose, 180 sucrose, 10 HEPES; pH 7.2), mushroom bodies (MBs) were transferred back to L15-500 medium (4 MBs/200 μ l) and finally dissociated by gentle trituration with a 100 μ l siliconized Eppendorf pipette. Cells were then plated in 25 μ l aliquots into 100 μ l droplets of L15-500 medium on uncoated Falcon plastic dishes and allowed to attach for at least 15 min. Thereafter, the dishes were filled with 2.5 ml of L15-500 and were kept at 29°C in an incubator at high humidity. Under these conditions the cells can survive for up to 10 d in culture (Kreissl, 1992).

Electrophysiological techniques. Whole-cell gigohm seal recording was performed at room temperature following the methods described by Hamill et al. (1981). Recordings were made using a Axopatch 1D amplifier (Axon Instruments). Pulse generation, data acquisition, and analysis were carried out using a TL-1 interface in conjunction with pCLAMP programs (Axon Instruments) running on an AT-type microcomputer. Pipette and membrane capacitance were compensated, and series resistance compensation (80%) was routinely employed. Currents were low-pass filtered with a 4-pole Bessel filter at 2–5 kHz and digitally sampled at 4–20 kHz, depending on the current under investigation. Voltages were corrected for liquid junction potential; leakage currents were not subtracted. Electrodes were pulled from borosilicate glass capillaries (GC 150-15, Clark, Reading) and had tip resistances between 4 and 6 M Ω in standard external solution (see below). For statistical analysis of the data we used ORIGIN (MicroCal Inc.). Where appropriate, data are presented as mean $\pm$ standard error of the mean (SEM), unless stated otherwise.

Solutions. The bath was continuously perfused at 2 ml/min with a standard external solution that consisted of (in mM) 130 NaCl, 6 KCl, 4 MgCl₂, 5 CaCl₂, 160 sucrose, 25 glucose, 10 HEPES/NaOH; pH 7.2. In some experiments Ca²⁺ was replaced by Ba²⁺. For the analysis of outward currents 200 nM tetrodotoxin (TTX) and in some cases 50 μ M CdCl₂ was added. The standard internal solution contained (in mM): 100 K-aspartate, 40 KF, 20 KCl, 2.5 MgCl₂, 1 EGTA, 3 ATP, 160 sucrose, 10 HEPES; pH 7.2. For the recording of inward currents KCl was replaced by TEA-Cl and the remaining K⁺ was substituted by Cs⁺. Totally synthetic, HPLC-purified charybdotoxin was obtained from Bachem (Heidelberg, Germany). All other chemicals were purchased from Sigma (St. Louis, MO).

Results

Based on the size of their cell bodies, two subtypes of Kenyon cells can be distinguished: one 10 μ m, the other 7 μ m in diameter (Fig. 1*b,c*). While the larger cell bodies are already present in

the larval and early pupal stages, the smaller-diameter somata appear not before the second half of pupal development, after neurogenesis of Kenyon cells has been completed. Judged by their location in the center of the calycal cups, the 7 μ m cells are most likely the latest progeny of Kenyon cell neuroblasts (S. Eichmüller and S. Schäfer, unpublished observations). In contrast to Kenyon cells of younger pupae, which in the dish usually grow a single long and thin process within a few hours after dissociation, the cells of late-stage pupae used in the present study hardly differentiate within the first 2 d. Recordings were made only from 10 μ m cells without processes, 12–36 hr after dissociation. The membrane capacitance estimated from the capacitance compensation settings on the amplifier was 2.6 ± 0.5 pF (mean $\pm$ SD; $n = 91$) and did not change with the time the cells had spent in culture. As the cells did not show any inward rectifying potassium currents, or any other gated currents at voltages below -60 mV, their input resistance was determined either from the slope of the current–voltage relationship between -60 and -120 mV, or, simply, by calculation from the response to a step to -90 mV from a holding potential of -60 mV. The input resistance was 6.6 ± 3.3 G Ω (mean $\pm$ SD; $n = 31$). With the exception of a transient sodium current, which could not be detected during the first 18 hr, all of the other currents described below were also seen in cells that were studied immediately after dissociation ($n = 8$). However, with these cells it was extremely difficult to obtain a whole-cell configuration.

In response to depolarizing voltage steps from a holding potential of -65 mV the cells showed a fast and transient inward current followed by a much larger outward current (Fig. 2). Both inward and outward currents are a combination of several components that can be separated by the use of pharmacological tools and the application of appropriate pulse protocols.

Outward currents

The outward current is composed of a rapidly inactivating and a noninactivating phase. In most of the cells examined, the $I-V$ curve for the outward current had a characteristic N-shape with a halfway peak around $+50$ mV (Fig. 2, inset). Alterations in the external K⁺ concentration and measurements of tail-current reversal potentials indicate that the outward current is primarily carried by K⁺ (not shown). Further analysis, performed in the presence of 200 nM TTX in the external solution to block Na⁺ currents, revealed that the outward current is composed of at least three different potassium currents.

Calcium-activated K⁺ currents. Eliminating calcium currents by addition of 50 μ M cadmium to the bath solution dramatically altered the size and shape of the outward current; both transient and sustained phase were considerably reduced in size, and the inactivation of the transient component became faster. Furthermore, the N-shape of the $I-V$ relation was completely abolished (Fig. 3). Switching to an external solution containing 2 mM cobalt or exposure to a calcium-free saline had the same effect. As these observations are indicative for the presence of a calcium-activated potassium conductance, we tested whether charybdotoxin (ChTX), a blocker for a subtype of calcium-activated potassium channels, also changes size and shape of the outward current. Indeed, ChTX (1–100 nM) blocked a transient outward current that activates at potentials above -30 mV and has a maximum around $+50$ mV (Fig. 4). Since the current gradually decreased with time and repeated application of depolarizing pulse protocols, its block by ChTX could be shown

Figure 2. Whole-cell current in the absence of any blocking agents. The membrane potential was stepped from a holding potential of -65 mV to a prepulse potential of -95 mV for 100 msec and then depolarized for 250 msec to command potentials between -65 and $+85$ mV in 10 mV increments. Voltage steps were applied every 5 sec. The I - V curves for the peak outward (■) and sustained outward current (▲) are shown in the inset.

to be reversible only for a few cells in which it was exceptionally large at the very beginning. In addition to this transient current, ChTX in some cells also affected a small, sustained component of the outward current (Fig. 4*d,e*) that, in contrast to the transient calcium-activated potassium current, continuously increased with increasing depolarization. As its time course resembled the delayed rectifier-type current described below, this component of the ChTX-sensitive outward current may be due to an unspecific action of the toxin.

A-type current. Superfusing the cells with an external solution containing TTX, CdCl₂, and quinidine (200 nM, 50 μ M, and 100 μ M, respectively) revealed a very rapidly activating and inactivating outward current (Fig. 5). The time course and sensitivity to 4-aminopyridine (4-AP; Fig. 6) indicate this to be an A-type current (Rudy, 1988). The block is voltage independent since 4-AP reduced the current by the same percentage over a large voltage range. To reveal the dose-response relationship we applied activation pulse protocols before and in the presence of three different concentrations of 4-AP (0.2, 1, and 5 mM). The relative current persisting at a given concentration was expressed as the mean of the values determined for five consecutive voltage steps (to 10, 25, 40, 55, and 70 mV). The inset of Figure 6 shows that the block is half-maximal at 0.8 mM, and is nearly complete at 5 mM 4-AP.

Voltage activation curves were determined by measuring the peak current activated by a given voltage (Fig. 7*a,b*). From these values the conductance (g) was calculated, using the relationship

$$g = I/(E - V). \quad (1)$$

For E we used the potassium equilibrium potential (-85 mV)

Figure 3. Effect of 50 μ M cadmium on the outward current in the presence of 100 nM TTX. I - V curves for the peak outward current before (▲) and after (●) cadmium. ♦ indicate the I - V relation of the cadmium-sensitive current obtained after subtracting the traces obtained in the presence from those before cadmium. The inset shows the response to a step to $+45$ mV.

determined according to the Nernst equation, assuming the potassium concentration within the cell to be identical to that of the pipette filling solution. Even though the absolute size of the A-type current was different from cell to cell (Fig. 7*b*), the respective curves for the relationship between the fraction of conductance activated ($g/g_{\max}$) and voltage were almost identical. From the normalized g - V curves a mean was calculated and fitted (Fig. 7*c*) with a Boltzmann distribution of the form

$$g/g_{\max} = 1/\{1 + \exp[(V - V_{1/2})/S]\}, \quad (2)$$

where $V_{1/2}$ is the voltage at which half of the current is activated and S is a factor determining the slope of the curve. The resulting values were $+10.7$ mV ($V_{1/2}$) and -16.76 (S). Steady state inactivation curves were obtained by measuring the peak current in response to a test pulse ($+30$ mV) from different preconditioning voltages (Fig. 7*d,e*). The mean inactivation curve and its Boltzmann fit are shown in Figure 7*f*. The $V_{1/2}$ and S values resulting from the fit were -42.33 mV and 8.15, respectively. For a description of the kinetics of the current we measured time to peak for the activation, and examined the decay of the current by fitting a single exponential to the falling phase at each voltage. The time to peak ranged from 5–23 msec near threshold to about 2 msec at $+40$ mV and above. The first-order time constant for the inactivation was 1–4 msec above $+40$ mV.

Delayed-rectifier current. Elimination of the A-type current by depolarizing prepulses to -25 mV uncovered a third type of outward current (Fig. 8) that slowly activates with depolarizing pulses above -30 mV. It was not affected by 10 mM 4-AP, a concentration at which the A-type current was blocked completely. The effect of externally applied tetraethylammonium (TEA) was somewhat variable: in some cells the current was reduced only slightly by 20 mM TEA, and could not be completely blocked even at a concentration of 100 mM; in other cases 20 mM TEA reduced the current to one-fourth or one-fifth of the original size. In general, the sensitivity to TEA was

Figure 4. Effect of charybdotoxin (ChTX) on outward currents. The insets show the I - V curves for the peak outward current. The cell was held at -65 mV, and after a 100 msec hyperpolarizing prepulse to -95 mV it was depolarized for 250 msec to command potentials between -80 and 100 mV, in 15 mV increments. *a*, Before application of ChTX the peak outward current has a maximum at $+55$ mV. *b*, In the presence of ChTX the peak outward current becomes apparently more transient and its I - V curve loses the typical N-shape. *c*, The effect is partially reversed after a 2 min wash. *d* and *e*, The time course of the ChTX-sensitive current obtained by subtracting *b* from *a* (*d*), and *b* from *c* (*e*).

related to the size of the current: the larger the current before application, the stronger the TEA effect. On the basis of its relatively slow activation, and because it showed only little inactivation even with depolarizations lasting as long as 2 sec, we have classified it as a delayed rectifier-type current. Since we observed some variability in the activation speed and inactivation of the current, we believe that there are at least two subtypes of channels with different activation and inactivation kinetics underlying the total current. However, as we were not able to separate these components of the current any further, we have not analyzed its kinetics in detail. Like its counterparts in other insect preparations (Singh and Wu, 1990; Hardie, 1991), it is sensitive to quinidine, being completely abolished with 100 μ M

(Fig. 5). In contrast to the A-type current, which persisted without change in recording sessions of over 30 min, the delayed rectifier-type current showed massive rundown already within the first 2 min after establishing the whole-cell configuration. Whether this reflects the existence of another subtype of delayed rectifier-type channel, which may require intracellular conditions that cannot be maintained during whole-cell recording, remains to be seen.

Inward currents

For an analysis of the inward current, outward currents were blocked by substituting KCl with TEA-Cl and KF with CsF in the pipette solution. Under these conditions the remaining whole-

Figure 5. Effect of quinidine on outward currents. Same pulse protocol as in Figure 4. Quinidine significantly reduces a sustained outward current component and leaves the very rapidly inactivating outward current largely unaffected. Inward currents and calcium-activated outward currents were blocked with 100 nM TTX and 50 μ M cadmium.

Figure 6. 4-Aminopyridine sensitivity of the A-type current. The current was elicited by a step to +40 mV following a hyperpolarizing prepulse to -95 mV from a holding potential of -65 mV. It was recorded in the presence of TTX, Cd, and quinidine to block all other currents. The concentrations of 4-AP tested were 0.2, 1, and 5 mM. The inset shows the dose-response relation obtained from three different cells.

Figure 8. Delayed rectifier-type outward current and its partial block by TEA. The current was recorded in the presence of 100 nM TTX and 50 μ M Cd. The A-type current was totally inactivated by a 200 msec depolarizing prepulse to -25 mV. Test pulses to -50, -35, -20, -5, and +10 mV. *a* and *b* show the same traces with different sweep speed. *c*, In this example 20 mM TEA reduced the current to 40% of its original size. Time scale: *a*, 8 msec; *b* and *c*, 50 msec.

Figure 7. Characterization of the A-type current. The current was isolated by application of 100 nM TTX, 50 μ M Cd, and 50 μ M quinidine. *a-c*, Activation of the A-type current. Same pulse protocol as in Figure 4. *d-f*, Steady state inactivation determined by applying preconditioning voltage pulses between -115 and +5 mV that preceded a test pulse to +25 mV. For further description, see Results.

Figure 9. Total inward current. The pipette was filled with CsCl and TEA to block potassium currents. The inward current has at least two components: one activating and inactivating rapidly, and one that decays more slowly. The *inset* shows the I - V curves for the peak (□) and the late (○) inward current.

cell current was mainly inward and showed a transient and a more slowly inactivating component (Fig. 9), the latter of which is normally masked by the massive outward currents. Both components are first activated by pulses to -40 mV and peak near -10 mV. They can be separated pharmacologically and in ion substitution experiments.

Sodium currents. Since we expected some of the inward current to be carried by calcium, we blocked calcium currents with $50 \mu\text{M}$ cadmium to study sodium currents in isolation. Under these conditions only a transient inward current remained, which was completely blocked with 50 – 100 nM TTX (see Fig. 11*b*) and disappeared in Na^+ -free external solution, indicating that this component of the inward current was indeed a sodium current. Of all the currents examined, this current was the only one for which we noticed an obvious correlation between the size of the current and the time the cells had spent *in vitro* after dissociation: during the first 18 hr the current could not be detected at all and thereafter seemingly increased with the time the cells had been in culture. Since we restricted our investigations to cells that had not developed any processes, the appearance of the transient sodium current does not seem to require any morphological differentiation.

Figure 10*a–c* shows an example of this inward sodium current and the I - V relation for 14 measurements. The current is activated at command potentials more positive than -45 mV

Figure 10. Transient sodium currents recorded in the presence of $50 \mu\text{M}$ Cd to block calcium currents. *a–c*, To measure the activation of the current, cells were held at -70 mV and depolarized to command potentials between -60 and $+60$ mV, in 10 mV increments. *d–f*, Steady state inactivation determined by application of preconditioning voltage pulses between -95 and -30 mV, in 5 mV increments, followed by a test pulse to -10 mV. For further information, see Results.

Figure 11. Effect of 100 nM TTX on the inward current. Barium was substituted for calcium in the external solution. Both *a* and *b* show the response to a test pulse to -10 mV from a holding potential of -70 mV. *a*, Application of TTX blocks an inward current that has a transient and a persistent component (lower trace). *b*, After application of cadmium only a transient inward current, which is blocked by TTX (lower trace), is observed.

with a maximum around -10 mV. With more positive test pulses the current decreases as the potential approaches the sodium equilibrium potential ($+55$ mV). Since we did not routinely use leak subtraction protocols, the sodium current is opposed by leakage and incompletely compensated capacitive currents, both of which are outward at the command potentials applied. Therefore, the reversal potential of the current differed from cell to cell, and deviated from the sodium equilibrium potential, especially when the sodium current was relatively small. In a few cases we did apply on-line leak subtraction using a *P/N* protocol, resulting in *I-V* curves that reversed very close to the sodium equilibrium. Neither activation threshold nor voltage at which the current was maximal differed from the values given above. Steady state inactivation was examined by applying 1 sec prepulses to voltages between -95 and -30 mV in 5 mV increments (Fig. 10*d,e*). From a set of nine normalized inactivation curves ($I/I_{\max}$), the mean was calculated and fitted (Fig. 10*f*) with a Boltzmann distribution (Eq. 2) giving a $V_{1/2}$ of -53 mV and a slope factor *S* of 5.4.

The time course of the sodium current was characterized by measuring time to peak (t_p) and inactivation rate. For the average of 14 cells measured, t_p decreased from 2.8 ± 0.3 at -40 mV to 0.3 ± 0.02 at $+30$ mV. The inactivation time constant (t_h) was determined from the fit of a single exponential to the falling phase of the sodium current. It decreased with potential from 3.7 ± 0.4 at -40 mV to 0.3 ± 0.05 at $+30$ mV.

Without Cd in the bath, application of TTX not only abolished the transient sodium current, but also eliminated a sustained component of the inward current (Fig. 11*a*). Superfusing the cells with an Na^+ -free solution had the same effect, indicating the existence of a persistent sodium current in addition to the transient sodium current described above. Since we never

Figure 12. Barium currents recorded in the presence of 100 nM TTX to block sodium currents. Cells were held at -70 mV and depolarized to command potentials between 0 and $+60$ mV, in 10 mV increments. *b*, *I-V* curves for the peak barium current obtained from 20 different cells. *c*, Mean barium current activation curve ($\pm$ SEM).

Figure 13. Effect of calcium channel blockers on the barium current. The same cell was consecutively exposed to verapamil (100 μ M; *a*), cadmium (10 μ M; *b*), and nifedipine (100 μ M; *c*) with a 5 min wash between the applications. Upper traces show the response to a depolarizing pulse to -10 mV (holding potential -70 mV) before and after application of the drug. Lower traces were obtained after subtraction, and depict the time course of the verapamil-, cadmium-, and nifedipine-sensitive current, respectively.

observed a persistent component of the inward current in 50 μ M Cd (Fig. 11*b*), this persistent sodium current could either be Cd sensitive or require calcium influx into the cell for its activation.

Calcium current. In an external solution containing 200 nM TTX, an inward current was observed that activates and inactivates more slowly than the transient sodium current. It was completely blocked by 50 μ M cadmium (Fig. 11*b*) and also disappeared when calcium in the external solution was removed or replaced by cobalt (not shown). We therefore regard this current to be a calcium current.

With calcium as a charge carrier, the current rapidly decreased to one-half to one-fifth of its original size within a few minutes. When calcium was substituted by barium this rundown was much less pronounced, suggesting that the calcium current may be inactivated or regulated in a calcium-dependent manner. However, this process must be fairly slow since the inactivation kinetics, as judged from the 50 msec pulses, were not obviously altered after switching to a barium-containing external solution. Replacing calcium with barium also resulted in an instantaneous increase of the current without a change in the reversal potential, indicating that the calcium channels underlying the whole-cell current are more permeable to barium than to calcium.

Figure 12 gives an example of the barium current and shows the I - V relations for the peak current derived from 20 different cells from a holding potential of -70 mV. An average ($\pm$ SEM) of all 20 measurements is shown in Figure 12*c*. The current is first activated at -40 mV and peaks between -10 and 0 mV. Changing the holding potential to more negative values increased the current significantly. The barium current was maximal at -100 mV, reduced to three-fourths to two-thirds at -70 mV, and was fully inactivated at -30 mV. Lowering the holding potential to -100 mV did not uncover a second type of calcium (or barium) current. It simply increased the size of the overall current without affecting its kinetics or the shape of the I - V relation.

For a further pharmacological characterization we studied the effects of organic calcium channel blockers verapamil (a phenylalkylamine) and nifedipine (a dihydropyridine), both of which, in the 20 nM to 50 μ M range, are thought to select for the L-type calcium channel in vertebrates (Tsien et al., 1988; Hille, 1992). At 100 μ M, verapamil significantly reduced (by 50–90%) the barium current in every cell tested ($n = 14$; see Fig. 13*a* for an example). The effect of 100 μ M nifedipine (Fig. 13*c*) was less pronounced (up to 50%); in several cases no effect was observed at all. Since we did not try any concentrations other than 100 μ M we do not know whether this is due to a difference in the dose-response relationships of the two compounds. A compar-

ison of the I - V curves of the nifedipine-sensitive current with that of the current that persisted in nifedipine did not reveal any difference with respect to the voltage at which the current activated, and where the current had its maximum. Furthermore, multiplying the current persisting in nifedipine with a variable factor would give a current with a time course that was more or less identical to that of the current before the application of the drug. Thus, it seems unlikely that there are two different calcium currents, one of which is blocked by nifedipine and one of which is not.

Discussion

Recordings were performed on Kenyon cell somata isolated from the brain of honeybee pupae that had nearly completed adult development. Using the same dissection and culturing protocol, Kreissl (1992) showed that by injection of depolarizing current the cells can be induced to spike. As the pupal cells do not noticeably differ from adult Kenyon cells in their composition of ionic currents (unpublished observations), we believe that the complement of ion channels expressed in Kenyon cells is complete at the end of the pupal stage. The inward and outward currents observed are similar to those described in other insect neuronal (Byerly and Leung, 1988; Solc and Aldrich, 1988; Baker and Salkoff, 1990; Saito and Wu, 1991) or muscle preparations (Gho and Mallart, 1986; Zagotta et al., 1988; Singh and Wu, 1989, 1990). In contrast to the former, which included a variety of different cell types dissociated from entire brains or ganglia, the preparation introduced here allows the selective study of a distinct neuronal population. Investigations were restricted to cells that had not grown any processes during their short-term culture lasting up to 2 d. Even though the pupal Kenyon cell somata have roughly the same diameter and whole-cell capacitance as *Drosophila* embryonic neurons (Byerly and Leung, 1988; O'Dowd and Aldrich, 1988), their currents are about one order of magnitude larger. This may be because we studied a very special neuronal type, or, more likely, because embryonic neurons express fewer ionic channels than pupal neurons. With the Kenyon cell preparation this question may be addressed, because it allows investigation of the expression of ionic currents in this particular neuronal population not only in the late pupa, but also at earlier stages of postembryonic development, and in the adult.

In *Drosophila* muscle two types of calcium-activated potassium currents have been described: a fast transient current (I_{CF} or I_{ACD}) and a slow noninactivating current (I_{CS} or I_C) (Salkoff, 1983; Elkins et al., 1986; Gho and Mallart, 1986; Singh and Wu, 1989). Neither one appeared to be affected by apamin, a bee venom polypeptide (Gho and Mallart, 1986) that has been

shown to block selectively a special type of calcium-activated potassium channel (see Cook and Quast, 1990, for review). I_{CF} has been reported to be blocked by charybdotoxin, a peptide from scorpion venom (Elkins et al., 1986). Because of its time course and its sensitivity to charybdotoxin, the transient calcium-activated current described here closely resembles I_{CF} .

The sustained component of the ChTX-sensitive current seen in Figure 4 may be due to an unspecific block of some other outward current. In contrast to the transient ChTX-sensitive current, the I - V relation of which has an N-shape characteristic for calcium-dependent currents, the I - V curve for the sustained component is more or less linear. In some cells ChTX has been reported not to be perfectly selective, affecting not only calcium-activated potassium channels but also delayed rectifier currents (Cook and Quast, 1990). This may also be the case in our preparation.

In addition, Kenyon cells may possess a slow, noninactivating calcium-activated potassium current: in many cells the I - V relation for the persistent component of the outward current also had a characteristic N-shape (Fig. 1). However, this component of the sustained outward current, and hence the N-shape of its I - V curve, usually disappeared within a few minutes after establishing the whole-cell configuration (see, e.g., Fig. 4). Therefore, we were not able to characterize this current further. Recently, Wegener et al. (1992) described a calcium-dependent potassium channel in antennal receptor neurons of the locust that is inhibited by millimolar concentrations of intracellular ATP and is blocked neither by apamin nor by charybdotoxin. As our pipette solution contained 3 mM ATP, inhibition by ATP of a calcium-activated potassium current would explain the rundown of a sustained outward current with N-shaped I - V relation. So far, we have not performed any experiments without ATP in the pipette solution in order to see whether under such conditions this particular component of the outward current would be more stable.

In an immunological study Schwarz et al. (1990) showed that *Drosophila* mushroom bodies express high levels of *Shaker*-encoded potassium channels. Thus, it was not surprising for us to find a prominent A-type current in honeybee Kenyon cells that resembles the A-current of *Drosophila* muscle encoded by the *Shaker* potassium channel gene, with respect to both its kinetic parameters and its voltage operating range (Salkoff and Wyman, 1983; Wu and Haughland, 1985; Zagotta and Aldrich, 1990). This is not the case in most other insect neuronal preparations in which the operating range of this current appears to be shifted by about 30 mV negative compared to that in *Drosophila* muscle (Solc and Aldrich, 1988; Hardie and Weckström, 1990; Hardie, 1991; Hardie et al., 1991; Saito and Wu, 1991). It remains to be seen whether the A-type current found in Kenyon cells differs with respect to its voltage operating range from that of other neurons in the bee brain.

In pupal *Drosophila* neurons Baker and Salkoff (1990) reported a considerable variation in the inactivation kinetics and voltage dependence of the A-type current, and suggested that in some cells two populations of channels coexist. The shape of some of the inactivation curves (e.g., solid diamonds in Fig. 7e) is indeed suggestive of the presence of a second type of A-type current with a more negative voltage operating range. Further experiments are necessary to substantiate this impression.

The second type of voltage-activated outward current encountered in our preparation is characterized by a slow activation, only little inactivation during 250 msec depolarizing

pulses, and sensitivity to TEA. It is very similar to the delayed rectifier current found in other insect neurons (Byerly and Leung, 1988; Solc and Aldrich, 1988; Hardie and Weckström, 1990; Hardie, 1991; Saito and Wu, 1991). The variability of its activation rate and the degree of inactivation observed during 250 msec test pulses suggest that this current is composed of at least two kinetically different conductances. Several potassium channels have been described that may contribute to sustained outward currents in insect neuronal and muscle preparations (Solc and Aldrich, 1988; Zagotta et al., 1988; Gorczyca and Wu, 1991; Hardie, 1991). In the absence of single-channel data it is difficult to decide which and how many types of channels underlie the delayed rectifier-type current in Kenyon cells. As the current showed only little inactivation during 2 sec voltage steps, a noninactivating potassium channel (K_1 or K_O), in addition to the slowly inactivating K_D , must significantly contribute to this current.

The most striking feature of the delayed rectifier-type current was its susceptibility to washout, which occurred within a few minutes after establishing the whole-cell configuration. The fraction of the current that persisted beyond this time was usually stable for the rest of the recording session. This observation, and the variability in the sensitivity of the current to TEA, could be explained with the presence of two subtypes of delayed rectifier channels, one of which is more sensitive to TEA and is readily "washed out," the other being only weakly sensitive to TEA, but resistant to washout. We have recently started to apply the perforated-patch technique to our cells using Amphotericin B as an ionophore. Under these conditions the delayed rectifier-type current was stable for a much longer period. Further experiments must now be performed to characterize the current in greater detail.

The transient sodium current described here is virtually indistinguishable, in terms of voltage operating range and kinetic parameters, from that of *Drosophila* embryonic neurons (O'Dowd and Aldrich, 1988). Since we studied only cells without any apparent processes, the source of the current is obviously the soma membrane. The fact that the sodium current does not arise in neurites (which might have been very thin and therefore overlooked) can also be seen in the current records: we never observed a significant delay between the onset of the test pulse and rising phase of the sodium current, which should occur, if there was improper voltage control over processes.

It is unlikely that Kenyon cell somata generate sodium-driven action potentials *in vivo*, as is the case for the majority of insect nerve cell bodies. We observed no sodium currents immediately after dissociation and up to 18 hr thereafter. The same occurs with isolated adult DUM neurons, where the development of the transient sodium current, following axotomy and deafferentation, can be abolished by protein synthesis inhibitors (Tribut et al., 1991). Immunocytochemical studies indicate that sodium channels in insect neurons are primarily located in axon membranes, but restricted to the cytoplasm in cell bodies (French et al., 1993). Our observations suggest that isolated Kenyon cells retain their ability to synthesize sodium channels, which are, however, incorporated into the soma membrane in the absence of an axon. In contrast to the transient sodium current, all other currents described here did not change with time after dissociation. We thus conclude that the channels underlying these currents are also present and functional in Kenyon cell somata *in vivo*.

Persistent, TTX-sensitive sodium currents have been found

in cockroach (Christensen et al., 1988; Lapied et al., 1989) and *Drosophila* (Saito and Wu, 1991) neurons. A similar current also seems to be present in Kenyon cells. However, we were not able to study this current in detail, since it could not be effectively separated from the other inward currents. The persistent sodium current was absent when the bath contained micromolar concentrations of cadmium to block the calcium current; substituting calcium by cobalt had the same effect. Isolating both sodium currents together by the use of a calcium-free external solution was also impracticable, as recordings were instable under these conditions. Therefore, we do not know whether the persistent sodium current differs with respect to activation threshold and steady state inactivation from the transient sodium current, as is the case in *Drosophila* neurons (Saito and Wu, 1991).

Calcium currents have been described in *Drosophila* embryonic neurons (Byerly and Leung, 1988; Leung and Byerly, 1991; Saito and Wu, 1991), *Manduca* larval and pupal motoneurons (Hayashi and Levine, 1992), and locust adult (Pearson et al., 1993) and embryonic interneurons (Laurent et al., 1993). In *Drosophila*, calcium currents with different inactivation kinetics have been observed (Byerly and Leung, 1988; Saito and Wu, 1991), which were differentially affected by two spider toxins, indicating the existence of two different types of neuronal calcium currents (Leung et al., 1989). In contrast, the waveform of the calcium current described here was rather consistent from cell to cell, presumably because we recorded only from Kenyon cells, and not from a variety of different neuronal types. Laurent et al. (1993) made a similar observation studying calcium currents in nonspiking interneurons from the locust.

Substituting calcium with barium had two effects. First, it increased the current without changing the reversal potential, indicating that the underlying calcium channels, like those present in adult locust neurons (Pearson et al., 1993), are more permeable to barium than to calcium. Second, it very much slowed the rundown of the current, which would normally result in a loss of the calcium current within 5–10 min, possibly because of a calcium-dependent inactivation or regulation of the current. Barium, however, could not reverse rundown once it had occurred. Unlike *Drosophila* embryonic neurons (Saito and Wu, 1991), barium did not change the waveform of the current, indicating that the inactivation observed during 50 msec depolarizing pulses is most likely not calcium dependent, and that the calcium-dependent regulation of the calcium current leading to rundown must be a rather slow process.

Organic calcium channel blockers, such as verapamil and nifedipine, which block certain types of vertebrate calcium channels, have been shown to have little effect on insect calcium currents (Byerly and Leung, 1988; Hayashi and Levine, 1992). Thus, it was not surprising to find that, even at a concentration as high as 100 μM , both verapamil and nifedipine reduced the calcium current to some extent, but would not abolish it completely. Pearson et al. (1993) reported that 1 μM verapamil reduced the calcium current in adult locust neurons by 85%. This was about the maximal effect of verapamil seen in our preparation with 100 μM . However, we did not test any concentrations lower than that. At the same concentration, nifedipine, a dihydropyridine that is also thought to select for L-type channels in vertebrates (Tsien et al., 1988), was even less efficient. The strongest blocking effect was seen with cadmium, which completely abolished the calcium current at 50 μM and significantly reduced it at concentrations well below 10 μM . The calcium

current seems to undergo steady state inactivation at fairly negative holding potentials, being 100% available at -100 mV and below, about 70% available at -70 mV, and fully inactivated at -30 mV and above. As is the case with all other insect preparations studied so far, the calcium current first activates at -40 mV and peaks between -10 and 0 mV. Given that the partial block by 100 μM verapamil and nifedipine may be a nonspecific effect, the calcium current described here has many characteristics in common with the vertebrate "N-type" calcium current, such as sensitivity to very low concentrations of cadmium, activation at relatively high voltages, and steady state inactivation at rather negative holding potentials.

It is yet unknown whether and how the above described currents are modulated, and how such a modulation may be related to neural plasticity. Indications of such have, however, emerged from studies on the *Drosophila* learning and memory mutants *dunce* (*dnc*) and *rutabaga* (*rut*), both of which are characterized by abnormal levels in intracellular cAMP (Byers et al., 1981; Livingstone et al., 1984). A clue as to how these altered cAMP levels might cause deficient learning and memory was provided by an electrophysiological study (Zhong and Wu, 1991) which showed that in contrast to the wild type, *dnc* larval neuromuscular junctions do not exhibit facilitation and posttetanic potentiation. As Delgado et al. (1992) demonstrated, this kind of synaptic plasticity can be restored by application of potassium channel blockers. *Drosophila* larval muscle fibers express a cAMP-activated potassium channel, which is persistently activated in *dnc* (Delgado et al., 1991). In the larval muscle fibers of *dnc*, both A-current and delayed rectifier are increased, possibly as a result of elevated cAMP levels (Zhong and Wu, 1993).

Obviously, the same mechanisms underlying lack of synaptic plasticity of the *dnc* neuromuscular junction may also be relevant in the CNS. In the brain of *Drosophila*, both *dnc* and *rut* gene products are preferentially expressed in the intrinsic elements of the mushroom bodies, the Kenyon cells (Nighorn et al., 1991; Han et al., 1992). In the fly, however, the role of the cAMP pathway in these cells has so far not been investigated. Due to their small size (≤ 5 μm) they appear to be inaccessible to electrophysiological studies *in vivo*. Furthermore, because of anatomical constraints, a preparation of the fly mushroom bodies and hence an isolation of their intrinsic elements does not seem feasible. The honeybee preparation described here allows selective study of this cell type, not only with electrophysiological but also with biochemical and other techniques. A potential mechanism underlying olfactory learning and memory in insects may be a change in Kenyon cell excitability through a cAMP-mediated modulation of ion channels. Most of the currents described here have been shown to be modulated by cAMP in other preparations (Rudy, 1988; Tsien et al., 1988). The effect of cAMP on ion channels may be direct, or involve the activity of a cAMP-dependent kinase (PKA), an enzyme that is also found in the bee brain (Altfelder and Müller, 1991). In a biochemical assay, dopamine, noradrenaline, and octopamine have been shown to stimulate adenylyl cyclase activity in bee brain tissue, which is particularly high in the mushroom bodies (Menzel et al., 1991). With immunocytochemical techniques, dopamine- and octopamine-containing neurons were found, which project into the main input regions of the mushroom bodies, the calyces (Schäfer and Rehder, 1989; Kreissl et al., 1991). Most if not all of the octopamine immunoreactivity within the calyces could be attributed to a neuron, which in an electrophysiological study has been shown to mediate the reward in

olfactory learning (Hammer, 1993). Furthermore, there is good evidence that acetylcholine may be transmitter of a subset of neurons conveying olfactory information from the antennal lobes to the mushroom bodies (Kreissl and Bicker, 1989). Thus, future studies will be directed toward determining the action of transmitters and neuromodulators on Kenyon cell currents, and whether cAMP is involved as a second messenger.

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