Forskolin Mimics the Dopamine-Induced K^+ Conductance Increase in Identified Neurons of *Aplysia kurodai*

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Abstract The ionic mechanism of the effect of micropressure ejection of dopamine (DA) and forskolin on the membranes of identified inhibition of long duration (ILD) neurons of *Aplysia kurodai* was investigated with conventional voltage-clamp and ion-substitution techniques. Ejection of DA and forskolin onto the neuropil of the ILD neuron produced a slow outward current (5–20 nA in amplitude; 10–60 s in duration) associated with a conductance increase. The outward currents induced by DA and forskolin were consistently a linear function of the holding potential, reversing near the predicted potassium equilibrium potential (-80 mV), and were sensitive to changes in the concentration of extracellular K^+ but not to extracellular Cl^- . Bath-applied isobutylmethylxanthine ($50 \mu M$) prolonged the outward current induced by both DA and forskolin, but imidazole (5 mM) had an opposite effect on these currents. Tolbutamide (1 – 5 mM), a protein kinase inhibitor, reduced the current induced by both DA and forskolin. A antagonist for DA receptors in molluscan neuron, ergometrine maleate, ($10 \mu M$) completely abolished the outward current induced by DA, but the current induced by forskolin persisted in the presence of the DA-antagonist. Guanosine 5'-O-(2-thiodiphosphate) (GDP β S, 5 – $10 \mu M$), an analogue of GDP inhibited the outward current induced by both DA and forskolin. These results suggest that dopamine acts on the ILD neurons of *Aplysia kurodai* by increasing K^+ conductance. Forskolin can mimic the DA action, suggesting that the effect of dopamine is mediated by increased intracellular cAMP.

Key words: *Aplysia*, forskolin, dopamine, cAMP, ionic mechanism.

In the abdominal ganglion of *Aplysia kurodai*, three neurons have been identified that responded with inhibition of long duration (ILD) to a single stimulation of the siphon nerve and to bath and iontophoretic application of dopamine (DA). The ILD is a long-lasting (15–60 s) hyperpolarizing potential,

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typically associated with a K^+ conductance increase. Intracellularly injected cAMP increases the ILD (SAWADA *et al.*, 1980).

Adenosine 3',5'-cyclic monophosphate (cAMP) probably mediates many transmitter-induced diminutions in K^+ conductance (CASTELLUCCI *et al.*, 1980; DETERRE *et al.*, 1982; MADISON and NICOLL, 1982; SIEGELBAUM *et al.*, 1982). In the report of MADISON and NICOLL (1982), the K^+ conductance diminished by cAMP is activated by intracellular Ca^{2+} . Furthermore, in the bag cells of *Aplysia*, cAMP appears to reduce a Ca^{2+} -activated K^+ conductance (KACZMAREK and STRUMWASSER, 1984) as well as the rapidly inactivating, voltage-gated A-current (STRONG, 1984). On the other hand, serotonin, dopamine, and stimulation of the branchial nerve all hyperpolarize neuron R15 of *Aplysia* and stop its bursting (DRUMMOND *et al.*, 1980). The stimulation of adenylate cyclase activity seems to be a prerequisite for the response to 5-HT, since an inhibitory substance to this enzyme, guanosine 5'-O-(2-thiodiphosphate) (GDP β S), can block the increase in K^+ conductance caused by 5-HT (LEMON and LEVITAN, 1984).

The diterpene forskolin, obtained from the roots of *Coleus forskohlii*, is a potent nonhormonal activator of adenylate cyclase in many systems, the functions of which have been proposed to be dependent on cAMP as a second messenger (SEAMON and DALY, 1982; SEAMON *et al.*, 1983). Forskolin appears to directly activate the catalytic subunit of adenylate cyclase, bypassing hormone or transmitter receptors, guanine nucleotide regulatory protein, and guanine nucleotides (SEAMON *et al.*, 1981).

In the present investigation we have examined the possibility that changes in the intracellular levels of cAMP may mediate the DA-induced K^+ conductance increase in the ILD neuron of *Aplysia kurodai*. We have performed physiological studies in which the receptor activation stage of the process is bypassed by artificially raising intracellular cAMP levels using forskolin. Such experiments should mimic the effect of activating adenylate cyclase by the DA receptors if cAMP is directly involved in mediating the responses of these receptors.

METHODS

Approximately 120 *Aplysia kurodai* weighing from 100 to 350 g were used in these experiments. Abdominal ganglia were dissected from the animals, pinned ventral side up on the Sylgard base of a recording chamber, and constantly (4 ml/min) superfused with *Aplysia* Ringer (587 mM Na^+ , 12 mM K^+ , 671 mM Cl^- , 14 mM Ca^{2+} , and 52 mM Mg^{2+} , a slight modification of the *Aplysia* Ringer originally made by SATO *et al.*, 1968). The effective perfusing volume of the chamber was 0.3 ml. The pH was adjusted to 7.8 with tris(hydroxymethyl)-aminomethane and HCl. The low K^+ solutions were made by mixing appropriate amounts of K^+ -free *Aplysia* Ringer with normal *Aplysia* Ringer. When the Ringer solution required a low Cl^- ion concentration, the Cl^- ion was replaced by an acetate ion. The connective tissue sheath of the ganglion was removed by dissection and the

underlying cells were directly exposed to the perfusing media. Two glass microelectrodes filled with 4 M K-acetate were inserted into an identified ILD neuron. One electrode recorded the membrane potential, and the other was used for current injection. An Ag-AgCl electrode with an agar-seawater bridge was immersed in the bath and served as the indifferent electrode and virtual ground. The membrane potential and the current were monitored by a dual beam oscilloscope (VC-9A, Nihon Kohden) and a four-channel pen recorder (RECTI HORIZ-8K, San-ei) with selected signal filtering.

The neuron was voltage-clamped conventionally with a two-electrode system (using the voltage-clamp amplifier, CEZ-1100, MEZ-7101, Nihon Kohden) and held at the resting membrane potential (from -45 to -55 mV). In order to evaluate the changes in the membrane conductance, the resting membrane was repeatedly hyperpolarized every 5 s by a voltage pulse of 10 mV with a duration of 1 s, and the current required for this constant hyperpolarizing pulse was recorded before and during the responses to dopamine and forskolin, ejected onto the neuropil of the identified ILD neuron. Dopamine and forskolin were applied by micropressure ejection from the third double-barrelled electrode filled with 100 mM dopamine HCl and 2.5 mM forskolin (Calbiochem) positioned near the neuropil. Ejections were made by constant pressures ranging from 1 to 2 kg/cm² for 100–900 ms.

Drugs applied extracellularly were added to the artificial *Aplysia* Ringer. 3-Isobutyl-1-methylxanthine (IBMX) was dissolved in ethanol and dispersed in the artificial *Aplysia* Ringer; the final ethanol concentration was less than 1%. Tolbutamide was dissolved in 10% acetone in distilled water and dispersed in the artificial *Aplysia* Ringer; the final acetone concentration was less than 1%. Control solutions with 1% ethanol and 1% acetone alone produced little effect on the membrane current. The following drugs were used: dopamine hydrochloride (Sigma); water soluble 7-O-hemisuccinyl-7-deacetyl forskolin (Calbiochem); 3-isobutyl-1-methylxanthine (IBMX, Aldrich); imidazole (Nakarai); tolbutamide (Sigma); ergometrine maleate (Nakarai); and GDP β S (Sigma).

Experiments were normally conducted at 17–20°C.

RESULTS

Identification of the ILD neurons

Three identified ILD neurons are usually in the ventral right caudal quarter of the abdominal ganglion near the base of the branchial nerve (SAWADA *et al.*, 1980). The ILD neurons are yellow, transparent, and 30–60 μ m in diameter. The resting membrane potential of the identified ILD neurons was -46.2 ± 6.3 mV (mean $\pm$ S.D., $n=42$), and the neurons had an irregular firing pattern and spontaneously occurring ILD responses.

DA-induced slow outward current

Clamping the ILD neuron at its resting potential level (-45 mV) and

micropressure ejecting DA onto the neuropil caused a transmembrane outward current associated with an increase in input conductance (Fig. 1). The change in input conductance produced by DA was directly measured by applying constant voltage hyperpolarizing pulse while clamping the neuron at its resting membrane potential in this and subsequent experiments. In an initial series of experiments we examined the sensitivity of the DA-induced outward current to changes in extracellular K^+ concentration. Decreasing extracellular K^+ from the control level of 12 to 1.2 mM increased the DA-induced outward current to about 170% ($172.8 \pm 19.2\%$, mean $\pm$ S.D., $n = 3$) of the control (Figs. 1A and 5C, and Table 1).

Fig. 1. Effects of $[K^+]_o$ -deficient *Aplysia* Ringer (A) and $40 \mu M$ forskolin (B) on the DA-induced outward current recorded from an ILD neuron. Holding potential was -45 mV. Dopamine (DA) was micropressure ejected onto the neuropil of the ILD neuron using a single constant pulse (800 ms, 2.1 kg/cm^2). Upper trace shows membrane current; middle trace, holding potential; the lowest trace, pressure monitor in each set of records. Constant hyperpolarizing command pulses with durations of 1 s were applied every 5 s and the current required for these command pulses was recorded for measurement of membrane conductance. A: upper, control; middle, 8 min after the ILD neuron was exposed to $[K^+]_o$ -deficient (0.1 normal K^+) *Aplysia* Ringer; bottom, 16 min after washout. B: upper, control; middle, 12 min after the ILD neuron was exposed to forskolin; bottom, 20 min after washout. NSW represents normal *Aplysia* Ringer in this and subsequent figures. Straight bar in B, middle shows the baseline holding current. Note that amplitude of the DA-induced outward current was potentiated after 12 min of exposure to forskolin.

If DA is acting through a cyclic nucleotide second-messenger system, then agents that alter cyclic nucleotide metabolism should modulate the DA-induced outward current. To test this hypothesis, DA was applied by micropressure ejection onto the neuropil of the ILD neuron bathed in *Aplysia* Ringer containing the adenylate cyclase activator, forskolin. The amplitude of the DA-induced outward current was potentiated by about 47% of the control and the rate of rise of the current also increased in the presence of 40 μ M forskolin (Fig. 1B). It can be seen that forskolin applied to the bath at low concentration produced a slight outward shift (2 nA) in the baseline holding current (Fig. 1B, middle).

Bath-applied forskolin (20 μ M) itself induces the inward current similar to the 5-HT-induced inward current in *Helix* neuron (DETERRE *et al.*, 1982) and also induced an inward current similar to the FMRF-amide-induced inward current in identified molluscan neuron (COLOMBAIONI *et al.*, 1985). These observations led us to eject forskolin onto the neuropil of the ILD neuron directly using one barrel of a double-barrelled electrode positioned near the neuropil while continuously recording the DA-induced outward current induced by DA-ejection onto the neuropil using the other barrel of the electrode.

Comparison of the DA-induced responses and the forskolin-induced responses

Micropressure-ejected DA and forskolin onto the neuropil both caused marked hyperpolarizations in the non-clamped ILD neuron (Fig. 2A). Clamping the neuron at its resting potential level (-45 mV) and ejection of DA and forskolin induced outward currents (Fig. 2C). The slow outward current responses to ejected DA and forskolin were observed in over 42 ILD neurons studied. The responses to successive ejections of DA were markedly reduced under non-clamped and voltage-clamped conditions when the ejections were repeated at short intervals (20 s) (Fig. 2B and D). The forskolin-induced hyperpolarization and outward current persisted after the DA receptor of the neuron was desensitized by the repeated ejection of DA at short intervals (Fig. 2B and C). To further compare the DA-induced outward current with the forskolin-induced outward current, DA and forskolin were micropressure-ejected onto the same neuropil of the ILD neuron by double pulses (Fig. 3A and B). A second ejection of DA at a short interval (20 s) produced the reduced outward currents (Fig. 3Ac). On the other hand, forskolin repeatedly ejected at the same interval induced a summated outward current (Fig. 3Ad). Furthermore, micropressure-ejected forskolin at short enough intervals to desensitize the DA receptors induced an outward current (Fig. 3Bc) comparable to the control forskolin-induced outward current (Fig. 3Bb). These results suggest that forskolin can induce an outward current after the DA receptors on the membrane on the ILD neuron are desensitized by repeated ejection of DA at short intervals, so mechanisms underlying the forskolin-induced outward current bypass the receptor activation stage.

Fig. 2. DA-induced responses and forskolin-induced responses recorded from the ILD neuron in the unclamped condition (resting membrane potential, -45 mV; A and B), and in the voltage-clamped condition (holding potential, -45 mV; C and D). DA and forskolin were micropressure-ejected onto the neuropil of the ILD neuron at arrows using one each of a double-barrelled electrode. Numbers represent the duration of the pressure pulse (ms) in this and subsequent figures. Micropressure-ejected DA and forskolin onto the same neuropil region hyperpolarized the membrane (A), and produced a slow outward current in the ILD neuron voltage-clamped at -45 mV (B). DA and forskolin (F) were micropressure-ejected by a single constant pulse (100 ms, 2 kg/cm 2 and 900 ms, 2 kg/cm 2 in A and B; 900 ms, 2 kg/cm 2 and 800 ms, 2 kg/cm 2 in C and D). Note that the forskolin-induced hyperpolarizing response and slow outward current could be obtained during desensitization of DA receptors after repeated ejections of dopamine onto the neuropil (B and C).

Fig. 3. Comparison of the DA-induced and the forskolin-induced outward current when the ILD neuron was ejected with these drugs twice onto the same neuropil region. Same nomenclatures as in Fig. 1. DA was micropressure-ejected by a single constant pulse (150 ms, 0.8 kg/cm² in A; 300 ms, 1.2 kg/cm² in B). Forskolin was ejected by a single constant pulse (900 ms, 0.5 kg/cm² in A; 500 ms, 0.5 kg/cm² in B). Aa and Ba, and Ab and Bb were control responses produced by micropressure ejection of DA and forskolin, respectively. Note that no desensitization was observed after the repeated ejections of forskolin and subsequent ejection of forskolin after DA ejection.

Ionic mechanisms of the DA-induced outward current and the forskolin-induced outward current

Effect of holding potential. We explored the voltage sensitivity of the DA-induced and the forskolin-induced outward current by clamping the ILD neuron to holding potentials ranging from -45 to -105 mV and measuring the amplitudes of these currents. Figure 4A illustrates the changes in holding potential of the DA-induced outward current. The DA-induced outward current decreased with hyperpolarized holding potentials and reversed at holding potentials more negative than -80 mV. The reversal potential of the DA-induced outward current was -80.1 mV. Under voltage clamp, micropressure-ejected forskolin produced an outward current that decreased with hyperpolarized holding potentials and reversed at hyperpolarized holding potentials more negative than -85 mV. The reversal potential of the forskolin-induced outward current was -78.5 mV (Fig. 4B). The conductance changes produced by DA and forskolin showed voltage dependency in the range of membrane potentials examined (from -45 to -105 mV; Fig. 4A and B). Both the DA-induced outward current and the forskolin-induced outward current reversed at ca. -80 mV, suggesting that these currents were due to an increase in K^+ conductance. To further test this hypothesis, the external ionic compositions were altered.

Fig. 4. The DA-induced outward currents (A) and the forskolin-induced outward currents (B) at different holding potentials, and voltage sensitivity of these currents (right) recorded from the same ILD neuron. Constant hyperpolarizing command pulses with duration of 1 s were injected every 5 s for measurement of the membrane conductance (left). DA and forskolin were micropressure-ejected by a single constant pulse (400 ms, 1.8 kg/cm² in A and 200 ms, 1.8 kg/cm² in B). The reversal potential of the DA-induced and the forskolin-induced outward current was -80.1 and -78.5 mV, respectively. Note that the relative membrane conductance increases measured at the peak of the DA-induced and forskolin-induced responses show voltage-dependency.

Fig. 5. Lack of effect of $[Cl^-]_o$ -deficient seawater on the DA-induced outward current (A), on the forskolin-induced outward current (B), effect of $[K^+]_o$ -deficient seawater on the DA-induced outward current (C), and on the forskolin-induced outward current (D). Holding potential was -45 mV. DA and forskolin were ejected by a single constant pulse (400 ms, 1.8 kg/cm² in A and C; 200 ms, 1.8 kg/cm² in B and D), respectively. A: left, control; center, 6 min after the ILD neuron was exposed to $[Cl^-]_o$ -deficient (0.5 normal Cl^-) seawater; right, 12 min after washout. B: left, control; center, after 5 min of exposure to $[Cl^-]_o$ -deficient seawater; right, 12 min after washout. C: left, control; center, 6 min after the ILD neuron was exposed to $[K^+]_o$ -deficient (0.2 normal K^+) seawater and 6 min after further exposure to $[K^+]_o$ -deficient (0.1 normal K^+) seawater; right, 12 min after washout. D: left, control; center, 6 min after the ILD neuron was exposed to $[K^+]_o$ -deficient (0.1 normal K^+) seawater; right, 7 min after washout.

Low $[Cl^-]_o$ effects. The effects of reducing the extracellular chloride concentration ($[Cl^-]_o$) on the DA-induced outward current and the forskolin-induced outward current are illustrated in Fig. 5A and B. Reducing $[Cl^-]_o$ from 671 to 335 mM (replaced with acetate) caused little change in the DA-induced outward current or the forskolin-induced outward current recorded at holding potential of -45 mV.

Low $[K^+]_o$ effects. The effects of reducing the extracellular potassium concentration ($[K^+]_o$) on the DA-induced outward current and the forskolin-induced outward current are illustrated in Fig. 5C and D. If the outward current induced by DA or forskolin was simply due to an increase in K^+ conductance, we would expect that the current recorded at a holding potential of -45 mV would increase during exposure of the neuron to a $[K^+]_o$ -deficient solution. Reducing $[K^+]_o$ from 12 to 1.2 mM caused a marked increase in the DA-induced outward current ($172.8 \pm 19.2\%$ of the control, mean $\pm$ S.D., $n=3$) and in the forskolin-induced outward current (156.8% of the control). These results suggest that both the DA-induced outward current and the forskolin-induced outward current are due to an increase in K^+ conductance but not in Cl^- conductance.

Effects of IBMX, imidazole, tolbutamide, and ergometrine maleate

3-Isobutyl-1-methylxanthine (IBMX) was used to inhibit the breakdown of intracellular cAMP by phosphodiesterase (PDE). The half decay times of the DA-induced outward current and the forskolin-induced outward current were prolonged during 6 min exposure the ILD neuron to $50 \mu\text{M}$ IBMX. The effects of IBMX completely disappeared 24–26 min after the cessation of IBMX perfusion (Fig. 6A and B, Table 1).

Imidazole, an activator of PDE, has been reported to block some hormonal and transmitter actions mediated by cAMP (NAKANO *et al.*, 1970; TAKAGI *et al.*, 1972). Within 6 min after adding 5 mM imidazole, the DA-induced outward current and the forskolin-induced outward current were reduced to about 45% of the control ($43.8 \pm 7.9\%$, mean $\pm$ S.D., $n=5$) and to about 65% of the control ($64.8 \pm 4.2\%$, mean $\pm$ S.D., $n=3$), respectively (Fig. 7B and E, Table 1).

Tolbutamide has been shown to inhibit cAMP-dependent protein kinase activity (KANAMORI *et al.*, 1976). Tolbutamide applied in the bath at a concentration of 5 mM for 5 min caused a marked decrease in the DA-induced outward current to about 30% of the control ($29.2 \pm 9.4\%$, mean $\pm$ S.D., $n=3$) (Fig. 7C, Table 1). Similarly 1 mM tolbutamide bath-applied for 6 min reduced the forskolin-induced outward current to about 34% of the control ($33.8 \pm 6.0\%$, mean $\pm$ S.D., $n=3$) (Fig. 7F, Table 1). These results suggest that both the DA-induced outward current and the forskolin-induced outward current might be due to activation of cAMP-dependent protein kinase.

We further examined the DA-induced outward current and the forskolin-induced outward current in the presence of the DA antagonist, ergometrine maleate, which has been shown to block the DA receptor in molluscan neurons

Fig. 6. Effects of 50 μ M IBMX on the DA-induced outward current (A) and on the forskolin-induced outward current (B). Holding potential was -45 mV. DA and forskolin were ejected by a single constant pulse (400 ms, 1.9 kg/cm² in A; 600 ms, 1.9 kg/cm² in B), respectively. A: top, control; middle, 6 min after the ILD neuron was exposed to IBMX; bottom, 26 min after washout. B: top, control; middle, 6 min after the ILD neuron was exposed to IBMX; bottom, 24 min after washout. Note that the half decay times of the DA-induced and the forskolin-induced outward current was prolonged when the neuron was exposed to IBMX.

(ASCHER, 1972). Figure 7A and D illustrates typical records of the DA-induced outward current and the forskolin-induced outward current in the presence of 10 μ M ergometrine maleate. The DA-induced outward current was completely abolished by low concentrations of bath-applied ergometrine maleate. (Fig. 7A, middle). On the other hand, the forskolin-induced outward current persisted in the presence of ergometrine maleate (Fig. 7D, middle).

Effect of GDP analogue

Recently, ECKSTEIN *et al.* (1979) synthesized a GDP analogue, GDP β S, which can be phosphorylated or hydrolyzed very slowly. This analogue binds to the GDP binding site on the regulatory subunit of adenylate cyclase and prevents the stimulation of the cyclase by hormones or neurotransmitters (CASSEL *et al.*, 1979). Micromolar concentration of GDP β S inhibits *Aplysia* adenylate cyclase activity and also selectively inhibits the K⁺ conductance increase elicited by 5-HT (LEMON and LEVITAN, 1984). Therefore, we studied its effect on the DA-induced outward current and the forskolin-induced outward current. Adenylate cyclase inhibitor,

Fig. 7. Effects of 10 μ M ergometrine maleate (A and D), 5 mM imidazole (B and E), and 1–5 mM tolbutamide (C and F) on the DA-induced outward current and on the forskolin-induced outward current. Holding potentials were -50 mV in A, -40 mV in B–F. DA was pressure-ejected by a single constant pulse (100 ms, 2.0 kg/cm² in A; 400 ms, 2.0 kg/cm² in B; 600 ms, 2.0 kg/cm² in C). Forskolin was pressure-ejected by a single constant pulse (400 ms, 1.9 kg/cm² in D–F). A: top, control; middle, 6 min after the ILD neuron was exposed to ergometrine maleate; bottom, 12 min after washout. B: top, control; middle, 6 min after the neuron was exposed to imidazole; bottom, 15 min after washout. C: top, control; middle 5 min after the neuron was exposed to 5 mM tolbutamide; bottom, 12 min after washout. D: top, control; middle, 5 min after the neuron was exposed to ergometrine maleate; bottom, 12 min after washout. E: top, control; middle, 6 min after the neuron was exposed to imidazole; bottom, 12 min after washout. F: top, control; middle, 6 min

Table 1. Summary of the properties of the DA-induced response and the forskolin-induced response.

	DA-response	F-response
E_{reversal}	-80.1 mV	-78.5 mV
$1/10 [K^+]_o$	Increase $172.8 \pm 19.2\%$ ($n=3$)	Increase 156.8% ($n=1$)
$1/2 [Cl^-]_o$	No effect ($n=3$)	No effect ($n=3$)
Ergometrine maleate	Blocked ($n=3$)	No effect ($n=3$)
Imidazole	Decrease $43.8 \pm 7.9\%$ ($n=5$)	Decrease $64.8 \pm 4.2\%$ ($n=3$)
IBMX	Half decay time Increase $150.1 \pm 6.3\%$ ($n=3$)	Half decay time Increase $157.3 \pm 11.3\%$ ($n=3$)
Tolbutamide	Decrease $29.2 \pm 9.4\%$ ($n=3$)	Decrease $33.8 \pm 6.0\%$ ($n=3$)
GDP β S	Decrease $60.7 \pm 13.5\%$ ($n=3$)	Decrease $67.6 \pm 1.6\%$ ($n=3$)

Values are mean $\pm$ S.D.

GDP β S, applied in the bath at a concentration of $5 \mu\text{M}$ for 8 min caused a decrease in the DA-induced outward current to about 60% of the control ($60.7 \pm 13.5\%$, mean $\pm$ S.D., $n=3$) and in the forskolin-induced outward current to about 67% of the control ($67.6 \pm 1.6\%$, mean $\pm$ S.D., $n=3$) (Fig. 8A and B, Table 1).

DISCUSSION

In a previous paper (SAWADA *et al.*, 1980), we identified three neurons in the abdominal ganglion of *Aplysia kurodai* that responded with ILD to a single stimulation of the siphon nerve and to bath and iontophoretic application of DA, and reported that the ILD is due to an increase in K^+ conductance mediated by cAMP. The results presented in this study using the same identified ILD neurons under voltage-clamp confirmed this.

after the neuron was exposed to 1 mM tolbutamide; bottom, 12 min after washout. Note that $10 \mu\text{M}$ ergometrine maleate completely blocked the DA-induced outward current but the forskolin-induced outward current persisted in the presence of ergometrine maleate.

Fig. 8. Effects of $5 \mu\text{M}$ GDP β S (GDP analogue, adenylate cyclase inhibitor) on the DA-induced outward current (A and B left) and on the forskolin-induced outward current (B right). Holding potentials were -45 mV in A, -50 mV in B. DA was pressure ejected by a single constant pulse (400 ms, 1.9 kg/cm^2 in A; 100 ms 1.8 kg/cm^2 in B left). Forskolin was pressure ejected by a single constant pulse (800 ms, 1.8 kg/cm^2 in B right). A: top, control; middle, 8 min after the ILD neuron was exposed to $10 \mu\text{M}$ GDP β S; bottom, 21 min after washout. B left: top, control; middle, 8 min after the neuron was exposed to $5 \mu\text{M}$ GDP β S; bottom, 28 min after washout. B right: top, control; middle 8 min after the neuron was exposed to $5 \mu\text{M}$ GDP β S; bottom, 29 min after washout.

The present study demonstrates that micropressure ejection of DA onto the neuropil of the ILD neuron induces an outward current associated with an increase in K^+ conductance. Forskolin directly ejected onto the same neuropil induces similar current, and these currents might be due to activation of a cAMP-dependent protein kinase. The hypothesis that the dopaminergic receptors on the membrane of the ILD neuron mediate their actions on a K^+ conductance via an increase in intracellular cAMP levels caused by activation of a cAMP-dependent protein kinase is supported by the following observations. (1) Bath-applied forskolin, a potent activator of adenylate cyclase (SEAMON *et al.*, 1981) potentiated the DA-induced outward current that is sensitive to the extracellular K^+ concentration. (2) Forskolin ejected onto the neuropil of the ILD neuron mimicked the DA-induced

response, producing an outward current associated with an increase in K^+ conductance. (3) Both the DA-induced outward current and the forskolin-induced outward current are affected by a PDE inhibitor (IBMX) or activator (imidazole) in the perfusing *Aplysia* Ringer as well as inhibitors (tolbutamide) of cAMP-dependent protein kinase (KANAMORI *et al.*, 1976). (4) GDP β S, an inhibitory substance to the adenylate cyclase (ECKSTEIN *et al.*, 1979; LEMOS and LEVITAN, 1984), inhibited both the DA-induced outward current and the forskolin-induced outward current.

The DA-induced conductance increase in the ILD neuron of *Aplysia kurodai* described in this paper is analogous to that observed in the medial pleural neurons of *Aplysia californica* (ASCHER, 1972) and in the *Aplysia* burster R15 (CHESNOY-MARCAIS, 1984). In the experiments of CHESNOY-MARCAIS (1984), short iontophoretic pulses of DA induce an increase in K^+ conductance rather than a blockade of the slow inward current (SIC) that has been proposed by LEWIS *et al.* (1984) to explain the effect of DA on R15. Ligation experiments of the axon and simultaneous monitoring of $[Ca^{2+}]_i$ by the intracellular Arsenazo III absorbance have clearly shown that DA reduced calcium influx into the axodendritic tree of R15 during small depolarizing commands and the reduction in $[Ca^{2+}]_i$ in the axodendritic region could actually be quite large and could account for a significant portion of the net SIC (LEWIS *et al.*, 1984). CHESNOY-MARCAIS (1984) has reported furthermore that 1-min exposure to 100 μ M DA increased a resting K^+ current in R15. The inhibitory responses mediated by an increase in K^+ conductance following DA application in *Aplysia* cerebral ganglion neurons were found to be dependent on extracellular Na^+ and Ca^{2+} as well as intracellular Na^+ and Ca^{2+} concentrations (ASCHER and CHESNOY-MARCAIS, 1982; CHESNOY-MARCAIS and ASCHER, 1983), but the mechanism of this slow current has not been analyzed. However, for the long duration response like the ILD described in this paper, it is most likely that a specific conductance increase might be mediated by intracellular metabolism of cAMP to produce a physiological response.

The nervous system of molluscs contains a dopamine sensitive adenylate cyclase (CEDAR *et al.*, 1972; TREISTMAN and LEVITAN, 1976; OSBORNE, 1977; DETERRE *et al.*, 1982). Recently, using a single-cell microassay, Deterre and his coworkers have demonstrated that 5-HT and DA stimulate the adenylate cyclase activity in identified neurons of *Helix*, and concluded that the cAMP-mediated decrease in K^+ conductance by both 5-HT and DA resulted from the activation of two independent receptors (DETERRE *et al.*, 1982). Furthermore, cyclic nucleotides mediate a long-lasting synaptic hyperpolarization in the *Aplysia* bursting neuron and are capable of inducing bursting pacemaker activity in the usually silent metacerebral giant neuron (DRAKE and TREISTMAN, 1981).

The effects of activating the DA receptors of the ILD neuron, either directly by the micropressure ejection of DA onto the neuropil or indirectly by stimulation of the siphon nerve, can be potentiated by low doses of both IBMX and forskolin: IBMX would increase the effective amount of cAMP generated following receptor

activation by inhibiting the PDE that metabolizes cAMP; forskolin, on the other hand, would bring about the same effect by increasing the sensitivity of the adenylate cyclase complex to receptor activation, thus increasing the amount of cAMP generated by a given dose of agonist (SEAMON and DALY, 1982; SEAMON and WETZEL, 1984). Furthermore, increasing cAMP levels by forskolin, an adenylate cyclase activator, that do not involve receptor activation should also mimic the effect of DA. In view of the unique properties of forskolin as an activator of adenylate cyclase, SEAMON *et al.* (1981) proposed that the mimicking of the effect of an agonist by forskolin should be considered as a new criterion for cAMP mediation.

Since the described DA-induced outward current and the forskolin-induced outward current are both sensitive to the presence of PDE inhibitor (IBMX) or activator (imidazole) in the medium as well as tolbutamide, an inhibitor of protein kinase reaction (KANAMORI *et al.*, 1976), it is likely that the mechanism underlying the development of these currents includes the activation of a cAMP-dependent protein kinase, the phosphorylating action of which is necessary for the appearance of the corresponding ionic conductance on some functionally important proteins. Although we cannot be absolutely sure that DA stimulates the adenylate cyclase activity in the ILD neuron without using a single-cell cAMP microassay the action of DA on the described membrane conductance might represent the final part of the second messenger response by which the action of transmitter is finally transformed to electrical signals of the membrane of the ILD neuron. Recently it has been shown that extracellular application of PDE inhibitor (theophylline or IBMX) increased the amplitude of the cAMP-induced inward current. Imidazole, tolbutamide, and lowering the temperature reduced the amplitude of this current (KONONENKO *et al.*, 1983; HARA *et al.*, 1985). From such evidence it has been concluded that the cAMP-induced inward current is due to activation of a cAMP-dependent protein kinase in an identified *Helix* neuron (KONONENKO *et al.*, 1983) and in an unidentified neuron L5 of *Aplysia* (HARA *et al.*, 1985). Forskolin mimics the effects of DA by increasing the activity of adenylate cyclase through a direct activation of its catalytic subunit (SEAMON and WETZEL, 1984). WALSH and BYRNE (1984) observed that actions of low concentrations of forskolin mimic a 5-HT-sensitive decrease in K^+ conductance in tail sensory neurons of *Aplysia*. Furthermore, extracellular application of forskolin induces an inward current in the FMRF-amide-sensitive neuron of *Helix* (COLOMBAIONI *et al.*, 1985) and in identified neurons of the viscerio-abdominal ganglion of *Helix* sensitive to 5-HT (DETERRE *et al.*, 1982). However, CONNOR and HOCKBERGER (1984) have shown that bath application of forskolin (100 μ M) had no effect on the *Archidoris* neurons that respond with induction of an inward current to intracellular injection of cAMP.

In many preparations, receptor-activated long-term changes in the properties of ion channels can be shown to be mediated via the actions of second messengers such as nucleotides or calcium (SIEGELBAUM and TSIEN, 1983). In the squid Schwann cell, EVANS *et al.* (1985) have reported that the PDE inhibitor, theophylline, the

adenylate cyclase activator, forskolin, and a cyclic nucleotide analogue, CPTcAMP, all mimicked the long-lasting hyperpolarization induced by activation of the acetylcholine receptors. STRONG (1984) had studied the modulation of K^+ current kinetics in bag cell neurons of *Aplysia* by forskolin and reported that the bath application of forskolin and PDE inhibitor to *Aplysia* cultured bag cells markedly speeds up the inactivation kinetics of the A-current at all potentials, while having little effect on the steady-state parameters of inactivation or on the activation process.

On the other hand, DE PEYER *et al.*, (1982) reported that addition of the catalytic subunit of cAMP-dependent protein kinase to internally perfused molluscan neurons increased a Ca^{2+} -dependent net outward current. Furthermore, EWALD and ECKERT (1983) have recently demonstrated that cAMP analogues potentiated a rapid K^+ current activated by an intracellular injection of Ca^{2+} . In the experiments of MADISON and NICOLL (1982), the K^+ conductance diminished by cAMP was activated by intracellular Ca^{2+} . In bag cells of *Aplysia*, cAMP appears to diminish a Ca^{2+} -activated K^+ conductance (KACZMAREK and STRUMWASSER, 1984). At the present time it is not known whether the increased cAMP levels release calcium from intracellular stores, and thereby activate the K^+ conductance in the ILD neuron. We have not addressed the role of Ca^{2+} in the ILD response. Thus, the simplest explanation we can provide for our results is that the DA receptor of the ILD neuron mediates its effects via the activation of adenylate cyclase.

The adenylate cyclase is inactivated by the hydrolysis of GTP to GDP by a GTPase closely allied to the N protein, and $GDP\beta S$ apparently inhibits the cyclase by competing with both GTP and GDP for the guanyl nucleotide binding site on this protein (CASSEL *et al.*, 1979). At micromolar concentrations, $GDP\beta S$ inhibits the stimulation of adenylate cyclase by 5-HT in particulate fractions from *Aplysia* ganglia and inhibits the increase in K^+ conductance normally elicited by 5-HT in *Aplysia* neuron R15 (LEMON and LEVITAN, 1984). The findings obtained by using an adenylate cyclase inhibitor, $GDP\beta S$, are consistent with adenylate cyclase activation being a necessary step in the sequence of events leading to activation of a specific K^+ conductance by DA in the ILD neuron. Forskolin interacts with several components of adenylate cyclase: the regulatory subunits Ni and Ns, and the catalytic subunit or a closely associated protein (BAROVSKY *et al.*, 1984; SEAMON and WETZEL, 1984). This could explain why bath-applied $GDP\beta S$ at a concentration of $5\mu M$ also inhibits an increase in K^+ conductance induced by forskolin (Fig. 8B, Table 1).

It is unlikely that a change in K^+ conductance is the only response to an increase in the intracellular levels of cAMP in the ILD neuron. It seems more likely that the changes in cAMP level co-ordinate changes of other candidates of second intracellular messengers, inositol trisphosphate and diacylglycerol (BERRIDGE, 1984; JOSEPH *et al.*, 1984; ORON *et al.*, 1985).

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