J. exp. Biol. ***189****, 37–54 (1994)*
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MODULATION OF IONIC CURRENTS BY DOPAMINE IN AN INTERNEURONE OF THE RESPIRATORY CENTRAL PATTERN GENERATOR OF *LYMNAEA STAGNALIS*

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Accepted 30 November 1993

Summary

Dopamine elicits alternating bursts of activity in the respiratory interneurones of the snail *Lymnaea stagnalis*. One of the neurones (VD4) was isolated in culture, and the effects of dopamine on both membrane voltage and current were studied utilising the whole-cell tight-seal recording technique. Dopamine had little effect on resting potentials near −60 mV, nor did it affect spike threshold or input resistance measured near −60 mV. However, it did alter the excitability of the cell, changing the response to current injection from one of repetitive spiking to one of rapid accommodation. Under voltage-clamp, VD4 responded to dopamine (EC_{50}=92 nmol l^{-1}) with increased net outward current at all potentials more positive than −60 mV. This was due primarily to an increase in voltage-gated potassium current and a decrease in calcium current. A reduction of Cd^{2+}-sensitive outward current, possibly calcium-gated potassium current, was also evident at potentials more positive than +60 mV. The physiological actions of dopamine on these cells *in vivo* are consistent with the inhibitory mechanisms presented in this study.

Introduction

Many rhythmic behaviour patterns are controlled by networks of neurones known as central pattern generators (CPGs). The emergent properties of these networks depend upon the synaptic connections between, and the intrinsic membrane properties of, member neurones (Kristan, 1980; Selverston, 1985). An example of a well-characterized CPG is the network of central neurones that controls respiration in the pond snail *Lymnaea stagnalis* (Janse *et al.* 1985; Syed and Winlow, 1991; Syed *et al.* 1991). Respiratory behaviour is controlled primarily by three identified interneurones: the giant dopamine cell (right pedal dorsal 1, RPeD1) and two respiratory interneurones, input 3 interneurone (IP3I) and visceral dorsal 4 (VD4). The interneurones VD4 and IP3I make mutually inhibitory connections and VD4 controls inspiration, while IP3I controls expiration (Syed and Winlow, 1991). The giant dopamine cell, responsible for the initiation and maintenance of respiration, elicits alternating bursts of activity in the two

Key words: ion channel modulation, dopamine, inhibition, snail, *Lymnaea stagnalis.*

interneurones (IP3I and VD4) by releasing dopamine (Syed *et al.* 1990). The network interactions between these neurones have been well characterized *in vivo* and have been found to be appropriate, sufficient and necessary to control respiratory rhythmogenesis (Syed and Winlow, 1991; Syed *et al.* 1992). The CPG has also been reconstructed *in vitro* by co-culturing the three component neurones (Syed *et al.* 1990). Since RPeD1 and exogenously applied dopamine were found to be sufficient to initiate patterned activity in the cultured network, we sought to determine the ionic mechanism of dopamine's actions.

Here we demonstrate that dopamine exerts an inhibitory action on VD4 by modulating, in opposing fashion, two primary ionic currents: calcium current (I_{Ca}) is decreased and voltage-dependent potassium current [$I_{K(V)}$] is increased. The role of dopaminergic inhibition of excitability in neurone VD4 of *Lymnaea stagnalis* is discussed. These cells offer the benefits of being both identifiable and isolatable and of having a clearly defined role in CPG activity (Syed *et al.* 1990). Some of these results have been presented previously in abstract form (Barnes *et al.* 1991).

Materials and methods

Isolation of cells

Identification and isolation of cell VD4 from *Lymnaea stagnalis* were performed as previously described (Ridgway *et al.* 1991) with the following modifications. Regarding enzyme treatment, ganglia were treated with 1.5 mg ml^{-1} collagenase/dispase (Boehringer Mannheim) and 0.5 mg ml^{-1} trypsin (Sigma type III) for 30–40 min in defined medium (modified Liebowitz-15), and then with 2 mg ml^{-1} trypsin soybean inhibitor (Sigma) for 10 min, also in defined medium. The cells were plated in 35 mm plastic culture dishes (Corning 25000) at a density of one cell per dish and maintained for 2–24 h at room temperature in defined (rather than conditioned) medium to prevent neurite outgrowth.

Solutions

For recording, the bathing medium was changed to normal saline containing (in mmol l^{-1}): 51.3 NaCl, 1.7 KCl, 1.5 $MgCl_2$, 4.1 $CaCl_2$ and 5 Hepes (pH 7.9). Where specified in the text and figure legends, the following ionic substitutions and additions were made to the bathing solutions: (1) 0.1 mmol l^{-1} Ca^{2+}, 18.9 mmol l^{-1} Mg^{2+}, (2) 51 mmol l^{-1} choline, 0 mmol l^{-1} Na^+, (3) 20 mmol l^{-1} tetraethylammonium (TEA^+), (4) 5 or 10 mmol l^{-1} 4-aminopyridine (4-AP), and (5) 0.1, 1 or 2 mmol l^{-1} Cd^{2+}. Patch pipettes contained either (in mmol l^{-1}) 50 KCl or 50 CsCl, each with 5 EGTA, 5 $MgCl_2$ and 5 Hepes, pH 7.4. A 10 mmol l^{-1} stock of dopamine (3-hydroxytyramine; Sigma) was prepared before each recording in 1 % sodium metabisulphite and then diluted to its final concentration. All recordings were made at room temperature (21–24 ˚C).

Whole-cell recording and analysis

Whole-cell pipettes were pulled from glass haematocrit tubes (Western Scientific, Richmond, BC) in two steps on a vertical pipette puller (Kopf model 730, Tujunga, CA). When filled with the CsCl pipette solution, they had resistances of 2–10 MΩ measured in

the normal bath. The bath reference electrode consisted of an agar bridge filled with bath solution with a chlorided silver wire immersed in the normal bath solution in the bridge holder. The isolated cells were observed under an inverted microscope (Nikon Diaphot) and held near −60 mV under whole-cell voltage-clamp with an Axopatch 1-C or 200 (Axon Instruments). Series resistance compensation was made at the 50–100 % level together with capacitance current subtraction at the time of recording. Current recordings were usually filtered at 1 kHz (−3 dB, four-pole low-pass Bessel), digitized and stored at 2 kHz (12-bit resolution) with a BASIC-Fastlab System (Indec Systems, Sunnyvale, CA) incorporating an 80 kHz LabMaster A/D board with opto-isolation and a 386 computer. In some recordings, several successive data points were averaged when the response changed slowly with time in order to reduce storage requirements. In the illustrations, points at the peak of saturating capacitance current transients have been removed from some recordings.

Pipette and bath electrode junction potentials were measured against 3 mol l^{-1} KCl-filled electrodes of high resistance (200 MΩ). Pipette potentials relative to the bath were about −2 mV for the CsCl pipette/NaCl bath combination and about −1 mV for the KCl pipette/NaCl bath combination. Reported voltages are uncorrected for these errors.

Once the whole-cell patch-clamp configuration had been achieved by rupturing the patch of membrane, two major alterations of whole-cell current occurred, depending on which pipette solution was being used. Blocking of outward currents as CsCl from the pipette solution diffused into the cell was complete within about 10 min of breakthrough. Rundown of calcium currents, with a concomitant reduction of calcium-dependent outward currents, occurred within about 15 min. After this, the magnitude of calcium current was fairly stable for the duration of the recordings, usually 30–60 min. Calcium currents did not fully run down within the duration of a recording, which lasted a maximum of 145 min.

Results

Dopamine modifies the spike discharge pattern evoked by current injection

The response of neurone VD4 to current injection was modified by dopamine. As illustrated in Fig. 1A, prior to dopamine application, small current injections (200–600 pA) caused this cell to spike repetitively with moderate accommodation. This result was typical of the five cells tested. Spike threshold was near −32 mV. The rate of firing was between 4 and 6 Hz over this current injection range, but larger current injections (700–900 pA) caused rapid accommodation, typically producing only a single action potential followed by membrane potential oscillations.

Application of 10 μmol l^{-1} dopamine (Fig. 1B) hyperpolarized the cell by 4 mV, from a resting potential of −58 mV to −62 mV. The threshold for action potential generation, in the region of −29 mV, was little changed, but twice as much current was required to reach this value (400 pA, rather than 200 pA). With larger current injections, the cell fired only one spike, even though the current injection was maintained for 1 s. Membrane potential oscillations followed the single spike in response to the largest current injections (800 and 900 pA).

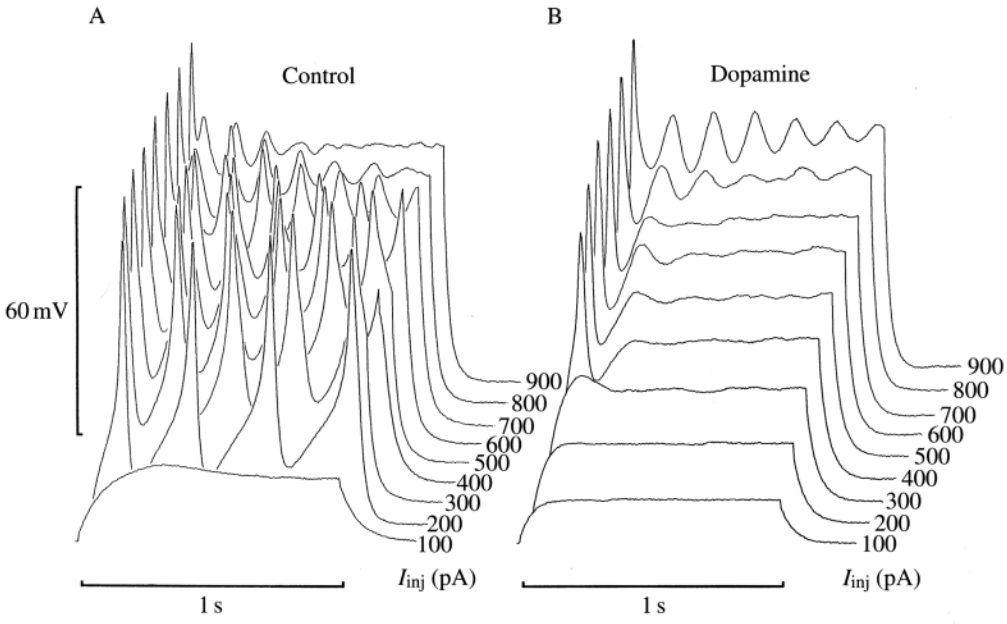

Fig. 1. Voltage responses of cell VD4 to depolarizing current injections in absence of (A) and presence of (B) 10 μmol l^{-1} dopamine. In each case, current injections (I_{inj}) in multiples of 100 pA were made ranging from 100 to 900 pA. The traces are offset vertically and horizontally, by the same amounts in A and B, for clarity.

Dopamine modulates whole-cell current under voltage-clamp

We found evidence that at least four ionic currents have roles in the membrane potential behaviour described above. These include a calcium current, I_{Ca}, a voltage-gated potassium current, $I_{K(V)}$, a transient potassium current, I_A, and a transient sodium current, I_{Na}. All are typical of the currents found in a variety of molluscan cells (Adams *et al.* 1980). Each was seen in response to the voltage-clamp paradigm of Fig. 2 and each is described below in subsequent sections of this paper.

The VD4 cell in Fig. 2, which is representative of 40 cells, was held at −60 mV and membrane potential was stepped first by −20 mV to −80 mV and then by increments of +20 mV to potentials between −40 mV and +80 mV. The current traces labelled control, measured before dopamine application, provide an overview of membrane currents present in these cells. Control current responses to 20 mV steps made to potentials more positive and negative than −60 mV showed little time-dependence and yielded a slope conductance in this voltage range of 1.3 nS (760 MΩ). The step to −20 mV revealed a small inactivating outward current and was similar to I_A in terms of its activation and inactivation kinetics (described later). A fast transient inward current was evoked as the membrane potential was stepped to 0 mV. This current is attributable to Na^+ channel activity since it was abolished when choline was substituted for sodium in the bath (described later). This current was, however, poorly blocked even by high concentrations of tetrodotoxin (TTX) (44 % reduction with 10 μmol l^{-1} TTX, average of three cells). Less obvious at this potential was a sustained inward current, shown below to be calcium-

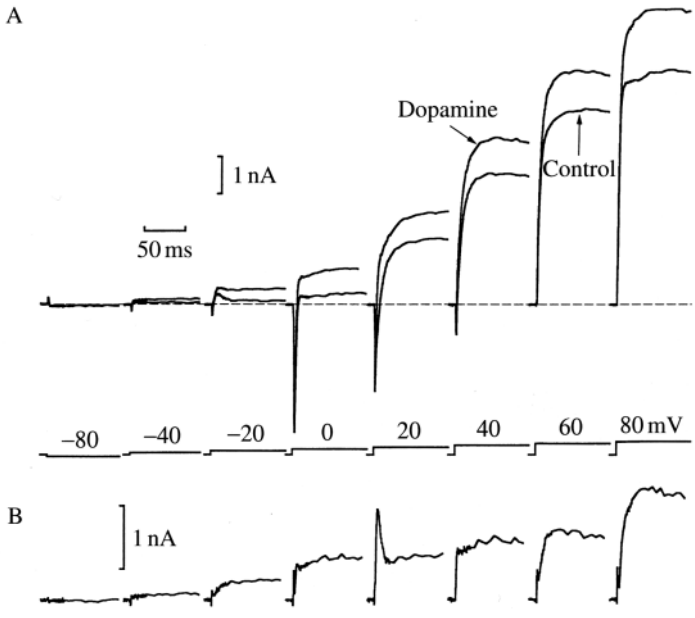

Fig. 2. Voltage-clamp response of an isolated VD4 neurone to 10 μmol l^{-1} dopamine. (A) Whole-cell currents elicited by the voltage-clamp steps were composed of at least four components, many of which are apparent in the control recordings (the lower of the two recordings at each voltage) and each of which is described by text and figures in subsequent experiments. Dopamine increased net outward current at all potentials more positive than −60 mV (the upper of the two recordings at each potential). (B) Digital subtractions of the recordings made with and without dopamine at each potential. No change in current magnitude was seen in response to dopamine at −80 mV. The cell was held at −60 mV.

dependent, and an outward current carried by potassium. The step to +20 mV (before application of dopamine) also began with a transient inward current, although the current trajectory decayed more slowly when compared with the 0 mV step. In response to this step to +20 mV and to those at +40 mV, +60 mV and +80 mV, more sustained outward current developed.

Dopamine, tested at a concentration of 10 μmol l^{-1} in 35 cells, altered the properties of ionic currents only at potentials more positive than −60 mV. In the presence of dopamine, the slope conductance measured between −80 and −60 mV remained at 1.3 nS, but with each greater depolarizing voltage step, an increase in net outward current was seen. As shown later, dopamine evoked an increase in outward current as well as a decrease in inward current. These current changes are especially clear in the digitally subtracted current traces shown in Fig. 2B. Note that, at a membrane potential of +20 mV, dopamine reduced transient and sustained inward calcium current in addition to increasing sustained outward current (described in more detail below). Dopamine exerted its actions over a range of concentrations with an average EC_{50} value of 92 nmol l^{-1} (Fig. 3). The maximal increase in outward current occurred near 10 μmol l^{-1}, and applications of concentrations higher than 100 μmol l^{-1} (maximum 1 mmol l^{-1} tested) produced smaller effects.

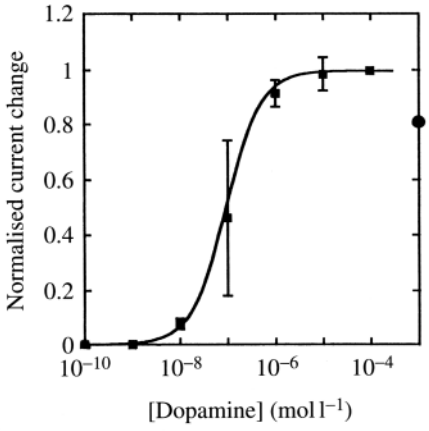

Fig. 3. Sensitivity of cell VD4 to dopamine. Responses were obtained from three cells, normalised to the response at 100 μmol l^{-1} dopamine, with means shown as squares and standard deviations indicated by the bars. The smooth curve is the average of the dose–response relationships determined for each cell and has an EC_{50} value of 92 nmol l^{-1}. The responses were measured as steady-state increases in outward current at +40 mV in response to different concentrations of dopamine between 100 pmol l^{-1} and 1 mmol l^{-1}. Application of 1 mmol l^{-1} dopamine evoked a smaller increase in outward current than did lower concentrations (shown as a filled circle, measured in one cell, but measured in two other cells from which full dose–response curves were not obtained).

Inward current reduction is not due to Na^+ channel modulation

We investigated whether dopamine acted on the transient sodium current in cell VD4 (27 cells). The presence of the transient sodium current was demonstrated by replacing sodium with choline (Fig. 4A,B). We then isolated the transient sodium current by blocking K^+ channels with 20 mmol l^{-1} TEA^+ and Ca^{2+} channels with 2 mmol l^{-1} Cd^{2+}. When these channels were blocked, application of dopamine had no effect on the sodium current (Fig. 4C). This result suggests that the steady-state increase of net outward current induced by dopamine did not involve these Na^+ channels.

Ca^{2+} current is reduced by dopamine

To examine Ca^{2+} currents and their possible modulation by dopamine, we reduced outward currents by dialysing cells with Cs^+. In seven cells tested under these conditions, dopamine reduced inward current over the same limited range of potentials (Fig. 5). Internal Cs^+ eliminated much outward current (compare Figs 2 and 5A). Net inward current began to flow at −30 mV and reached a peak of about 1.2 nA at 0 mV. Dopamine applied at 10 μmol l^{-1} reduced the inward current over the voltage range −30 to +60 mV, an effect that was reversible. The reduction of current at its peak was just under 800 pA, i.e. about 500 pA of inward current persisted in the presence of dopamine. In other experiments, higher concentrations of dopamine did not produce further block, indicating that the modulatory actions of dopamine in reducing the inward current does not affect all of the available channels. Following these responses in normal saline, the cell was superfused with low-Ca^{2+}, high-Mg^{2+} saline. Nearly all of the inward current was

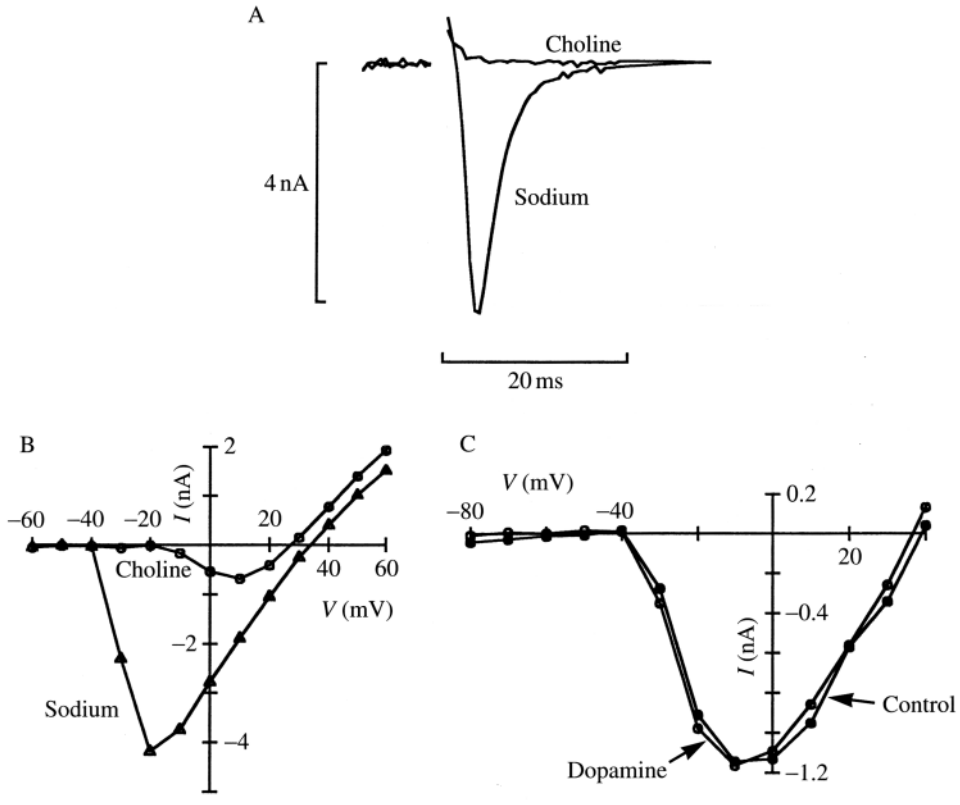

Fig. 4. Lack of effect of dopamine on transient sodium current. (A) Elimination of the transient sodium current with choline-containing saline. Pipette solution contained KCl. Current responses to a single voltage step to −20 mV from a holding potential of −60 mV, before and after superfusion of the cell with saline containing no sodium. Capacitance current was reduced for illustration by not plotting the current for 2 ms immediately following the voltage step. (B) *I–V* relationships made in normal (triangles) and choline-containing saline (circles) from the same cell. Elimination of the sodium current, which activates at voltages more positive than −40 mV, reveals an inward current that activates at voltages more positive than −20 mV. (C) Isolation of the sodium current and the effect of dopamine. Pipette solution contained KCl. The bath contained 20 mmol l^{-1} TEA^+ and 2 mmol l^{-1} Cd^{2+}. The curve obtained in the presence of 10 μmol l^{-1} dopamine (open circles) is nearly identical to that obtained before dopamine application (filled circles).

eliminated by this ionic substitution and, under these conditions, dopamine had little effect (Fig. 5B).

We confirmed that dopamine modulated sustained inward currents carried by ions other than sodium. Choline-substituted saline eliminated transient sodium currents (Fig. 4A,B) and could be expected to eliminate sustained sodium currents as well. Under these conditions, dopamine had actions similar to those seen in normal saline, e.g. it reduced the peak inward current by hundreds of picoamperes, but did not fully eliminate it (Fig. 5C). Together, these results indicate that the inward current reduced by dopamine

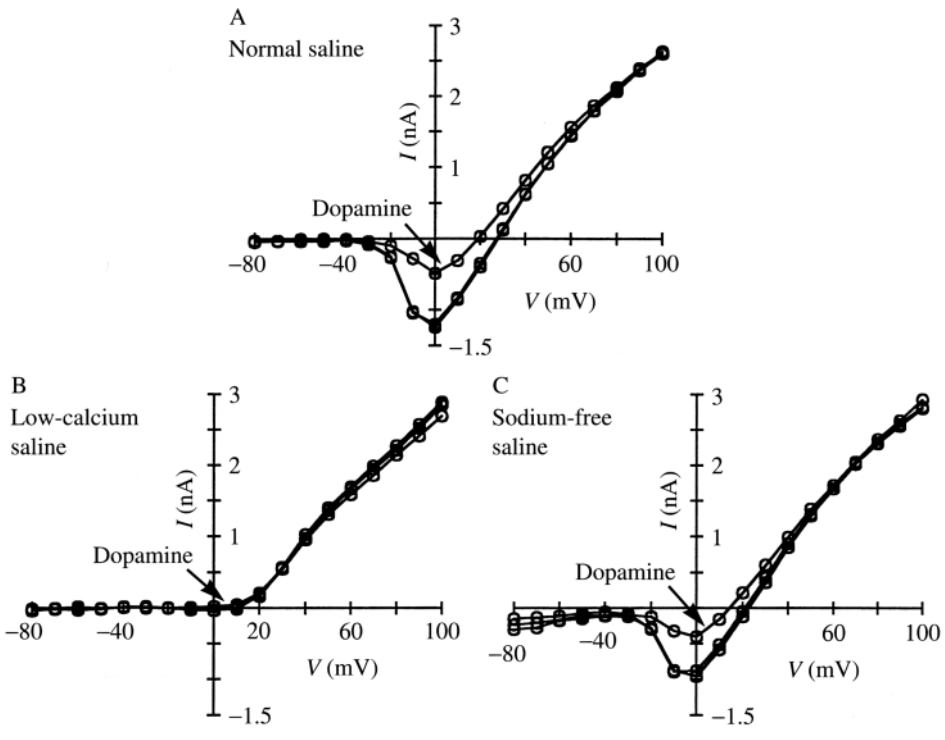

Fig. 5. Reduction of calcium current by dopamine. The pipette contained the CsCl solution and the bath solution was changed to reveal the ionic dependence of the dopamine-reduced current. In each case, control recordings produced before and after 10 μmol l^{-1} dopamine application are superimposed, indicating complete reversibility of the response. (A) In normal saline, dopamine caused a reduction of inward current in the voltage range −30 to +60 mV. (B) With the bath containing low-Ca^{2+}, high-Mg^{2+} saline, dopamine had no effect. Note that nearly all inward current was eliminated by this ionic replacement. (C) The cell was superfused with choline-containing (sodium-free) saline. Leak current increased at negative potentials, but inward current was reduced over the same voltage range as in A when dopamine was applied. Current magnitudes were measured near the end of individual 110 ms steps to the voltages indicated. Voltage steps were applied every 2 s from a holding potential of −60 mV.

was a sustained calcium current and not, for example, a sodium current or a non-specific cation current.

Outward current increase reflects modulated K^+ channels

The most pronounced action of dopamine was to increase net outward current at potentials more positive than −60 mV (Fig. 2). We have shown above that some of the increase in net outward current was due to a reduction of inward current, but that this action occurred over a limited range of membrane potentials between −30 and +60 mV. Here we show that the dopamine-induced increase in outward current results from an increase of potassium current in voltage-gated K^+ channels. We suggest that a small decrease of potassium current in Ca^{2+}-gated K^+ channels (or some other Cd^{2+}-sensitive outward current) could also occur at potentials more positive than +60 mV.

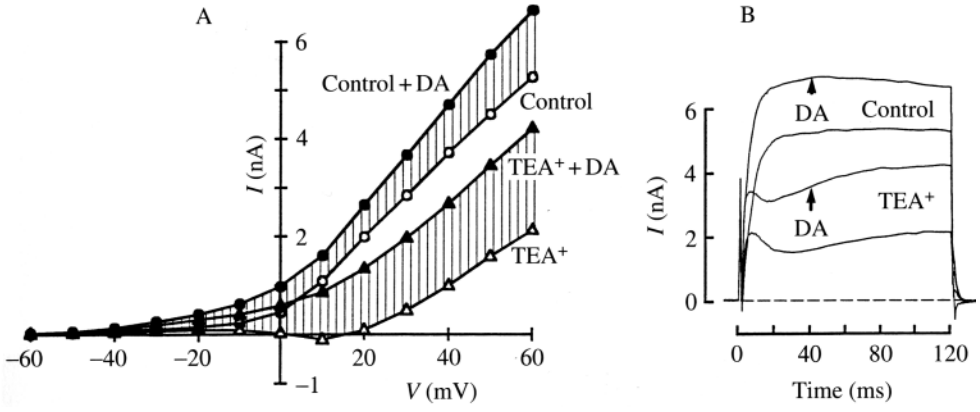

Fig. 6. Enhancement of the dopamine-induced increase of outward currents at potentials more positive than −60 mV by TEA^+. Individual steps lasting 110 ms to the voltages indicated were applied every 2 s from a holding potential of −60 mV. The pipette contained KCl solution. (A) The *I–V* relationship labelled control (circles) was made in normal saline, while that measured in 10 μmol l^{-1} dopamine (filled circles) is labelled control+DA. Vertical shading indicates the increase in outward current. The *I–V* relationships drawn with triangles were made in the presence of 20 mmol l^{-1} TEA^+. That drawn with filled triangles shows the effects of 10 μmol l^{-1} dopamine. (B) Currents recorded at +60 mV before and during the application of 10 μmol l^{-1} dopamine in normal saline and in saline with 20 mmol l^{-1} TEA^+. Recordings made before and after dopamine application are superimposed for both the normal and TEA^+-containing bathing solutions.

Evidence that the cells express voltage-activated potassium currents was obtained from several experimental approaches. As already shown, a great deal of outward current was eliminated when recordings were made with CsCl-filled electrodes (e.g. compare 3 nA at +100 mV in Fig. 5 with 6 nA in Fig. 8), indicating that this current was carried through K^+ channels. Under these conditions, dopamine had little or no effect on whole-cell current at potentials more positive than +60 mV.

The K^+ channel blocker TEA^+ proved useful and interesting when tested on 12 cells. Fig. 6 shows that, in normal saline (labelled control), dopamine increased outward currents at all potentials more positive than −60 mV. In this cell, the increase was 1.5 nA at +60 mV and 0.5 nA at both 0 and at +20 mV. In the 12 cells tested with TEA^+, dopamine increased outward current by 1.54±0.29 nA (mean ± S.D.) at +60 mV. Following TEA^+ application, the *I–V* relationship of the cell showed a substantial decrease in outward current, consistent with the known action of TEA^+ blocking a variety of K^+ channel types. The range of membrane potentials over which TEA^+ blocked outward current was the same as the voltage range over which dopamine increased current, i.e. it was limited to potentials more positive than −60 mV. In this cell, outward currents were reduced by 3.2 nA at +60 mV, 1.8 nA at +20 mV and 0.4 nA at 0 mV. The average reduction of outward current by TEA^+ at +60 mV was 3.02±0.64 nA (N=12). However, in the presence of TEA^+, dopamine produced a greater increase in outward current at the positive end of the voltage range. Outward current increased in this cell by

2.2 nA at +60 mV, 1.36 nA at +20 mV and 0.6 nA at 0 mV. The average increase in the 12 cells sampled was 1.94±0.14 nA at +60 mV. A possible explanation of this is that dopamine simultaneously increased one and decreased another outward current component. For example, suppose that TEA^+ preferentially blocked the decreasing component. Then, in the presence of TEA^+, dopamine would act on the increasing component alone and would produce a greater increase of outward current than it had in control conditions, where the decreasing component had subtracted from the net outward current increase. In the following series of experiments, we test the hypotheses that dopamine increases $I_{K(V)}$ and decreases calcium-gated potassium current [$I_{K(Ca)}$] and that the two effects can be differentiated using pharmacologial blockers of $I_{K(V)}$ and $I_{K(Ca)}$.

First, to investigate the possible involvement of Ca^{2+} and Ca^{2+}-activated currents in the result described above, we tested the effects of dopamine in the presence and absence of Cd^{2+} in 16 cells. The increase in outward current induced by dopamine under both conditions was both voltage- and time-dependent, as shown by the selected traces in Fig. 7A. Cd^{2+} reduced inward current over the specific voltage range −30 to +60 mV (Fig. 7B), a result also observed in the absence of K^+ currents in Fig. 5. There was a decrease in outward current at potentials more positive than +60 mV, however, that could reflect the reduction of $I_{K(Ca)}$ (or some other Ca^{2+}-dependent outward current) concomitant with the reduction of Ca^{2+} current. When dopamine was applied in the presence of Cd^{2+}, there was a larger increase in outward current observed at very positive potentials (Fig. 7C), while there was less of an increase in outward current in the range between −30 and +60 mV. In other words, in the absence of Cd^{2+}, in this cell dopamine increased outward current by 2.6 nA at +100 mV, while in the presence of Cd^{2+}, dopamine increased outward current by 3 nA at this voltage. Overall, dopamine increased outward current by 0.96±1.29 nA (mean ± S.D., N=16) at +100 mV before Cd^{2+} had been added, compared with 1.57±1.08 nA in the presence of Cd^{2+}. At +10 mV, a voltage where Ca^{2+} channel activity was high, dopamine increased net outward current by about 1.3 nA in control conditions, while in the presence of Cd^{2+}, with Ca^{2+} channels blocked, dopamine increased outward current by only about 0.4 nA. In the 16 cells tested, dopamine increased outward current by 0.59±0.40 nA at +10 mV before the addition Cd^{2+}, compared with 0.20±0.18 nA in the presence of Cd^{2+}.

The action of dopamine in the presence of 4-AP was quite different from its action in the presence of TEA^+ or Cd^{2+}. Fig. 8 shows that the application of 4-AP reduced outward current markedly in the voltage range more positive than +50 mV, a typical response observed in seven cells tested. This gave rise to a partially N-shaped *I–V* relationship, suggesting that Ca^{2+}-gated K^+ channel activity persisted in the presence of 4-AP. While dopamine reduced inward current in the Ca^{2+} channels (appearing as an increase in net outward current) between −20 and +30 mV, we observed a small *decrease* in outward current at potentials more positive than +30 mV in the presence of 4-AP. On average, dopamine reduced outward current by 0.35±0.13 nA (N=7) at +60 mV in the presence of 4-AP. If 4-AP were blocking voltage-gated potassium currents, this experiment revealed on outward current that was reduced by dopamine, which, considering the experiments with TEA^+ and Cd^{2+} presented above, might be a Ca^{2+}-activated potassium current.

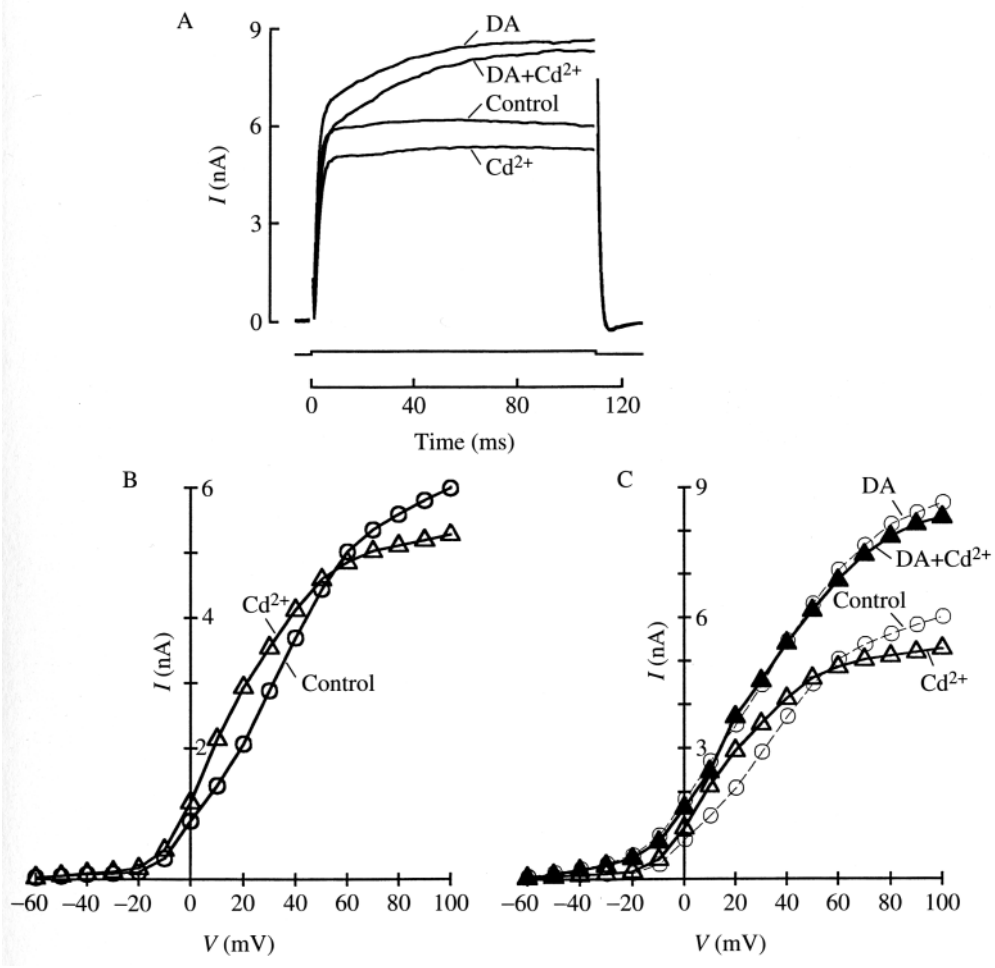

Fig. 7. Increase of outward currents induced by dopamine (DA) in the presence and absence of the Ca^{2+} channel blocker Cd^{2+}. (A) Currents in response to voltage steps to +90 mV from a holding potential of −60 mV lasting 110 ms. The pipette contained KCl solution. The recordings show increases in outward current induced by 10 μmol l^{-1} dopamine in control conditions with normal saline and with 100 μmol l^{-1} Cd^{2+} bath solution. (B) *I–V* relationships from the same cell as illustrated in A, measured at the end of each voltage step, showing the effects of 100 μmol l^{-1} Cd^{2+}. (C) *I–V* relationships from the same cell as illustrated in A and B, showing the effects of 10 μmol l^{-1} dopamine on currents measured with and without Cd^{2+}. The lightly drawn dashed lines and circles show currents in control conditions and with dopamine before addition of Cd^{2+}. The solid lines show currents measured in the presence of Cd^{2+} before (open triangles) and after (filled triangles) dopamine application.

Transient potassium current is not modulated by dopamine

Increased outward current appeared in response to dopamine at potentials as negative as −50 mV, a potential at which, in control recordings, no sustained voltage-gated K^+ current was detected. Our next experiments focused on this negative voltage range to determine whether the gating properties of the transient potassium current, I_A, were

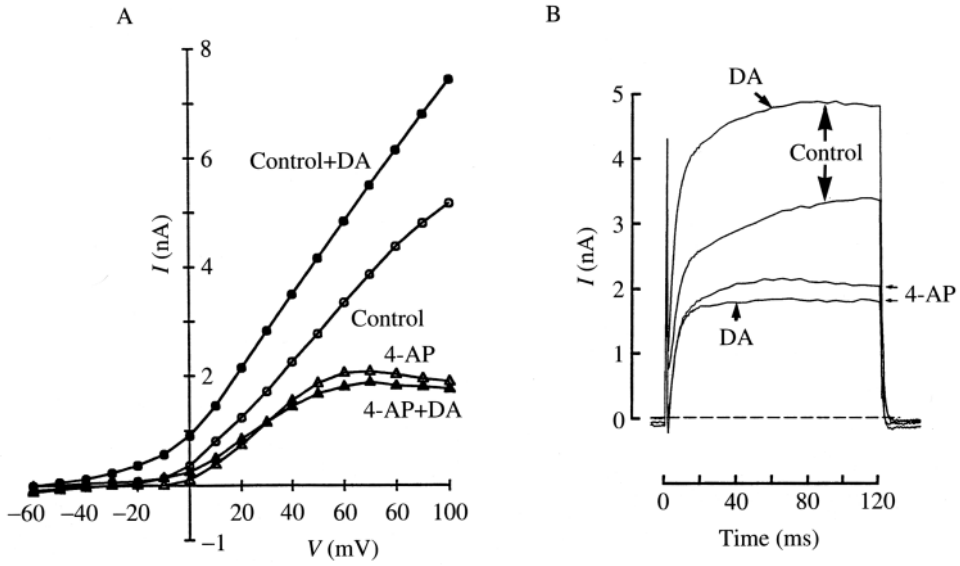

Fig. 8. Dopamine-induced decrease of outward currents at potentials more positive than +30 mV in the presence of 4-AP. Individual steps lasting 110 ms to the voltages indicated were applied every 2 s from a holding potential of −60 mV. The pipette contained KCl solution. (A) The *I–V* relationship labelled control (circles) was made in normal saline, and that made in the presence of 10 μmol l^{-1} dopamine is labelled control+DA (filled circles). The *I–V* relationships drawn with triangles were made in the presence of 5 mmol l^{-1} 4-AP. That drawn with filled triangles shows the effects of 10 μmol l^{-1} dopamine. Dopamine increased net outward current between −20 and +30 mV in the presence of 4-AP while decreasing outward current at potentials more positive than +30 mV (filled triangles). (B) Currents recorded at +60 mV before and during the application of 10 μmol l^{-1} dopamine in a control with normal saline and with 5 mmol l^{-1} 4-AP. Recordings made before and after dopamine application are superimposed for both the control and 4-AP bathing solutions.

modified such that the inactivation of current was slowed or eliminated. Alternatively, a different class of sustained K^+ channel could be recruited. Activation and inactivation voltage ranges for I_A (typical of six cells), shown in Fig. 9A, indicate that I_A would normally not be measurable at voltages more negative than −40 mV and that its inactivation would be nearly complete in a cell held or resting at potentials more positive than −30 mV. Fig. 9B shows that there were no sustained outward currents evoked by steps to −50 and −40 mV and that the sustained currents activated at −30 and −20 mV were very small (<10 pA). Application of dopamine (Fig. 9C) evoked sustained outward currents at all of these potentials. Fig. 9D shows difference currents obtained by digital subtraction of the two sets of recordings (Fig. 9C minus Fig. 9B). These indicate that there was no increase in the magnitude of the transient outward current, as would be expected if there were an elimination or pronounced slowing of inactivation. Also, holding the membrane potential at −30 mV to inactivate 90 % of I_A failed to alter the dopamine-induced sustained current seen at −50 mV (not shown). It seems likely that dopamine made available a rapidly activating sustained potassium current in this voltage range, possibly by shifting activation of $I_{K(V)}$ into this more negative region.

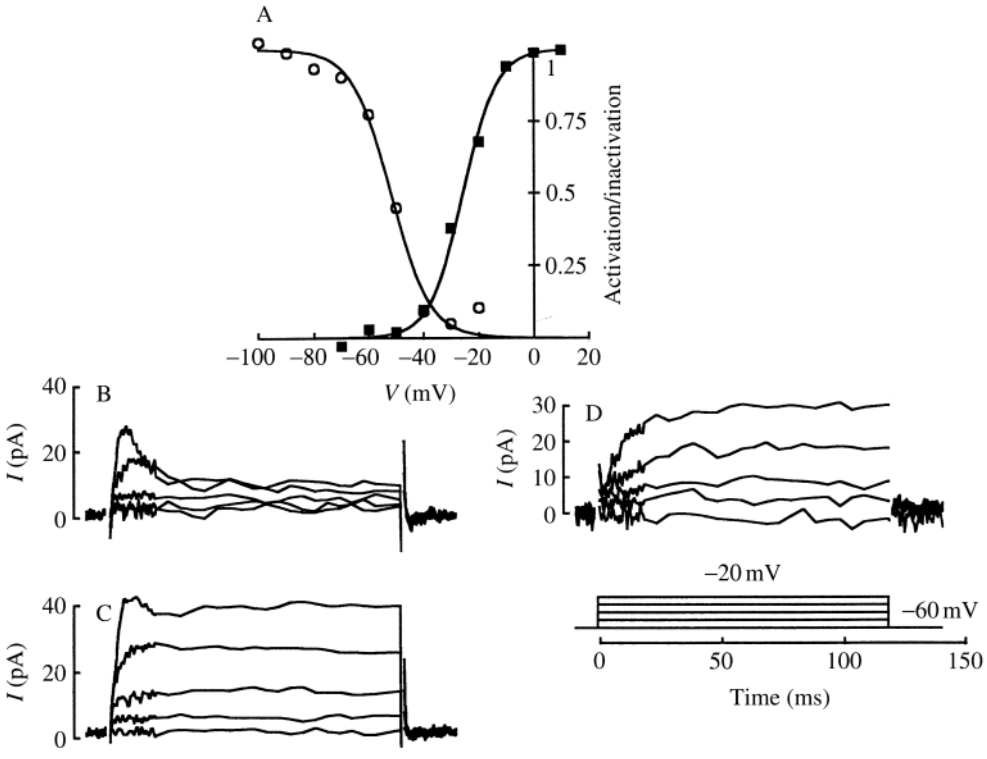

Fig. 9. Transient outward current (I_A) and its fate in the presence of 10 μmol l^{-1} dopamine. (A) Activation and steady-state inactivation curves for I_A were derived from a voltage-clamp paradigm with 200 ms prepulses given by the standard method. The current activates at potentials more positive than −50 mV (filled squares) and is fully inactivated by holding potentials more positive than −40 mV (open circles). (B) Current responses to voltage steps to −60, −50, −40, −30 and −20 mV from a holding potential of −60 mV before dopamine application. Activation followed by inactivation of the outward current can be seen at the steps to −30 and −20 mV in this cell. A small sustained outward current (<10 pA) develops at −40 mV and becomes larger at −30 and −20 mV. (C) During superfusion of 10 μmol l^{-1} dopamine, the same voltage steps elicit larger sustained outward currents, although there was no change in the current at −60 mV or at potentials more negative than this (see *I–V* relationships in Figs 2 and 7). A transient outward current can be seen at the beginning of the step to −20 mV. (D) Subtraction of the two sets of responses shows only sustained outward current elicited by dopamine. A few points (2 ms) immediately following the voltage steps have been deleted from each current recording to reduce the capacitance current for illustration. The voltage paradigm and time scale for B and C are shown in D.

Discussion

The respiratory central pattern generator of *Lymnaea stagnalis* is regulated by the dopaminergic neurone RPeD1 (Syed *et al.* 1990). We sought to determine the mechanism of the inhibitory action of dopamine on one of its targets in the CPG, neurone VD4. Dopamine application to neurone VD4 caused a striking increase in net outward current at all potentials more positive than −60 mV. Although dopamine did not modulate I_{Na} or

I_A, we found that it increased $I_{K(V)}$ and decreased I_{Ca} and $I_{K(Ca)}$. These physiological actions of dopamine account for the inhibitory action of cell RPeD1 on cell VD4.

Our results support the conclusion that dopamine modulates voltage-gated channels. We found no indication that dopamine directly gates ion channels. If dopamine had opened voltage-insensitive K^+ channels directly, we would have seen an increase in conductance at all potentials with current reversing at around −85 mV, the value of the potassium equilibrium potential under our conditions. Instead, we did not observe a change of conductance or current at potentials below −60 mV, because no voltage- or Ca^{2+}-gated channels were available for activation in this voltage range. A novel action of dopamine directly gating Cl^- channels would have been revealed as an increase in conductance and current with a reversal potential near 0 mV under the conditions tested here, a phenomenon that was not observed. Furthermore, no evidence for inward rectification of the membrane over the potential range from −60 to −100 mV was found. Slow inward rectifier currents, such as h-current (Bader *et al.* 1982) and chloride current (Chesnoy-Marchais, 1982), and faster forms of potassium inward rectifier activity (Hagiwara *et al.* 1976) were not observed in the absence or presence of dopamine.

Our interpretation of how dopamine has a greater effect in increasing outward current when Cd^{2+} or TEA^+ is present requires that Cd^{2+} and TEA^+ are more potent in blocking Ca^{2+}-dependent outward current than in blocking voltage-gated potassium current. The outward current reduced by dopamine could have been Cs^+ exiting the cell *via* Ca^{2+} channels (Lee and Tsien, 1984). Dopamine application, which reduced calcium channel activity, would thus reduce the outward flow of cations through the same channels. Alternatively, the outward current reduced by dopamine might be $I_{K(Ca)}$. In general, the literature comparing external TEA^+ block of voltage- and Ca^{2+}-gated potassium channels would support this possibility: EC_{50} values for TEA^+ acting on $I_{K(Ca)}$ are in the submillimolar range, but in the 10 mmol l^{-1} range for $I_{K(V)}$ (Rudy, 1988). With regard to molluscan K^+ channels, TEA^+ block of $I_{K(V)}$ is consistent: 50 % block occurs between 5 and 12 mmol l^{-1} for *Helix pomatia* neurones (Neher and Lux, 1972), at 8 mmol l^{-1} in *Tritonia diomedia* (Thompson, 1982) and at 6 mmol l^{-1} in *Aplysia californica* (Hermann and Gorman, 1981). For $I_{K(Ca)}$, predictions of external TEA^+-sensitivity based on the literature are difficult. For example, in *Tritonia diomedia*, 100 mmol l^{-1} TEA^+ blocks only 20 % of $I_{K(Ca)}$ (Thompson, 1982), while in *Aplysia californica* the EC_{50} value is 400 μmol l^{-1} (Hermann and Gorman, 1981). Thus, our use of 20 mmol l^{-1} TEA^+ in *Lymnaea stagnalis* could have blocked most of $I_{K(V)}$, but an unpredictable proportion of $I_{K(Ca)}$. The observations that, in the presence of 4-AP, an N-shaped *I–V* relationship was obtained and that some of the N shape (outward current around +60 mV) was reduced by dopamine suggests that the net increase of outward current induced by dopamine was due to a predominant increase in $I_{K(V)}$ and to a lesser decrease in $I_{K(Ca)}$.

Comparison of our results with the actions of dopamine in other cells

The actions of dopamine we describe differ only in detail from those in other molluscan and mammalian neurones. Reduction of I_{Ca} by dopamine has been reported previously for *Lymnaea stagnalis* neurones, where it was shown not to involve intracellular calcium or cyclic AMP (Akopyan *et al.* 1985). A similar reduction of I_{Ca} in *Helix pomatia* was

mediated by a GTP-binding protein (Harris-Warrick *et al*. 1988). In another study (Paupardin-Tritsch *et al.* 1985), *Helix pomatia* neurones responded to dopamine with a reduction of I_{Ca}, as in our results, but also with a reduction of potassium current, I_{KS}, an effect not seen in our work. The latter action differs from our observations in two respects. First, we saw an increase, rather than a decrease, in voltage-gated potassium current. Second, as a target of dopamine modulation, I_{KS} presents a different type of potassium current from $I_{K(V)}$ of *Lymnaea stagnalis*. I_{KS} is a cyclic-AMP-dependent, weakly voltage-dependent current (Klein *et al*. 1982), whereas $I_{K(V)}$ is strongly voltage-dependent, being entirely unactivatable at potentials more negative than −60 mV.

In *Aplysia californica* neurones, previous studies have implied that dopamine modulates calcium and potassium currents as a mechanism for reducing bursting activity (reviewed by Adams and Benson, 1985). Cell R15 responds to applied dopamine with a reduction of I_{Ca} (Lewis *et al*. 1984; Lotshaw and Levitan, 1988) and an increase of potassium conductance (Ascher, 1972; Chesnoy-Marchais, 1984), both actions leading to inhibition of regenerative activity, as seen in our work.

The modulatory actions of dopamine are remarkably similar in *Lymnaea stagnalis* cell VD4 and in one type of mammalian cell. In rat anterior pituitary cells, dopamine suppresses two types of Ca^{2+} channel activity and enhances three types of K^+ channel activity (Lledo *et al*. 1992). In this case, different G-proteins are used for coupling D_2 receptors to the Ca^{2+} and K^+ channels. In our study, the actions of dopamine on Ca^{2+} and K^+ channels appear to be parallel in terms of concentration and temporal properties. Nevertheless, it could be of value to determine whether Ca^{2+} and K^+ channel modulation involves different G-proteins and/or second-messenger systems since variable control of the channels could allow specific physiological tuning of cell VD4.

Physiological relevance of the actions of dopamine

The reduction in calcium current in cell VD4 would be a relevant physiological action for dopamine. Ca^{2+} channels in neurones are frequent targets of neurotransmitter modulation, with suppression or enhancement of Ca^{2+} channel activity being a common outcome, albeit *via* different mechanisms (Gerschenfeld *et al.* 1989; Yakel, 1991). Calcium current is readily activated in the normal physiological range of membrane potentials. Its modulation could therefore affect many calcium-dependent functions, even in relatively quiescent cells. For example, calcium current modulation could directly alter the synaptic output of the cells *via* actions on calcium-dependent neurotransmitter release.

The dopaminergic modulation of membrane potential excitability shown in Fig. 1 is readily explained in terms of the channel modulation described. Dopamine had little effect on the resting potential of the cell, causing it to hyperpolarize only from −58 to −62 mV, because these values lie at the negative range of all K^+ channel activation curves. The resting potential of an isolated cell can be set by the balance of extremely small (picoampere range) inward and outward currents. Thus, by increasing the outward current in these cells, the resting potential would move in the negative direction, but in doing so the outward current would become less activated until the fine balance between inward and outward currents was again reached. For the same reasons, the input

resistance measured within a few millivolts of rest would not be changed by the modulatory actions of dopamine on voltage-gated channels that are not activated at this voltage.

The threshold for action potentials changed from −32 to −29 mV when dopamine was applied. Since we could demonstrate no change in sodium currents with dopamine application, K^+ and Ca^{2+} channel modulation in the same range of voltage at which Na^+ channel activity first appears must affect threshold (Fig. 2). In the presence of dopamine, a much greater amount of depolarizing current, reflecting the increased conductance of the cells positive to rest, had to be injected to reach threshold.

Dopamine caused a prominent modification of repetitive spike activity in these cells. Dopamine increased accommodation of the cells, converting the firing pattern from one of tonic to one of phasic activity. The presumed explanation for this change is the increase in the magnitude of the voltage-gated potassium currents and the reduction in the voltage-gated calcium currents, which have very similar overall effects in reducing membrane excitability. Thus, by increasing the potassium current available, which represents an increase in conductance with a strongly hyperpolarizing influence, and by decreasing the calcium current, which eliminates a regenerative influence, repetitive activity would be subdued.

The results in this study clarify the mechanism by which dopamine inhibits neurone VD4 in the CPG. Clearly, the effect of dopamine on neurone VD4 will depend upon its resting potential. Although the resting potential of isolated VD4 neurones in this study was near −60 mV, these cells are more depolarized *in vivo*, resting between −40 and −50 mV (Syed and Winlow, 1991; McKenney, 1992), leading to the expectation that dopamine would have a stronger hyperpolarizing action *in vivo*. It is possible that the cells could reach action potential or burst threshold in the absence of dopamine and simply be inactive due to hyperpolarization when dopamine is present. However, in the CPG, neurone VD4 is also inhibited by neurone IP3I, whose transmitter is unknown. The bursts of action potentials in neurone VD4 therefore arise as a result of the integrated actions of two transmitters (dopamine from RPeD1 and an unidentified transmitter from IP31). Determining the mechanisms underlying burst generation in the respiratory CPG will, therefore, require a series of further studies.

This work was supported by the Medical Reseach Council of Canada (MRC), the Natural Sciences and Engineering Research Council (NSERC), the Alberta Heritage Foundation for Medical Research (AHFMR) and the Alberta Lung Association. S.B. is an AHFMR and MRC Scholar, N.I.S. is an AHFMR Scholar and Parker B. Francis Fellow and A.G.M.B. is an AHFMR Scientist.

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