Dopamine modulation of honey bee (*Apis mellifera*)

antennal-lobe neurons

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ABSTRACT

Primary olfactory centers (antennal lobes, ALs) of the honey bee brain are invaded by dopamine (DA)-immunoreactive neurons early in development (pupal stage 3), immediately prior to a period of rapid growth and compartmentalization of the AL neuropil. Here we examine the modulatory actions of DA on honey bee AL neurons during this period. Voltage-clamp recordings in whole-cell configuration were used to determine the effects of DA on ionic currents in AL neurons in vitro from pupal bees at stages 4 to 6 of the 9 stages of metamorphic adult development. In approximately 45 percent of the neurons tested, DA (5-50 × 10^{-5} M) reduced the amplitude of outward currents in the cells. In addition to a slowly-activating, sustained outward current, DA reduced the amplitude of a rapidly-activating, transient outward conductance in some cells. Both of the currents modulated by DA could be abolished by the removal of Ca^{2+} from the external medium, or by treatment of cells with charybdotoxin (2 × 10^{-8} M), a blocker of Ca^{2+}-dependent K^+ currents in the cells. Ca^{2+} currents were not affected by DA, nor were A-type K^+ currents (I_A). Results suggest that delayed rectifier-like current (I_{KV}) also remains intact in the presence of DA. Taken together, our data indicate that Ca^{2+}-dependent K^+ currents are targets of DA modulation in honey bee AL neurons. This study lends support to the hypothesis that DA plays a role in the developing brain of the bee.
INTRODUCTION

During metamorphosis, the central nervous system of the honey bee, *Apis mellifera*, undergoes dramatic growth and reorganization. Nowhere are the changes more striking than in the primary olfactory centers (antennal lobes, ALs) of the brain. Around pupal stage 2 of the 9 stages of metamorphic adult development, antennal sensory afferent neurons enter the ALs (Masson and Arnold 1984). Their arrival triggers the formation of prominent subunits of synaptic neuropil called glomeruli (Mobbs 1982; Pareto 1972; Suzuki 1975; see also, Boeckh and Tolbert 1993; Hansson and Anton 2000; Oland and Tolbert 1987, 1996; Rospars 1988; Salecker and Boeckh 1996), which are the functional subunits of the AL neuropil (Joerges et al. 1997; Sachse et al. 1999; Stopfer et al. 1997; see also Christensen and Hildebrand 1987; Hansson and Anton 2000). Each glomerulus contains the terminal arbors of antennal sensory afferent neurons, processes of local interneurons, dendrites of projection (output) neurons, and ramifications of centrifugal neurons that project to the ALs from other sites in the brain (Abel et al. 2001; Flanagan and Mercer 1989; Fonta et al. 1993; Homberg 1984; Müller et al. 2002; Sun et al. 1993).

Immediately prior to glomerulus formation (pupal stage 3), developing ALs are invaded by dopamine (DA)-immunoreactive processes that ramify extensively in the central neuropil of the lobes (Kirchhof et al. 1999). These processes originate from cell bodies located in the lateral deutocerebral soma rind, posterior to each AL (Kirchhof et al. 1999; Schäfer and Rehder 1989). The same cells extend processes into the dorsal
lobe of the deutocerebrum, as well as to the protocerebrum and suboesophageal ganglion
(Schäfer and Rehder 1989). Around pupal stage 4 there is a surge in DA levels in the
ALs, and rapid neurite outgrowth apparent in stage-5 AL neurons in vitro is enhanced
by exposure to DA (Kirchhof et al. 1999). While the identity of the receptors that
mediate the effects of DA has yet to be determined, mRNAs for 3 DA receptor genes,
\textit{Amdop1} (Blenau et al. 1998), \textit{Amdop2} (Humphries et al. 2003) and \textit{Amdop3}
(Beggs et al. 2005) have been detected in cells that surround the developing AL neuropil of the
bee (Beggs et al. 2005; Kurshan et al. 2003). The expression of these genes, in
particular \textit{Amdop2}, is strongly developmentally regulated (Kurshan et al. 2003),
suggesting that DA plays a central role in the developing brain of the bee (Kirchhof et

In adult worker bees, DA levels in the brain (Kokay and Mercer 1997;
Schulz and Robinson 1999; Taylor et al. 1992), and levels of dopamine receptor gene
expression (Humphries et al. 2003; Kokay and Mercer 1997) change markedly during
the lifetime of the bee. Intriguingly, regardless of age, DA levels in the antennal lobes of
foragers are higher than in the antennal lobes of bees performing nursing duties
suggesting that DA in antennal lobes is linked to behavioural state (Schulz and
Robinson 1999). While it has been suggested that biogenic amines such as DA might
influence response thresholds for task-related stimuli (Bicker and Menzel 1989;
Hammer 1993; Macmillan and Mercer 1987; Mercer and Menzel 1982; Scheiner et al.
2002; Schulz and Robinson 1999), relatively little is known about the mechanisms
through which this amine operates in the brain of the bee.
In this study, whole-cell voltage-clamp recordings are used to examine the modulatory actions of DA on ionic currents expressed by honey bee AL neurons in vitro. Our results reveal that Ca\(^{2+}\)-activated K\(^+\) currents are targets of DA modulation in these cells.
METHODS

Apis mellifera

Frames of honey bee brood collected from hives at the Department of Zoology, University of Otago were kept for periods of up to 1 week in a humidified incubator at 35 °C. Metamorphic adult development in the honeybee occurs over an 8 to 9 day period. Pupal honey bees at stages 4-6 (P4-P6) of the 9 stages of metamorphic adult development were collected from the brood frames. The stage of development was ascertained by using a method similar to that described by Jay (1962) based on external cues, such as eye colour and head pigmentation.

Cell cultures

Primary cell cultures were prepared from the antennal lobes (ALs) of pupal bees as described elsewhere (Kirchhof and Mercer 1997; Kreissl and Bicker 1992). The heads of 8 to 10 pupal bees were removed from their bodies, placed in a dish lined with sylgard (Dow Corning Corp., Midland, MI) and secured with insect mounting pins. The front of the head capsule and glandular tissue surrounding the brain were removed. The exposed brain was rinsed with modified Leibovitz L-15 culture medium (BL15, pH 7.2, 500 mOsm) supplemented with 4.0 g glucose, 2.5 g fructose 24.0 g sucrose and 3.3 g proline per liter (all purchased from Sigma, St. Louis, MO). ALs were removed with fine forceps and placed in fresh BL-15 medium. The remaining steps took place under sterile conditions. To aid dissociation of the tissue, ALs were exposed to a hyperosmotic culture medium (BL-15 supplemented with an additional 20.0 gl⁻¹ sucrose, 580 mOsm)
for 10 minutes and then transferred into a Ca\(^{2+}\) and Mg\(^{2+}\)-free honey bee ringer (in mM: NaCl 135, KCl 5, and Tris-HCl 114.5; pH 7.2, 460 mOsm) for the same period. The tissue was then rinsed briefly in culture medium (BL-15), excess fluid was removed and the ALs (6 per 250 µl) were dissociated by trituration. The dissociated cells were transferred in 100 µl aliquots to the center of an uncoated plastic culture dish (Falcon, 3001) and allowed to adhere to the substrate for 3-5 minutes. The dishes were then filled with 2 ml of culture medium (BL15) and placed in a humidified incubator at 28 ºC. Cells were maintained in culture for 4-5 days before use.

**Whole-cell recording**

Patch-clamp recordings in whole-cell configuration (Hamill et al. 1981) were used to examine dopamine (DA) modulation of ionic currents in honey bee AL neurons *in vitro*. The voltage-gated and Ca\(^{2+}\)-dependent currents examined in this study have been described in detail elsewhere (for descriptions in *Apis mellifera* see Grünewald 2003; Kloppenburg et al. 1999b; Pelz et al. 1999; Schäfer et al. 1994; for review see Wicher et al. 2001). These currents include a transient A-type K\(^+\) current (\(I_A\)), a sustained, delayed rectifier-like current (\(I_{KV}\)), Ca\(^{2+}\)-activated K\(^+\) currents (\(I_{KCa}\)), a Ca\(^{2+}\) current (\(I_{Ca}\)), and a rapidly-activating transient TTX-sensitive current carried by Na\(^+\) (\(I_{Na}\)). Cells were viewed under an IMT-2 microscope (Olympus) using phase-contrast optics. All experiments were conducted at room temperature. Recording electrodes (2-3 MΩ) were prepared from borosilicate glass (100-µl micropipettes, OD 1.71 mm, ID 1.32 mm, VWR Scientific, West Chester, CA) using a Flaming-Brown micropipette puller (P-87, Sutter Instrument Co, CA) and backfilled with a solution containing (in mM) K-aspartate 100, KF 40, KCl 20, MgCl\(_2\) 2.5, EGTA 1, sucrose 160, HEPES 10
(pH 7.2). Throughout the recording period cells were continuously superfused with artificial insect saline (AIS) containing (in mM) NaCl 130, KCl 6, MgCl₂ 4, CaCl₂ 5, sucrose 160, glucose 25, HEPES/NaOH 10 (pH 7.2, 500 mOsm). Junction potentials were nullified prior to seal formation. Recordings were made using an Axopatch 1D amplifier in conjunction with a CV4 1/100 headstage and Digidata 1200 interface (Axon Instruments, Union City, CA). Data were acquired using pClamp6 software (Axon Instruments, Union City, CA) run on a 486 PC computer. Cells were clamped at a holding potential of −70 mV and depolarizing voltage steps were used to activate voltage-gated channels in the cells. Membrane currents were filtered at 2 kHz using a low-pass 4-pole Bessel filter and sampled at intervals of 100 µs. In most recordings, a P/4 protocol (see Armstrong and Bezanilla 1974) was used for digital subtraction of linear leak currents and capacitance artifacts. No compensation was made for series resistance and therefore voltage errors may be present where currents measured were large. However, series resistance errors should not affect the central conclusions of this study.

Isolation of currents

Whole-cell current profiles were examined initially in the absence of any blocking agents. Components of the whole-cell current profile were then isolated using routine pharmacological techniques described elsewhere (for Apis neurons see Grünewald 2003; Kloppenburg et al. 1999b; Pelz et al. 1999; Schäfer et al. 1994; Wüstenberg et al. 2004). Na⁺ currents were blocked with tetrodotoxin (TTX, 10⁻⁷ M), whereas Ca²⁺ currents (and Ca²⁺-activated K⁺ currents) were blocked with 5 × 10⁻⁵ M CdCl₂. Rapidly-activating, transient (A-type) current (Iₐ) was blocked with 4-
aminopyridine (4-AP, $5 \times 10^{-3}$ M), quinidine ($5 \times 10^{-5}$ M) was used to block the delayed-rectifier-like current, $I_{KV}$, and $K^+$ currents collectively were blocked by substituting $K^+$ in the electrode solution with Cs$^+$. To reveal the contribution that $Ca^{2+}$-dependent $K^+$ currents ($I_{KCa}$) make to outward current profiles in *Apis* AL neurons, cells were exposed to $Ca^{2+}$-free saline in which $CaCl_2$ had been replaced with $MgCl_2$. Effects of the $I_{KCa}$ blocker charybdotoxin (CTX, $2 \times 10^{-8}$ M; reviewed by Garcia et al., 1995) were also tested in the cells. All chemicals were purchased from Sigma with the exception of TTX and CTX, both of which were purchased from Alomone labs, Israel.

**Dopamine application**

DA (dopamine hydrochloride, Sigma) was prepared in AIS immediately before use and pressure-ejected across the cell soma using a Picospritzer II (General Valve Corp., Fairfield, N. J.). DA was used at concentrations of between $5-50 \times 10^{-5}$ M (pipette concentration). At these concentrations, effects of DA on DA-sensitive cells were immediately obvious and the chances of observing at least some recovery during DA washout were maximized. DA was applied in 20 ms pulses delivered immediately prior to each depolarising voltage step. Continuous superfusion of the recording chamber with fresh AIS prevented the localized accumulation of DA around cells between individual voltage steps. The delivery pipette was then removed from the recording chamber and the cells were superfused with DA-free AIS to facilitate recovery from any DA effect. As a result of $Ca^{2+}$-current rundown in the cells, total outward current amplitudes decreased progressively over time. For this reason, effects of DA were examined over a 10-minute recording period and compared to time-dependent changes in current amplitudes occurring in control (untreated) cells.
Statistical analysis

Repeated-measures ANOVA was performed to determine the overall effect of DA application. This accounted for any serial correlation between data recorded from the same cell over time. In groups of cells recorded in the absence of blockers, and in cell groups recorded in the presence of TTX and 4-AP, small numbers of missing values at the +8 minute time point were estimated using multiple regression techniques, where the values of control or DA-treated groups recorded over the initial 6 minute recording period were used to predict missing 8-minute data points. Where significant overall, or group, effects were detected by ANOVA, Student’s t-tests for independent samples were performed post hoc to determine at which specific time points the current amplitudes of DA-treated and untreated cell groups were significantly different. Student’s t-tests were also used to assess statistical differences in time-to-peak data. Significance was accepted at p=0.05 except when multiple comparisons were made, where the level of significance was reduced according to Bonferroni’s correction. Mean values are expressed as mean ± standard error of the mean (mean ± S.E.). All analyses were performed using SPSS 11.0 (SPSS inc., Chicago, IL.).

RESULTS

Current profiles in *Apis* AL neurons

Based on the composition of outward currents, AL neurons examined in this study (n=51) could be grouped into 2 main categories (Fig. 1). In 71% of cells (n=36) the outward current profile exhibited a rapidly-activating transient component followed
by a sustained component that showed little or no inactivation during the voltage step (Fig. 1Ai). With voltage steps above approximately -30 mV, the amplitude of the sustained outward currents in these cells increased linearly with each voltage step (Fig 1Aii). Cells exhibiting this form of current profile are referred to here as Type 1 cells. The remaining 29% of cells (Type 2 cells, n=15) exhibited outward currents in which the rapidly-activating transient current seen in Type 1 cells appeared to be absent and the sustained component showed significant inactivation during the voltage step (Fig. 1Bi). In contrast to the outward current recorded in Type 1 cells, the I/V curve for outward current in Type 2 cells had a non-linear relationship at potentials above +40 mV (Fig. 1Bii). The current profiles in Type 2 cells are similar to those reported for AL projection (output) neurons (Grünewald 2003). We assume, therefore, that Type 1 current profiles originate from cells belonging to the second major category of AL neurons, namely local AL interneurons, but this has yet to be confirmed. Here, attention is focused predominantly on cells exhibiting Type 1 current profiles, although small numbers of Type 2 cells were found to be responsive to DA (see below).

**Effects of DA on outward current profiles**

Effects of DA on ionic currents were examined initially in the absence of any channel blockers. Changes in the amplitude of outward currents in cells exposed to DA (DA-Treated; n=13) were compared to those observed in cells receiving no DA treatment (Untreated, n=18). In the majority of DA-treated cells (54 %), DA had no effect on the amplitude of currents contributing to the whole-cell current profile. In 46 % of the DA-treated cells, however, exposure to DA caused a pronounced reduction in the amplitude of outward currents in the cells (Fig. 2Ai-iii). Among the DA-sensitive
cells there were 4 Type 1 cells and 2 Type 2 cells. Washing in DA-free saline partially reversed the effects of DA (see Figs. 2A-C), but current rundown over time generally worked against the recovery of current amplitudes to levels recorded prior to DA application. Comparing the current profiles of control (untreated) cells and cells treated with DA revealed significant treatment-related differences between these two groups (Fig. 2D; ANOVA, F=5.38, p=0.028). Immediately after DA treatment, the mean amplitude of outward currents in DA-treated cells (measured 105 ms after the onset of a voltage step from –70 mV to +50 mV) was significantly lower than in untreated cells (Student’s t-test, t=3.07, p=0.009, significance at p<0.013 after Bonferroni’s correction for 4 pair wise comparisons). After 2-6 minutes washing in DA-free saline, however, differences between the 2 groups of cells were no longer statistically significant.

Identification of outward currents modulated by DA

A-type K⁺ currents (Iₐ). Outward currents modulated by DA were examined more closely in Type 1 cells. Type 1 cells express a prominent, rapidly-activating, transient A-type current, Iₐ (Fig. 3A). To assess the effects of DA on the amplitude of this current, Iₐ was isolated and identified in 11 Type 1 cells. A series of 10 mV voltage steps from –100 mV to +90 mV were used to compare peak Iₐ recorded prior to DA application with peak current amplitudes recorded in the presence of DA (5 × 10⁻⁵ M (n=3) or 5 × 10⁻⁴ M (n=8), data pooled for analysis; Figs. 3B,C). Iₐ amplitude remained stable over time and was not affected by treatment with DA (Fig. 3C; ANOVA, F=0.82, p=0.451).

K⁺ currents other than Iₐ. Effects of DA on Type 1 cells were examined further using cells in which the transient A-type current, Iₐ, was blocked by 4-AP (5 ×
10^{-3}$ M; Fig. 4). Of the 9 cells examined under these conditions, 5 showed no response to DA ($5 \times 10^{-5}$ M). In the 4 remaining cells, DA caused an immediate reduction in the amplitude of outward currents in the cells (Fig. 4Ai,ii). The responses of DA-sensitive cells were found to be significantly different from those of cells that were unresponsive to this amine (ANOVA, $F=13.41$, $p<0.008$). Immediately following DA application, outward current amplitudes in DA-sensitive cells were significantly lower than in cells in which no DA response was observed (Student’s t-test, $t=5.70$, $p=0.006$, significance at $p<0.017$ after Bonferroni’s correction for 3 pair wise comparisons; Fig. 4B). However, 2 to 6 minutes after DA application, and with continuous rinsing in DA-free saline, current amplitudes in these 2 groups of cells were no longer significantly different (Fig. 4B).

The subtraction of currents recorded in DA-responsive cells immediately after DA treatment (Fig. 4Aii) from currents recorded in the same cells prior to DA application (Fig. 4Ai) revealed that DA modulated 2 currents: (i) a rapidly-activating transient outward current and (ii) a slowly-activating current that increased in amplitude throughout the 120 ms voltage step (Fig. 4Aiii). To assist with the identification of these currents, their activation properties (times to peak) were compared with those of the A-type current, $I_A$ (see Fig. 3A) and the delayed rectifier-like current, $I_{KV}$ (see Fig. 5Aii). Using depolarising voltage steps from $-70$ mV to $+20$ mV, the transient component of the DA-sensitive current reached peak levels in $2.28 \pm 0.45$ ms ($n=4$), significantly faster than the time-to-peak $I_A$ ($3.82 \pm 0.14$ ms, $n=34$) measured under the same conditions (Student’s t-test, $t=-3.30$, $p=0.046$). The relatively slowly-activating sustained current, $I_{KV}$, reached peak levels (also using voltage steps from $-70$ mV to
+20 mV) in 87.30 ± 10.30 ms (n=7), distinguishing \( I_{KV} \) also from the currents modulated by DA. As the kinetics of \( I_A \) and \( I_{KV} \) suggest that they are not the targets of DA modulation, we examined the possibility that \( Ca^{2+} \)-activated K\(^+\) currents in \textit{Type 1} AL neurons might be modulated by this amine.

**\( Ca^{2+} \)-dependent currents in \textit{Type 1} AL neurons**

\( Ca^{2+} \)-dependent currents in \textit{Type 1} neurons were identified using \( Ca^{2+} \)-free saline (Fig. 5). The amplitude of outward currents in the cells was dramatically reduced under these conditions (Fig. 5Ai,ii). Subtracting currents recorded in \( Ca^{2+} \)-free saline (Fig. 5Aii) from those recorded in the same cells under normal AIS (Fig. 5Ai) revealed that exposure to \( Ca^{2+} \)-free saline abolished a rapidly-activating transient component as well as a sustained outward current in \textit{Type 1} cells (Fig. 5Aiii). Analysis of the \( Ca^{2+} \)-sensitive currents (Fig. 5Aiii) suggested that the 2 components share a similar activation threshold (around −20 mV; Fig. 5C). To explore the possibility that these \( Ca^{2+} \)-dependent K\(^+\) currents may be targets of DA modulation, we compared the modulatory actions of DA (5 × 10\(^{-5}\) M) with those of CTX (2 × 10\(^{-8}\) M; Fig. 6), a potent \( I_{KCa} \) channel blocker (García et al. 1995). When bath applied for 2-4 minutes immediately after a baseline recording, the effects of CTX (Fig. 6Ai,ii) were strikingly similar to the effects of DA (Fig. 6Bi,ii). Like DA (Fig. 6Biii), CTX blocked a rapidly-activating transient outward current in \textit{Type 1} AL neurons (\( I_{KCa \text{ transient}} \)) with a time to peak current (using a depolarising voltage step from −70 mV to +20 mV) of 2.76 ± 0.16 ms (n=14), as well as a slowly-activating, sustained current (\( I_{KCa \text{ sustained}} \)) that increased in amplitude throughout the 120 ms voltage step (Fig. 6Aiii). Exposing cells to CTX at a concentration of 2 × 10\(^{-8}\) M had a greater impact on the amplitude of these currents than
5 × 10⁻⁵ M DA, suggesting that CTX is a more potent inhibitor of calcium-dependent K⁺ channels in these cells than DA. Nonetheless, the kinetics of the currents blocked by CTX closely resembled those of the DA-sensitive currents described above. I/V curves generated from the CTX- and DA-sensitive currents (Fig. 6Aiii, Biii, respectively) show that CTX-sensitive currents, like those modulated by DA, exhibit an activation threshold around –20 mV (Fig. 6Aiv, Biv). The I/V curves reveal also that transient currents blocked by DA and CTX exhibit a more strongly non-linear I/V relationship than sustained currents (see Figs. 6Aiv, Biv), suggesting that I_KCa transient and I_KCa sustained differ in their relative Ca²⁺ and voltage sensitivities.

To obtain additional evidence that DA modulates I_KCa, we examined the effects of DA (5 × 10⁻⁵ M) on cells exposed to CTX (2 × 10⁻⁸ M) prior to DA application (n=10). Cells treated with CTX alone served as controls (n=8). Cells were exposed to CTX from the moment they were placed in the recording chamber. Interestingly, the longer exposure time (ca. 10 minutes in total) produced effects on outward current amplitude similar to those of Ca²⁺-free saline (compare Figs. 5Aii, 6Ci). Brief pulses of DA applied to CTX-treated cells had no effect on the amplitude of outward currents in the cells (Fig. 6Cii). Measurements of current amplitude taken 105 ms after the onset of a voltage step from -70 mV to +50 mV (Fig. 6Ciii) show very little run-down in current amplitude over time in CTX-treated cells and no significant difference between control cells treated with CTX alone and cells treated with CTX plus DA (ANOVA, F=0.19, p=0.668). Taken together, these results suggest that Ca²⁺-dependent K⁺ currents in Type 1 AL neurons are the targets of DA modulation. One
alternative possibility, however, is that these currents may be blocked indirectly through DA modulation of Ca\textsuperscript{2+} currents in the cells.

*DA modulation of I_{Ca}.* To explore the possibility that DA affects Ca\textsuperscript{2+}-dependent K\textsuperscript{+} currents indirectly through modulation of I_{Ca}, we examined the effects of DA on isolated Ca\textsuperscript{2+} currents in Type 1 cells (Fig. 7). Eight cells were exposed to DA (5-15 × 10\textsuperscript{-5} M, pooled for statistical analysis) and 10 (untreated) cells served as controls. The Ca\textsuperscript{2+} currents in these cells reached maximum amplitude within 30 ms of the onset of a depolarising voltage step and then declined over the remainder of the voltage pulse (Fig. 7A). Whether or not cells were exposed to DA, I_{Ca} activated between –50 mV and –40 mV, peaked around -10 mV and reversed at approximately +45 mV (Fig. 7B). In all cells, there was a decrease in I_{Ca} amplitude over time (Fig. 7C). However, there was no significant difference in normalized I_{Ca} amplitudes between DA-treated cells and controls (ANOVA, F=0.68, p=0.797).
DISCUSSION

This study is the first to reveal the modulatory actions of DA on ionic currents in honey bee AL neurons. Our results show that a significant percentage of AL neurons are sensitive to DA, and that DA reduces the amplitude of Ca\(^{2+}\)-activated K\(^+\) currents in these cells. We show, in addition, that in Type 1 cells, I\(_{\text{Ca}}\), I\(_{\Lambda}\), and most probably also I\(_{\text{KV}}\), remain intact in the presence of this amine.

While the identity of cells responsive to DA has yet to be clearly established, our results suggest that Type 1 cells are likely to represent a subpopulation of local AL interneurons (LNs). In the ALs of the bee there are significantly more LNs than projection (output) neurons (PNs). Estimates of around 4000 LNs per lobe (Witthöft 1967) and 800 PNs (Bicker et al. 1993) have been reported. That a majority of the cells in vitro exhibited Type 1 current profiles suggests, therefore, that Type 1 cells are more likely to be LNs than PNs. Examination of the electrophysiological properties of the cells supports this view. Outward current profiles exhibited by Type 1 cells are markedly different from those reported for PNs (Grünewald 2003), which instead resemble current profiles observed in this study in cells of Type 2.

Type 1 AL neurons, in contrast to Type 2 cells (and PNs, Grünewald 2003), express a prominent A-type current. Our results show clearly that I\(_{\Lambda}\) remains intact in the presence of DA, and the kinetics of the DA-sensitive currents identified in Type 1 cells suggest indirectly that the delayed rectifier-like current, I\(_{\text{KV}}\), is also unaffected by the presence of this amine. In contrast, I\(_{\text{KCa transient}}\) and I\(_{\text{KCa sustained}}\) both appear to be
direct targets of DA modulation. The kinetic properties of the 2 DA-sensitive currents closely resemble those of $I_{KCa}^{transient}$ and $I_{KCa}^{sustained}$, and effects of DA on Type 1 cells are mimicked by the $I_{KCa}$ blocker, CTX. That no detectable DA modulation was observed in cells in which $I_{KCa}$ had been blocked with CTX prior to DA application, provides strong support for the conclusion that DA modulates $Ca^{2+}$-dependent $K^+$ currents in these neurons.

$Ca^{2+}$-dependent $K^+$ currents have been described in many insect species (Grolleau and Lapied 1995; Mercer and Hildebrand 2002b; Schäfer et al. 1994; Thomas 1984; Torkkeli and French 1995; Wegener et al. 1992; Zufall et al. 1991). Transient and sustained components of $I_{KCa}$ have been reported, for example, in cockroach dorsal unpaired median neurons and, as in Type 1 cells, both currents are blocked by CTX (Grolleau and Lapied 1995). The gene encoding the sustained $I_{KCa}$ channel in cockroach neurons has been identified (Derst et al. 2003) and in cells heterologously expressing this channel, sustained $I_{KCa}$ has an activation rate similar to that of the DA-sensitive sustained current observed in honey bee AL neurons.

Reports describing the outcome of $I_{KCa}$ modulation in other invertebrate systems provide clues as to likely effects of DA modulation of $Ca^{2+}$-activated $K^+$ currents on the excitability of Apis AL neurons. In molluscan central pattern generator neurons, serotonin-induced attenuation of spike after-hyperpolarization, attributed to a reduction in $I_{KCa}$ amplitude, promotes repetitive spiking in these neurons (Katz and Frost 1997). Applying DA to isolated pyloric dilator neurons in the lobster stomatogastric ganglion on the other hand, increases the amplitude of total $I_{KCa}$ contributing to an increase in interspike interval and a reduction in action potential frequency in these
neurons (Kloppenburg et al. 1999c). These results, together with reports in other systems (e.g. Baxter and Byrne 1989, 1999; Buchholtz et al. 1992), suggest that DA-induced attenuation of $I_{KCa}$ will increase the excitability of honey bee AL neurons.

In developing nervous systems, levels of electrical activity have a significant impact on neuronal growth and differentiation (e.g. Baines et al. 2001; Duch and Levine 2000, 2002; Gu and Spitzer 1980; Kater et al. 1988; Kater and Mills 1991; Schilling et al. 1991; Spitzer et al. 1995, 2002) and also on activity-dependent tuning of neuronal connections (e.g. Katz and Shatz 1996; Ruthazer and Stryker 1996; Shatz 1994; Sherrard and Bower 1998). DA-induced changes in cell excitability, therefore, could have developmental relevance in ALs of the bee. The early appearance of DA-immunoreactive processes in honey bee ALs (Kirchhof et al. 1999), evidence that DA-receptor genes in the bee brain are strongly developmentally regulated (Kurshan et al. 2003), and the identification in this study of DA-sensitive currents in developing AL neurons, all suggest a developmental role for DA, in addition to its functions in the adult brain. In ALs of the sphinx moth, Manduca sexta, modulatory actions of serotonin on developing AL neurons (Mercer et al. 1995; 1996a,b; Mercer and Hildebrand 2002a; Oland et al. 1995) strongly foreshadow the actions of this amine in the brain of the adult moth (Kloppenburg et al. 1999a; Kloppenburg and Heinbockel 2000; Kloppenburg and Hildebrand 1995).

In adult honey bees, olfactory information is encoded by spiking patterns in AL projection neurons (Abel et al. 2001; Müller et al. 2002; Stopfer et al. 1997) and patterns of activity across the glomerular array (Galizia et al. 1999; Sachse et al. 1999). Both are likely to be affected by DA-induced changes in the activity of local AL
interneurons and/or projection neurons. DA has the potential, therefore, to influence the
detection and discrimination of odors, and perhaps also olfactory learning and the
formation of odotopic memories. An early investigation of DA function in honey bee
ALs showed that DA applied to the lobes reduces the percentage of animals that
respond to a conditioned olfactory stimulus (Macmillan and Mercer 1987). One possible
target of DA modulation is the subpopulation of LNs that contain the inhibitory
neurotransmitter, γ-aminobutyric acid (GABA). Approximately 750 of the 4000 LNs in
the honey bee ALs are GABA immunoreactive (Schäfer and Bicker 1986; Witthöft
1967) and there is compelling evidence that these neurons play a role in AL functions
such as odor discrimination (Stopfer et al. 1997; Sachse and Galizia 2002). However,
DA may not target GABAergic pathways alone. In the vertebrate olfactory bulb, for
example, DA not only regulates GABA-ergic inhibitory processing (Brüning et al. 1999;
Davison et al. 2004; Duchamp-Viret et al. 1997), but also controls sensory input to the
olfactory bulb via a presynaptic action on olfactory nerve terminals (Berkowicz and
Trombly 2000; Ennis et al. 2001; Hsia et al. 1999; see also Duchamp-Viret et al. 1997).
DA is likely to act at multiple sites also in primary olfactory centers of the honey bee
brain. At least 2 DA receptor genes are expressed by deutocerebral neurons that
surround the ALs (Kurshan et al. 2003; see also Kokay et al. 1998) and in the present
investigation not only Type 1 cells, but also a small number of Type 2 AL neurons
responded to this amine.

While the mechanisms through which DA operates in the ALs have yet to
be fully resolved, the results of this study provide an important step towards this goal.
The aim of future studies will be to determine the functional consequences of DA-
modulation of Ca\textsuperscript{2+}-activated K\textsuperscript{+} currents, both in the developing ALs and in AL neurons of the adult worker bee.
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REFERENCES


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**FIGURE LEGENDS**

**FIG. 1.** *Ai* Whole-cell currents typical of *Type 1* AL neurons. Voltage was stepped from a holding potential of −70 mV to +70 mV in 10-mV increments. The current profile is dominated by large outward currents that include a rapidly-activating transient component and a sustained component that shows little or no inactivation during the voltage pulse. *Aii* I/V relationship measured 105 ms after voltage pulse onset (* in *Ai*). *Bi* Whole-cell currents typical of *Type 2* cells. Voltage was stepped from a holding potential of −70 mV to +90 mV in 10-mV increments. The current profile is dominated by large slowly-activating outward currents that show significant inactivation during the voltage pulse. *Bii* I/V relationship measured 105 ms after voltage pulse onset (* in *Bi*).

**FIG. 2.** Effect of DA on whole-cell currents recorded from a *Type 1* AL neuron. Voltage was stepped from a holding potential of −70 mV to +50 mV in 10-mV increments. *Ai* Currents recorded prior to DA application. *Aii* Currents recorded from the same cell in the presence of DA (5 × 10^−5 M). DA was applied in 20 ms pulses immediately prior to each depolarizing voltage pulse. *Aiii* Currents recorded after rinsing the neuron for 2 min in DA-free AIS. *B* I/V relationship measured at ◆ in *Ai*, * in *Aii* and ○ in *Aiii*, 10 ms after the onset of each depolarizing voltage pulse. *C* I/V relationship of profiles at ■ in *Ai*, ● in *Aii* and ▲ in *Aiii*, measured 105 ms after the onset of each depolarizing voltage pulse. Effects of DA were partially, but not fully reversible. *D* The effects of DA on mean outward current amplitudes. Voltage steps from a holding potential of −70 mV to +50 mV were used to compare the mean amplitude (± S. E.) of outward currents recorded in control (untreated) cells (n=18) and in cells exposed to 5 × 10^−5 M DA (DA-treated, n=13) over time. Measurements were taken 105 ms after the onset of each depolarising voltage pulse. Current amplitudes for each cell were normalized to the initial value recorded at time zero (0 min) and expressed as a fraction of that value. In cells receiving DA treatment, DA was applied 2 minutes after the first reading (arrow). Immediately after DA treatment, cells were washed in DA-free saline. Current amplitudes in DA-treated cells are compared with those recorded in control (untreated) cells. The
significance of changes in current amplitude within each group was tested using repeated measures ANOVA. Differences between groups at equivalent time points were tested for post hoc using independent samples Student’s t-tests. Bonferroni’s correction was made for multiple t-tests (n=4) and significance was accepted at p=0.013. NS = not significant.

FIG. 3. \( I_A \) recorded in Type 1 AL neurons. \( I_A \) was isolated using TTX (10\(^{-7}\) M) to block \( I_{Na} \), quinidine (5 \times 10^{-5} M) to block \( I_{KV} \) and \( Cd^{2+} \) (5 \times 10^{-5} M) to block Ca\(^{2+}\)-activated K\(^+\) currents in the cells (see Methods). A. Current traces showing effects of DA on \( I_A \). The holding potential was −70 mV. After prepulses to −100 mV (1 s), the voltage was stepped from −100 mV to +90 mV in 10-mV increments. \( Ai \) \( I_A \) prior to DA application. \( Aii \) \( I_A \) recorded in the presence of 5 \times 10^{-5} M DA. \( Aiii \) \( I_A \) recorded after rinsing the neuron in DA-free saline for 2 minutes. B I/V relationship of peak \( I_A \) amplitude in \( Ai-Aiii \). C. Effect of DA (5-50 \times 10^{-5} M) on mean peak \( I_A \) amplitude (± S.E., n=11) during a voltage step from −100 mV to +90 mV. Data from cells were normalized to their initial (0 minute) value and are expressed as a fraction of that value. The significance of changes in current amplitude within each group was tested using repeated measures ANOVA. There was no significant change in \( I_A \) amplitude over time, or as a result of DA application (p>0.05).

FIG. 4. Effects of DA on non-A-type outward currents in Type1 AL neurons. TTX (10\(^{-7}\) M) was used to block \( I_{Na} \), and 4-AP (5 \times 10^{-3} M) was used to block \( I_A \) (see Methods). The voltage was stepped from a holding potential of −70 mV to +70 mV in 10-mV increments. \( Ai \). Outward currents elicited prior to DA application. \( Aii \). Outward currents recorded from the same cell in the presence of DA (5 \times 10^{-5} M). \( Aiii \). DA-sensitive currents revealed by subtracting currents recorded in the presence of DA (\( Aii \)) from those recorded prior to DA application (\( Ai \)). B. Comparison of changes in mean current amplitude (± S.E.) over time in cells that were responsive to DA (○) versus cells that showed no response to this amine (‘unresponsive’ ■). Measurements of current amplitude were taken 105 ms after the onset of each depolarizing voltage pulse. Voltage steps from −70 mV to +50 mV were used for this purpose. The significance of changes in current amplitude within each group was tested using repeated measures ANOVA. Differences between groups at equivalent time points were tested for post hoc using independent
samples Student’s t-tests. Bonferroni’s correction was made for multiple t-tests (n=3) and significance was accepted at p=0.017. NS = not significant.

**FIG. 5.** Identification of Ca\(^{2+}\)-dependent currents in Type 1 AL neurons using Ca\(^{2+}\)-free saline. TTX (10\(^{-7}\) M) was used to block I\(_{Na}\), and 4-AP (5 \(\times\) 10\(^{-3}\) M) was used to block I\(_{A}\). Voltage steps were applied in 10-mV increments from a holding potential of –70 mV to +70 mV. *Ai.* Outward currents recorded in normal saline (AIS, see Methods). *Aii.* Outward currents recorded in the same cell after 4 minutes exposure to modified AIS in which Ca\(^{2+}\) had been substituted with Mg\(^{2+}\). *Aiii.* Ca\(^{2+}\)-sensitive currents obtained by subtracting the traces shown in *Aii* from the traces shown in *Ai.* *B.* I/V relationships of currents at ■ in *Ai* and ○ in *Aii*, 115 ms after the onset of each voltage step. There is a marked reduction in the amplitude of outward currents in Ca\(^{2+}\)-free saline. *C.* I/V relationship of the Ca\(^{2+}\)-sensitive outward current measured at peak amplitude (● in *Aiii*), and 115 ms after the onset of each voltage step (∆ in *Aiii*).

**FIG. 6.** Comparison of the effects of charybdotoxin and DA on non A-type outward currents in Type 1 AL neurons. TTX (10\(^{-7}\) M) was used to block I\(_{Na}\), and 4-AP (5 \(\times\) 10\(^{-3}\) M) was used to block I\(_{A}\). Voltage steps were applied in 10-mV increments from a holding potential of –70 mV to +60 mV. *A.* Effects of CTX (CTX, 2 \(\times\) 10\(^{-8}\) M). *Ai.* Outward currents recorded prior to CTX exposure. *Aii.* Outward currents recorded in the same cell after 4 minutes of exposure to CTX. *Aiii.* CTX-sensitive currents revealed by subtracting traces shown in *Aii* from the traces shown in *Ai.* CTX blocks a rapidly-activating transient current as well as a slowly-activating sustained current. *Aiv.* I/V relationships of CTX-sensitive currents. Peak transient current was measured at □ in *Aiii*. The sustained component was measured 105 ms after the onset of the voltage step (● in *Aiii*). *B.* Effects of DA (5 \(\times\) 10\(^{-5}\) M). *Bi.* Outward currents prior to DA application. *Bii.* Outward currents recorded in the same cell in the presence of DA. *Biii.* DA-sensitive currents revealed by subtracting traces shown in *Bii* from the traces shown in *Bi.* *Biv.* I/V relationships of DA-sensitive currents. Peak transient current was measured at □ in *Biii*. The sustained component was measured 105 ms after the onset of the voltage step (● in *Biii*). The effects of DA are strikingly similar to those of CTX. *C.* Effects of DA on outward
currents recorded in cells exposed to CTX (2 \times 10^{-8} \text{ M}) for ca.10 minutes. \textit{Ci.} Outward currents recorded in CTX prior to DA application. \textit{Cii.} Outward currents recorded in CTX plus DA (5 \times 10^{-5} \text{ M}). \textit{Ciii.} Voltage steps from a holding potential of \(-70 \text{ mV}\) to +50 mV were used to compare the mean amplitude (\pm S. E.) of outward currents recorded in control (untreated) cells (n=8) and in cells exposed to 5 \times 10^{-5} \text{ M} DA (DA-treated, n=10) over time. Measurements were taken 105 ms after the onset of each depolarising voltage pulse. Current amplitudes for each cell were normalized to the initial value recorded at time zero (0 min) and expressed as a fraction of that value. In cells receiving DA treatment, DA was applied 2 minutes after the first recording (arrow). Immediately after DA treatment, cells were washed in DA-free saline. Current amplitudes in DA-treated cells are compared with those recorded in control (untreated) cells. The significance of changes in current amplitude within each group was tested using repeated measures ANOVA. There was no significant change in outward current amplitude as a result of DA application (p>0.05).

\textbf{FIG. 7.} \textit{Ca}^{2+} \textit{currents in Type 1 AL neurons.} \textit{I}_{\text{Ca}} \text{ was recorded in the presence of TTX (10^{-7} \text{ M}), 4-AP (5 \times 10^{-3} \text{ M}) and quinidine (5 \times 10^{-5} \text{ M}). In addition, Cs}^{+} \text{ replaced K}^{+} \text{ in the recording electrode (see Methods). Voltage steps were applied in 10-mV increments from a holding potential of \(-70 \text{ mV}\) to +50 mV. A} \textit{I}_{\text{Ca}} \text{ recorded in a Type 1 AL neuron. B.} \textit{Representative} \textit{I}_{\text{Ca}} \text{ activation curves recorded 2 minutes after recording was initiated in an untreated (control) cell and a DA-treated cell. The curves are superimposed to show that DA had no apparent effect on} \textit{I}_{\text{Ca}} \text{ activation. C.} \textit{Changes in peak} \textit{I}_{\text{Ca}} \text{ amplitude (mean \pm S.E.) recorded over time in control (untreated) cells (n=10) and in cells (n=8) treated at the 2-minute time point (arrow) with DA (5 \times 10^{-5} \text{ M}). The significance of changes in current amplitude within each group was tested using repeated measures ANOVA. There was no significant change in outward current amplitude as a result of DA application (p>0.05).}
Figure 3

Ai. Control

Aii. DA

Aiii. Wash

B. Plot of current (nA) against command potential (mV)

C. Plot of normalized current against time (min)