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INHIBITORY AND EXCITATORY EFFECTS OF DOPAMINE ON *APLYSIA* NEURONES

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SUMMARY

1. Electrophoretic application of dopamine (DA) on *Aplysia* neurones elicits both excitatory and inhibitory effects, which in many cases are observed in the same neurone, and often result in a biphasic response.

2. The DA receptors are localized predominantly on the axons. Desensitization, which occurs after repeated injections or with bath application of DA, is more marked for excitatory responses.

3. Tubocurarine and strychnine block the DA excitatory responses without affecting the inhibitory ones, which can be selectively blocked by ergot derivatives. It is concluded that the excitatory and inhibitory effects are mediated by two distinct receptors.

4. The two DA receptors can be pharmacologically separated from the three ACh receptors described in the same nervous system.

5. In some neurones the dopamine inhibitory responses can be inverted by artificial hyperpolarization of the membrane at the potassium equilibrium potential, E_K, indicating that dopamine causes a selective increase in potassium permeability.

6. In other neurones the reversal potential of dopamine inhibitory responses is at a more depolarized level than E_K, but can be brought to E_K by pharmacological agents known to block the receptors mediating the excitatory effects of DA.

7. In still other neurones, the hyperpolarization induced by DA cannot be inverted in normal conditions, but a reversal can be induced by ouabain or by the substitution of external sodium by lithium. These results are discussed in terms of an hypothesis in which dopamine increases the potassium permeability of a limited region of the axonal membrane.

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8. It is concluded that a selective increase in potassium permeability probably accounts for all dopamine inhibitory effects in the neurones studied.

INTRODUCTION

Dopamine (DA) is the main catecholamine present in the nervous system of Molluscs (Sweeney, 1963; Dahl, Falck, Von Mecklenburg, Myhrberg & Rosengren, 1966) where it appears to meet several of the criteria proposed for the identification of a synaptic transmitter (Tauc, 1967). Most of DA effects observed on molluscan neurones (Kerkut & Walker, 1961; Gerschenfeld, 1964; Walker, Woodruff, Glaizner, Sedden & Kerkut, 1968), as well as on other neurones (cf. Hornykiewicz, 1966; Phillis, 1970), were described as inhibitory.

However, in *Aplysia*, the DA effects are both inhibitory and excitatory, and the first purpose of the present work was to characterize the receptors mediating these two effects. The second purpose was to determine the ionic mechanisms of the inhibitory responses. Initially (Ascher, 1968), a simple change in ionic permeability did not seem sufficient to explain the 'anomalous' behaviour of some of the DA inhibitory responses. The data presented below, however, suggest that these anomalies are due to the properties of the DA receptors, and not to a peculiarity of the ionic mechanism underlying the inhibition.

METHODS

Most experiments were performed on *Aplysia californica* obtained from Dr R. Fay, Pacific Bio-Marine Suppy Co. Some experiments used *A. fasciata* and *A. rosea*, obtained from the French coasts. No differences between species were observed with regard to DA responses.

The isolated visceral or pleural ganglia were pinned in a transparent chamber, and observed by transillumination. The neurone somata were exposed by cutting the outer connective tissue sheath along a lateral margin and reflecting it on one side (Otsuka, Kravitz & Potter, 1967). Exposure of the axonic region, when attempted, was obtained by removing with forceps the surrounding cell bodies and washing the preparation thoroughly. Half of the experiments were done on ganglia which had been kept 24 hr at 6° C. No difference was observed between these and freshly dissected ganglia.

The neurones were penetrated with double-barrelled micro-electrodes made with a de Fonbrune microforge. Both barrels of the micro-electrodes were filled with K_2SO_4 (0·5 M) and only electrodes showing minimal interbarrel coupling (< 50 kΩ) and having resistances of less than 10 MΩ were used. The 'polarizing barrel' was connected to a variable d.c. source and used to fix the membrane potential. Square hyperpolarizing current pulses of constant intensity could also be fed through this barrel and allowed an evaluation of the membrane resistance.

Injections of dopamine (DA) and acetylcholine (ACh) on the neuronal surface were made with double-barrelled micropipettes (similar to those used for intracellular recording and polarization) filled with DA HCl (200 mg/ml.) or ACh Cl (20 mg/ml.).

The injection currents (lasting 0·1–1·0 sec) were monitored on an oscilloscope; they were usually higher for the DA injections. A braking current of a few nA was applied to each pipette; its value was empirically chosen as the minimal value with which a stable response was obtained for injections separated by about 5 min. Adjustment of the braking current was of critical importance in the study of DA excitatory effects, which, as shown below, are extremely sensitive to repeated applications. In many Figures, the injection current is not illustrated, but its constancy can be judged from the constancy of the amplitude of the artifact recorded with the intracellular electrode.

Temporary increases in the intracellular chloride concentration $[Cl^-]_i$ were usually obtained by interbarrel injection (Eccles, Eccles & Ito, 1964) from electrodes filled with KCl (3 M) instead of K_2SO_4 (0·5 M) normally used to fill both the polarizing and recording electrodes. In a few experiments, chloride was injected from a single, KCl-filled micro-electrode temporarily introduced in a neurone already penetrated with a double-barrelled, K_2SO_4-filled micro-electrode. Intracellular injection of tetraethylammonium (TEA) was similarly made with micro-electrodes filled with TEA Cl (2 M).

The extracellular medium was usually artificial sea water containing (in mM): $Na^+ = 480$; $K^+ = 10$; $Cl^- = 610$; $Ca^{2+} = 10$; $Mg^{2+} = 50$. The pH was adjusted to 7·8 by addition of about 10 mM Tris HCl. Variations in the extracellular potassium concentration $[K^+]_o$ between 2·5 and 20 mM were obtained by omitting or adding solid potassium chloride, without compensating for the resulting variation in the extracellular chloride concentration $[Cl]_o$ and osmotic pressure. Variations in $[Cl^-]_o$ were made by substituting for sodium chloride the sodium salts of isethionate or sulphate (see Kehoe, 1972*a*, for the composition of the solutions). In experiments using sulphate, controls were made to test the effects of a reduction of $[Ca^{2+}]_o$: the inhibitory DA effects on medial pleural neurones and R 15 (see below) were not affected by lowering $[Ca^{2+}]_o$ to 0·5 mM. The same does not apply for the excitatory effects of DA, as will be reported elsewhere (P. Ascher, in preparation).

Antagonists of DA and ACh responses were dissolved in sea water just before use. Gifts from Sandoz Laboratoires (methyl-ergometrine hydrogenomaleate), Lilly Research Laboratories (ergometrine maleate), Ciba Laboratories (tolazoline, phentolamine), and Smith, Kline and French Overseas Co. (phenoxybenzamine) are gratefully acknowledged.

Neurones of the visceral ganglion are named according to the nomenclature of Frazier, Kandel, Kupfermann, Waziri & Coggeshall (1967). Neurones of the pleural ganglia are named according to the description of Kehoe (1972*b*).

RESULTS

Characteristic types of responses

Most of the pharmacological analyses presented below were performed on three groups of pleural neurones which provided models for three 'types' of responses: predominantly excitatory, inhibitory, and excitatory-inhibitory (biphasic).

The large neurones of the anterior plot of the pleural ganglia which have been labelled *anterior pleural neurones* are typically depolarized by DA. The shape and the time course of the response vary greatly with the position of the injection pipette and with the value of the injection current.

However, as a rule, the response becomes polyphasic when the pipette is brought closer to the cell (Fig. 1, I). Similarly, for a given position of the pipette, increasing the injection current often leads to a polyphasic depolarization, of which the initial components are usually those requiring the higher currents (Fig. 2). Possible interpretations of these polyphasic responses will be considered in the discussion. In most experiments, the position of the pipette and the injection current were chosen in such a way that the response had a simple shape (see, however, Fig. 12).

The *medial pleural neurones* respond to DA by a slow, smooth hyperpolarization (Fig. 1, II). Times to peak between 10 and 50 sec were the most commonly encountered. Half-times for the return to the original potential were usually 3–4 times longer. In contrast to the excitatory responses, the inhibitory ones never showed a tendency to break into distinct components when the pipette was moved (Fig. 1, II) or when the injection current was modified.

The *posterior pleural neurones* were selected for the pharmacological characterization of biphasic, excitatory-inhibitory responses to DA. This kind of response is illustrated in Fig. 1, III (see also Figs. 5, 7, 8). The relative amplitudes of the two components depend on the polarization level (the inhibitory component can be inverted by hyperpolarizing the neurone), upon the value of the injection current, and upon the position of the injection pipette. Fig. 1, III shows a case where the depolarizing component disappeared entirely when the pipette was drawn away from the neurone, whereas the hyperpolarizing component remained.

Functionally, biphasic responses corresponded to excitatory-inhibitory effects, and when tested on neurones presenting a regular discharge of action potentials, DA induced a brief acceleration of the discharge usually followed by a prolonged silent period.

The fact that the ratio of excitatory to inhibitory effects in the posterior pleural neurones depends on the parameters of injection (current, position) raised the problem of whether it was possible to classify the neurones according to their response. Indeed, in the anterior pleural neurones which were described above as excited by DA, a late hyperpolarizing component could often be revealed by careful examination (see Fig. 2), and when DA was applied on artificially depolarized anterior pleural neurones, the late effect was shown to be a very effective inhibition (see Fig. 18). However, repeated observation and various dissection procedures never revealed an excitatory component in the DA responses of medial pleural neurones; and the lability of the excitatory component observed in posterior pleural neurones (Fig. 1, III) was never observed with the anterior pleural neurones. Thus, even if a group of neurones cannot be linked with a single shape of DA response, it seems that each functional group can be characterized by

a certain 'pattern' of response, defined by the proportions of excitatory and inhibitory effects. Up to now, our observations are these: (1) no neurone was identified for which the DA effect was exclusively excitatory; (2) predominantly excitatory effects, as described in the anterior pleural neurones, were also observed in the 'giant neurone' of the left pleural

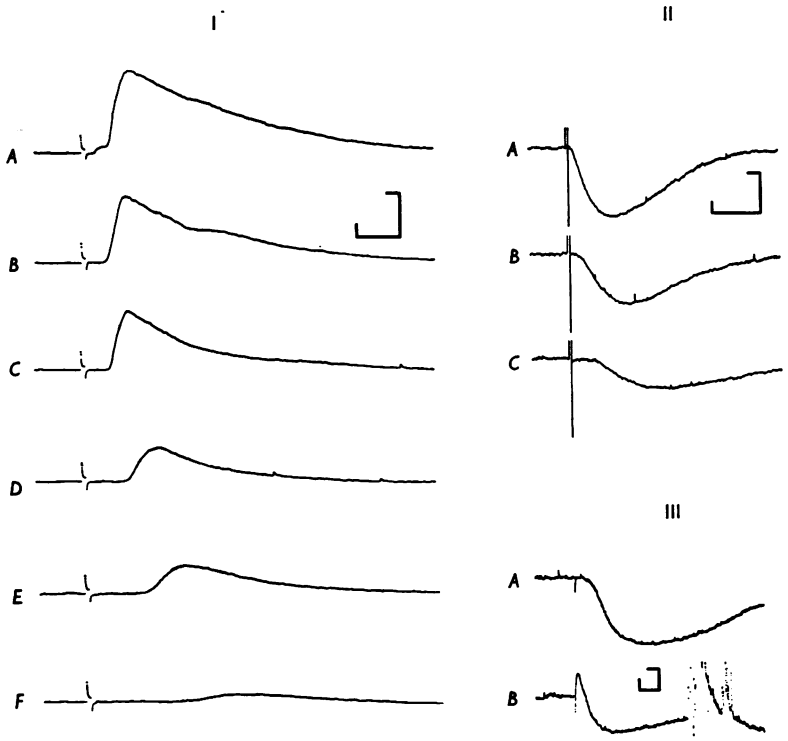

Fig. 1. Effects of distance on the DA responses. I. *Anterior pleural neurone.* From *A* to *F*, the injection pipette was withdrawn in steps of approximately 40 μ. In *B* the early inflection observed in *A* has disappeared. Initial membrane potential set at -80 mV. Calibration: 2 sec, 10 mV. II. *Medial pleural neurone.* From *A* to *C* the injection pipette was withdrawn in steps of 130 μ. Initial membrane potential -60 mV. Calibration: 10 msec, 5 mV. III. *Posterior pleural neurone.* From *A* to *B* the pipette was brought closer to the neurone. Initial membrane potential -60 mV. Calibration: 4 sec, 2 mV. The late part of the trace in *B* corresponds to spontaneous synaptic activity leading to the emission of spikes.

ganglion (Hughes & Tauc, 1961), in the giant neurone of the visceral ganglion (R 2 of Frazier *et al.* 1967) as well as in the visceral neurones LB and LC; (3) predominantly inhibitory effects are observed in the visceral RB neurones (the excitatory component being usually disclosed only after

pharmacological elimination of the inhibitory one – Fig. 10); (4) pure inhibitory effects, described above for medial pleural neurones, are also observed in the visceral neurone R 15.

Localization of the dopamine receptors

In early experiments, DA responses were seldom observed, whereas the responses to ACh injection were ubiquitous. It soon became clear that the difficulty in obtaining a response to DA injection was due to the fact that the DA receptors were localized predominantly, if not exclusively, on the axonal region, i.e. in the neuropile. The percentage of success in observing DA effects was greatly increased if extensive dissection of the neurones was carried out and, in these conditions, a response could often be obtained even if the pipette was pulled back rather far from the cell (Fig. 1, I). For most neurones, however, and especially for the larger ones like R 15 or R 2, dissection was not a sufficient condition, and had to be followed by a systematic search of an effective 'point'. As a rule this point was never close to the cell body. That was particularly clear in the largest neurones, like the 'giant' neurone of the left pleural ganglion, or the visceral neurone R 15. In these neurones, injection on the soma never produced a detectable response, even when the pipette was approached so closely that it eventually entered the cell during the current pulse.

That the sensitive spots correspond to some part of the axon was suggested by the fact that further advance of the pipette in the 'sensitive region' often resulted in a sudden depolarization, attributed to a penetration of the axon. This could be confirmed by visual observation in the case of the giant neurone of the left pleural ganglion, for it was possible to dissect the axon of this cell for some length under the microscope and to show that the sensitive region was always along the axon pathway. In the case of R 15 the axon was not seen; however, the sensitive spot was usually at nearly 1 mm from the cell body and situated in the region where Kandel, Frazier, Waziri & Coggeshall (1967) locate the contact between the axons of R 15 and L 10.

The need for a close positioning of the injection pipette introduced the danger of an 'electrical' artifact. This was often observed with the cell R 15, where DA normally elicits a purely hyperpolarizing response, but where, when the injection pipette was approached very close to the axon, a depolarization with no measurable latency often appeared in front of the hyperpolarizing response. That this early response was due to an electrical artifact was suggested by the fact that it inverted with reversal of the current pulse, and was unaffected by tubocurarine, which blocks the depolarizing effect of DA on other neurones (as well as those of ACh on this neurone).

Desensitization

Desensitization by repeated injections

The responses to successive injections of DA were markedly reduced when the injections were repeated at 'short' intervals, even when the two successive injections were delivered from two independent barrels of a double pipette. The effect was often detected for intervals of 3–5 min between the two successive injections.

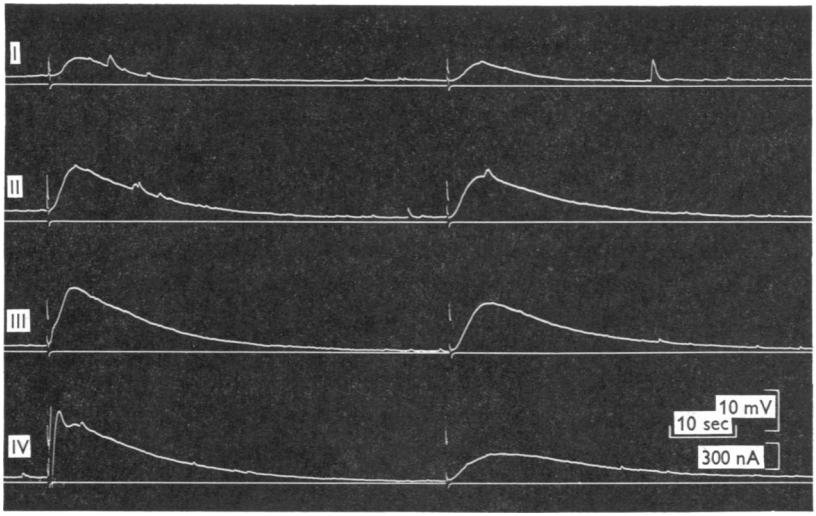

Fig. 2. Desensitization of DA receptors. Left pleural giant neurone. Two injections of DA from a single barrel were applied with an interval of 80 sec. When the injection current was increased (from I to IV), the complexity of the initial DA response increased. A fast-rising, short-latency component appeared for strength III; in IV this component triggered an action potential (of which the upper part is cut). In addition, the late hyperpolarizing component became more apparent from I to IV (the neurone was held at -60 mV between the injections). The behaviour of the second DA response indicates an increasing desensitization. In III the early component is nearly the only one reduced in the second injection. The later excitatory components are affected in turn for strength IV. The latest components of the response remain nearly unchanged, so that the half-time of the decay appears to increase.

The reduction of the second response could have been due to a persistent conductance change, since no DA response can be claimed to be devoid of a long-lasting inhibitory component. This interpretation could be excluded, however, for the reduction of excitatory effects: not only were the measured conductance changes very small after 10 or 20 sec, but, in addition, the

reduction of the second response was still observed after pharmacological blockade (see below) of the slow inhibitory component. Furthermore, the reduction of the DA response was selective; there was no effect on the ACh response, whether excitatory, inhibitory, or mixed.

The amount of desensitization in double-pulse experiments depends on the strength of the injection. This is illustrated in Fig. 2: desensitization is not detectable for the response to low current injections; it is most marked for the short latency components (see IV) which are brought into play for strong current injections.

Desensitization and bath application

The effects of bath application of DA mimic those of electrophoretic application when these are purely inhibitory. When they are mixed, the ratio of excitatory and inhibitory effects depends on the method of application.

Bath application of DA induces a long-lasting hyperpolarization of the *medial pleural neurones*. Up to concentrations of 10^{-6} M, the hyperpolarization shows little tendency to reduce with time. A slow return to the base line may start with higher concentrations, but the associated conductance increase persists (at least for 20 min, which was the longest period over which it was evaluated).

When the response to electrophoretically applied DA is tested on the background of DA perfusion, it appears to be strongly reduced, and nearly eliminated for concentrations of perfused DA of about 10^{-5} M. This cannot be explained simply by the increased membrane conductance, since this should equally affect the ACh potentials, which are only slightly reduced. This is illustrated in Fig. 3, where the ACh response is a potassium-dependent one (Kehoe, 1972*a*), i.e. is due to the same permeability change as the DA response (see below). On the other hand, the persistence of the conductance change produced by perfused DA suggests that the result is not due to a typical desensitization.

The *anterior pleural neurones*, which are predominantly excited by an injection of DA, are likewise excited by perfusion of DA. The excitatory response subsides rapidly, even with low (10^{-6} M) concentrations of DA (Fig. 4*B*), and the membrane resistance returns to its initial value. This still occurs when the superimposed inhibitory component has been eliminated by a specific antagonist, like methyl-ergometrine (see below). That the fading of the response is due to desensitization is further suggested by the fact that, if the DA perfusion is maintained, the depolarizing effects of injected DA are markedly reduced or even eliminated (Fig. 4*C*). For perfused DA concentrations of 10^{-6} M the reduction is much more marked than the corresponding effect observed with inhibitory responses.

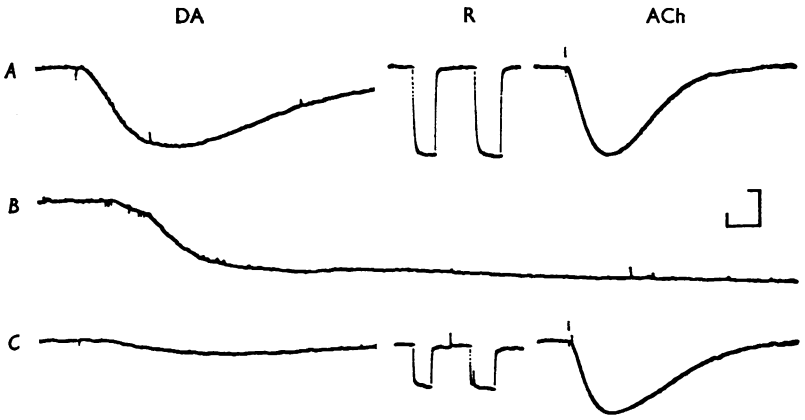

Fig. 3. DA perfusion on a medial pleural neurone. DA was applied electrophoretically in *A* and *C*, and perfused (10^{-5} M) in *B*. R: hyperpolarization elicited by a constant current square pulse. ACh: electrophoretic application of ACh. *A*: control. *B*: response to DA perfusion. *C*: as in *A*, but with DA in the bath. The membrane potential was held at -58 mV between the injections, i.e. at the reversal potential of the fast ACh response, leaving only the slow ACh response visible (see Kehoe, 1972*a*). Calibration; 10 sec, 2 mV.

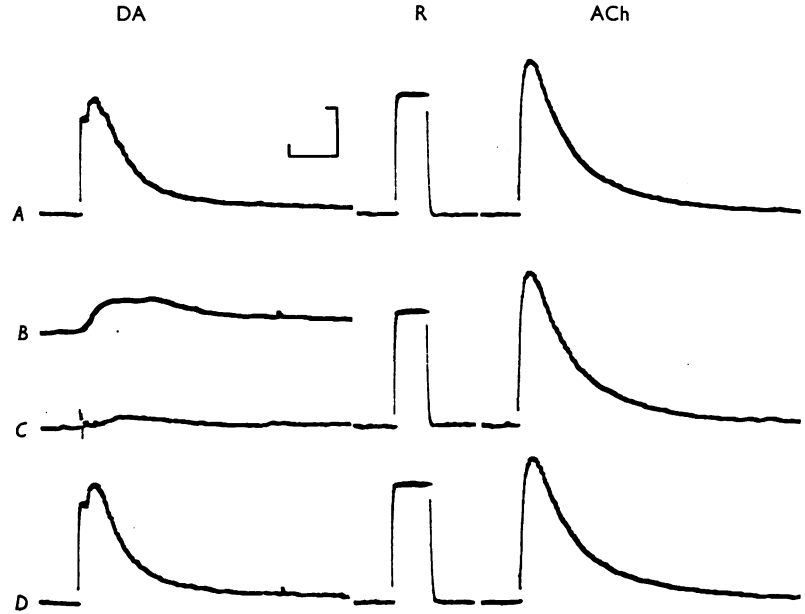

Fig. 4. DA perfusion on an anterior pleural neurone. DA was applied electrophoretically in *A*, *C* and *D*, and perfused (10^{-6} M) in *B*. *R*: depolarization elicited by a constant current square pulse ACh: electrophoretic application of ACh. The membrane potential was held at -80 mV between the injections. *A*: control; *B*: response to DA perfusion; *C*: as in *A*, but with DA in the bath; D: after washing. Calibration: 4 sec, 5 mV. Note the two peaks of the DA response.

When DA is perfused on the *posterior pleural neurones* (which show a biphasic response to an electrophoretic application of DA) one often observes only an inhibitory effect, as illustrated in Fig. 5. If the DA injection is repeated during the DA perfusion, the excitatory component of the biphasic response to injection appears to be reduced or eliminated. This indicates that the absence of an excitatory response to DA in perfusion is not due to an absence of effect of DA on the excitatory receptors,

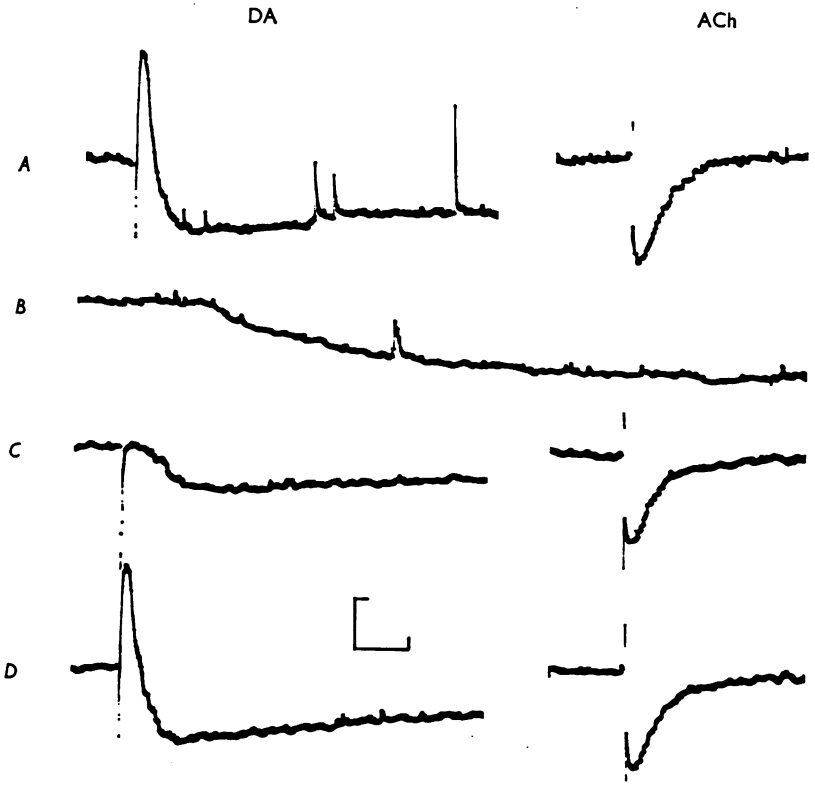

Fig. 5. DA perfusion on a posterior pleural neurone. *A*, control: electrophoretic applications of DA and ACh. The membrane potential was held at −45 mV between the injections. *B*: perfusion of DA (10^{-5} M) in the bath. *C*: as in *A*, but with DA (10^{-5} M) in the bath. *D*: as in *A*, after washing. Calibration: 4 sec, 2 mV.

but rather to the masking of receptor activation by another process (desensitization?). This process is more marked on excitatory receptors than on inhibitory ones (the excitatory response to injected DA was completely eliminated in the experiment of Fig. 5, while the inhibitory component was only reduced).

These results, which once more confirm the lability of excitatory effects elicited by DA, may explain why they were seldom seen before (see Discussion). In each of the experiments to be described here, intervals between injections were empirically determined in order to obtain stable responses. Five minute intervals were the most frequently used.

Pharmacological differentiation of two dopamine receptors

The main evidence suggesting that two different receptors mediate the excitatory and inhibitory effects of DA has been derived from the differential effects of two groups of compounds: curare (tubocurarine) and strychnine, on the one hand; ergot derivatives (ergometrine, methyl ergometrine, ergotamine) on the other.

It is known that the effects of *tubocurarine* and *strychnine* on molluscan neurones are not restricted to one type of receptor. Both curare and strychnine block two kinds of ACh receptors (Tauc & Gerschenfeld, 1962; Kehoe, 1972*b*); curare blocks as well certain serotonin receptors (Gerschenfeld & Stefani, 1966). On the other hand, the two drugs are not devoid of specificity for even at very high concentrations (10^{-3} M) they have no effect on other ACh or serotonin receptors (Kehoe, 1972*b*; Gerschenfeld, 1971).

Curare and strychnine show a similarly selective action on DA responses. A complete block of excitatory effects could be obtained without any alterations of the inhibitory effect. Fig. 6 shows the effects of increasing concentrations of tubocurarine on a pleural neurone excited by DA and inhibited by ACh. The two responses (recorded at a membrane potential where both were depolarizing) are reduced in similar proportions.

Although the complete elimination of either ACh or DA effects required concentrations often higher than 10^{-4} M strychnine, and still higher for curare, these same concentrations had no effect on the inhibitory DA responses of medial pleural neurones. That curare and strychnine are indeed selective antagonists of the excitatory effects of DA is further shown by their effects on biphasic responses, such as those illustrated in Fig. 7: both drugs transform the biphasic responses into purely inhibitory ones. This effect provides an analytical tool in the dissection of mixed DA responses, since it unmasks inhibitory components in responses which at first sight appear mainly excitatory (anterior pleural neurones), and reveals the complex character of seemingly simple inhibitory responses (RB neurones, Fig. 10).

Ergometrine has been reported by Walker *et al.* (1968) to antagonize the inhibitory effects of DA on snail neurones. When tested on *Aplysia* neurones, this compound and its methyl derivative were found to block the DA inhibitory effects, but to have no effect on the excitatory ones.

This selectivity is illustrated in Fig. 8 for methyl ergometrine. At a concentration of 10^{-5} M, the drug has no effect on the excitatory components of DA responses (on an anterior pleural neurone, II; on a posterior pleural neurone, III) whereas it eliminates the inhibitory response of the medial pleural neurone (I) as well as the inhibitory component of a biphasic

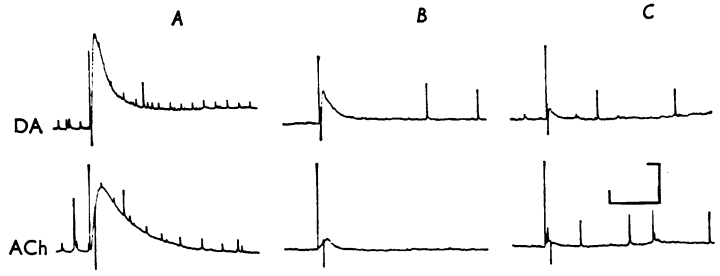

Fig. 6. Effects of tubocurarine on a visceral neurone excited by DA and inhibited by ACh. The membrane potential was held at −90 mV between the injections, so that the ACh response is depolarizing. *A*: control. *B*: tubocurarine 10^{-4} M. *C*: tubocurarine 10^{-3} M. Calibration: 4 sec, 5 mV.

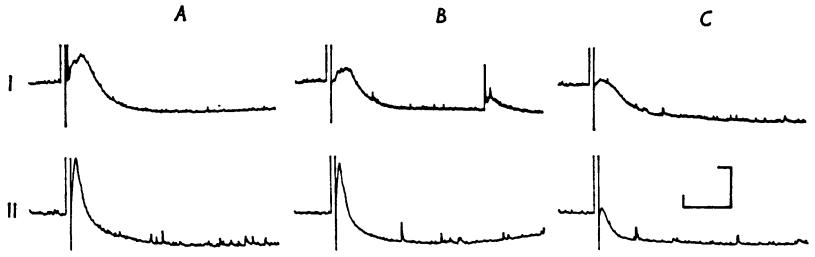

Fig. 7. Effects of tubocurarine and strychnine on biphasic DA responses of posterior pleural neurones. The membrane potential was held at −70 mV between the injections. (I) *A*: control. *B*: tubocurarine 10^{-4} M. *C*: tubocurarine 10^{-3} M. (II) *A*: control. *B*: strychnine 10^{-5} M. *C*: strychnine 10^{-4} M. Calibration: 10 sec, 5 mV.

response (III). These effects of ergometrine and methyl ergometrine appear after an exposure of a few minutes, are usually very difficult to reverse, and persist for more than 1 hr after washing.

Contrary to tubocurarine and strychnine, ergometrine and methyl ergometrine have agonist activity. Their blocking action is preceded by a small hyperpolarization, associated with a change of conductance. The change of conductance is likely to be the main cause of the non-specific reduction of DA excitatory responses, ACh excitatory and ACh inhibitory responses, which is observed for concentrations of ergometrine or methylergometrine above 10^{-4} M.

Particular attention was paid to the effects of methyl ergometrine on the neurone R 15 (Fig. 9) because of the suggestion that a characteristic synaptic input to this cell, known as inhibition of long duration (Tauc, 1960) might be due to a dopaminergic synapse (Ascher, Kehoe & Tauc, 1967; cf. Kerkut, Horn & Walker, 1969). R 15 is inhibited by DA and excited by ACh. Stimulation of the branchial nerve elicits a biphasic synaptic activity

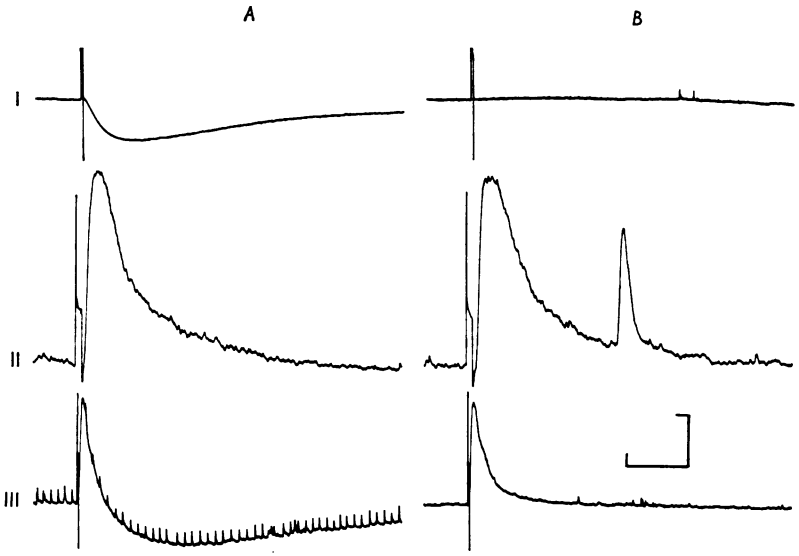

Fig. 8. Effects of methyl ergometrine on the DA responses of three different neurones: medial pleural neurone (I), anterior pleural neurone (II), and posterior pleural neurone (III). Initial membrane potential: −50 mV in I, −80 mV in II, and −60 mV in III. *A*: control. *B*: after application of methyl ergometrine 10^{-5} M. Calibration: 20 sec (I), 2 sec (II), and 10 sec (III); 5 mV.

known as ILD (Tauc, 1960), whereas stimulation of the left connective elicits a high amplitude excitatory synaptic potential which is presumed to be monosynaptic and cholinergic (Gerschenfeld, Ascher & Tauc, 1966). Methyl ergometrine causes a marked reduction of the DA response, while having no detectable effect on the ACh response. However, both the monosynaptic cholinergic synaptic potential and the biphasic ILD are reduced in about the same proportions. Further reduction of both synaptic inputs occurs with higher concentrations of methyl ergometrine. This suggests that methyl ergometrine has no specific effect on ILD, and that the reduction of the synaptic inputs is due to effects on 'non-synaptic' membranes of R 15 (note in Fig. 9 that methyl-ergometrine induces a change in the burst pattern) or of the presynaptic neurones.

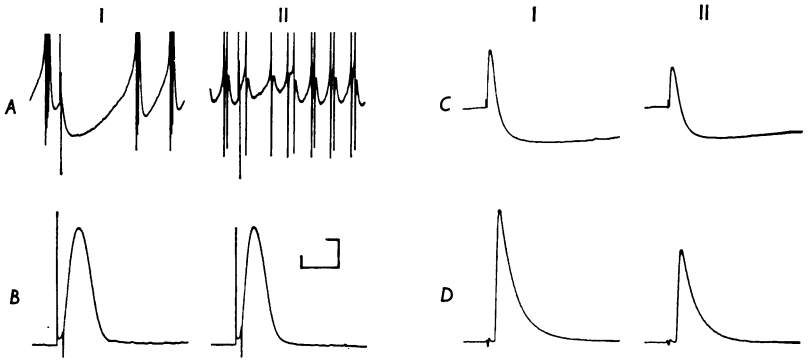

Fig. 9. Effects of methyl ergometrine on R 15. Four types of responses were analysed before (I) and after (II) addition of methyl ergometrine $2{\cdot}5 \times 10^{-5}$ M to the sea water perfusing the ganglion. These responses are the hyperpolarization triggered by a DA injection (*A*); the depolarization triggered by an ACh injection (*B*); the biphasic synaptic potential (ILD) triggered by stimulation of the branchial nerve (*C*); and the cholinergic e.p.s.p. triggered by stimulation of the right viscero-pleural connective (*D*). In *A* the neurone is not hyperpolarized and emits bursts of action potentials (of which the upper part is cut); the DA response starts at -45 mV. In *C* the initial membrane potential is -50 mV; in *B* and *D*, -90 mV. Calibration: *A*, 20 sec; *B*, 2 sec; *C*, 1 sec; *D*, 0·2 sec; ABD, 5 mV; *C*, 10 mV.

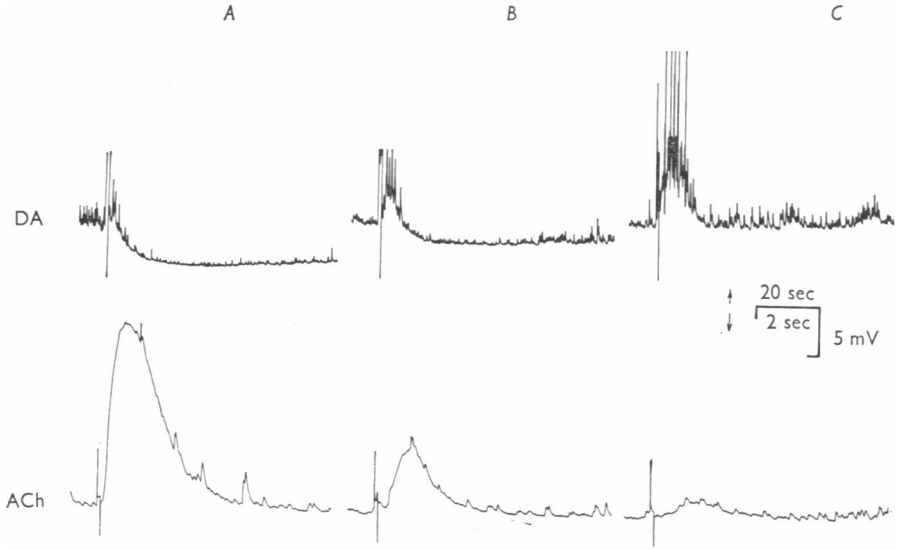

Fig. 10. Effects of ergotamine on a RB visceral neurone. The initial membrane potential was -60 mV for the DA electrophoretic injections, -80 mV for the ACh ones. *A*: control. *B*: ergotamine $7{\cdot}5 \times 10^{-6}$ M was applied for 50 min. *C*: after 1 hr under ergotamine plus 1 hr washing.

Ergotamine (10^{-6}–10^{5} M) mimicked the blocking effects of ergometrine, although with greater variability. Here again the blockade of inhibitory responses was irreversible. Fig. 10 illustrates the effects observed on the DA response of a RB visceral neurone. The response, which initially appeared to be purely inhibitory, was transformed by ergotamine into an excitatory response which was sufficient to trigger a burst of spikes.

Comparison of DA and ACh receptors

Like the effects of DA, the effects of ACh can be excitatory, inhibitory, or mixed (Tauc & Gerschenfeld, 1962; Wachtel & Kandel, 1971; Kehoe, 1972*c*). For the neurones where ACh and DA act in opposite directions, there is little doubt that they activate different receptors. Thus, for R 15

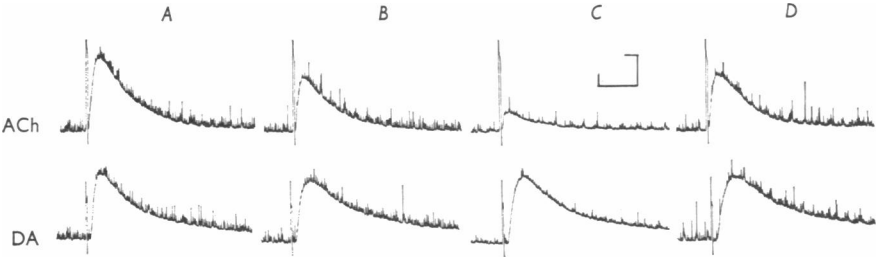

Fig. 11. Differential effects of hexamethonium on ACh and DA responses. Visceral neurone excited by ACh and DA. Initial membrane potential: −80 mV. *A*: control. *B*, *C*: hexamethonium chloride at 10^{-5} M (*B*) and 10^{-4} M (*C*). *D*: after washing. Calibration: 10 sec, 5 mV. Note in *C* the reduction of the spontaneous synaptic potentials, most of which are probably cholinergic.

and the RB neurones, the inhibitory effects of DA cannot be mediated by the same receptors as those responsible for the excitatory effects of ACh (Gerschenfeld, Ascher & Tauc, 1966; Frazier *et al.* 1967); conversely the excitatory effects of DA on LB neurones contrast with the inhibitory effects of ACh (Frazier *et al.* 1967). However, the possibility remained that some of the DA receptors might be slightly activated by ACh, or the reverse. This possibility was examined on two groups of neurones: the anterior pleural neurones (excited by DA and ACh) and the medial pleural neurones (inhibited by DA and ACh).

The excitatory effects of DA and ACh on the anterior pleural neurones were separated by hexamethonium and DA in perfusion. Hexamethonium, which is a selective antagonist of excitatory actions of ACh (Tauc & Gerschenfeld, 1962) had no effect on the excitatory DA responses up to the highest concentrations tested (10^{-3} M) (Fig. 11). Similarly DA in perfusion, which blocks the excitatory DA responses, had no effect on the excitatory ACh response (Fig. 4).

The inhibitory effects of DA on medial pleural neurones were similarly separated from the two inhibitory effects of ACh which were shown by Kehoe (1972*b*) to be respectively antagonized by curare and strychnine, on the one hand; tetraethylammonium and methylxylocholine, on the other. None of these compounds, at concentrations of 10^{-4} M, had any effect on the inhibitory effect of DA; conversely, methylergometrine suppresses the DA inhibitory effect without modifying the ACh response (see Kehoe, 1972*b*, Fig. 6).

Noradrenaline antagonists

Seven noradrenaline antagonists (phentolamine, tolazoline, phenoxybenzamine, chlorpromazine, pronethalol, dichloroisoprenaline, 1-(4-nitrophenyl)-2-iso-propylaminoethanol hydrochloride (INPEA)) were assayed in an attempt to characterize further the DA receptors. All these compounds were found to be better antagonists of the excitatory DA effects than of the inhibitory ones, although none was as selective in this respect as tubocurarine or strychnine. Although the seven compounds cause a fall in membrane resistance, this change is relatively small and does not account for the reduction of the DA responses. But in addition most of these compounds likewise reduce the ACh responses, whether excitatory or inhibitory.

Tolazoline is worth a special mention because at low concentration it enhances and prolongs ACh effects. This is illustrated in Fig. 12 for a chloride-dependent ACh inhibition, but it was also observed for the potassium-dependent ACh inhibition of the medial pleural cells, as well as for excitatory ACh effects. At doses higher than 10^{-4} M, and after prolonged application, a blocking effect is superimposed on the potentiation of the responses; this suggests that tolazoline shares with the other noradrenaline antagonists anti-ACh properties. The prolongation of the ACh responses is probably related to the anticholinesterasic effect described by Burn & Gibbons (1964).

Ionic mechanisms of the inhibitory effects

These mechanisms were analysed in three main groups of neurones: (1) the medial pleural neurones (Kehoe, 1972*b*); (2) the RB visceral neurones on which Gerschenfeld & Tauc (1964) first observed hyperpolarizing effects of catecholamines; (3) the R15 visceral neurone, which shares many synaptic inputs with the RB neurones, but presents a characteristic pattern of spontaneous activity ('parabolic burster' of Strumwasser, 1968).

All these neurones responded to a short DA injection (0·1–1·0 sec) by a prolonged hyperpolarization. On R15, the bursts were usually stopped,

then resumed with an altered pattern which often returned to normal only after many minutes.

In all neurones, DA could be shown to reduce the membrane resistance. The presence of 'anomalous rectification' (Kandel & Tauc, 1966) made it necessary to control that the reduction of resistance was not merely a consequence of membrane hyperpolarization. Thus, the resistance during the height of the DA induced hyperpolarization was always compared to that measured when the membrane was artificially hyperpolarized to the same level in the absence of DA injection. The relative change was usually more

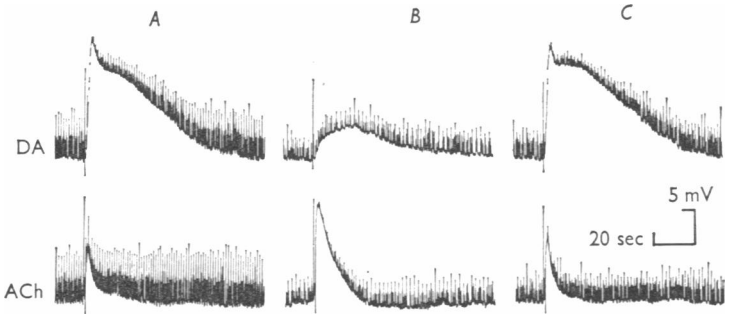

Fig. 12. Effects of tolazoline on a visceral neurone excited by DA and inhibited by ACh. Initial membrane potential: -90 mV. Pulse duration: 0·1 sec (DA) and 1 sec (ACh). *A*: control. *B*: tolazoline 10^{-4} M. *C*: after washing.

marked in medial pleural neurones than in R 15, a feature which may be linked with the distance between the DA receptors and the site of recording (soma).

The DA responses were not affected by variation of the chloride equilibrium potential (E_{Cl}). For example, substitution of half the external chloride by isethionate or sulphate did not induce any change in the DA response. Total substitution of chloride typically triggered a considerable synaptic 'noise' making it difficult to evaluate the DA response. On a few occasions, however, where reversal potentials could still be measured, they were found unchanged. Similarly, intracellular chloride injection did not change the characteristics of the DA response. A control of the efficacy of the injection was done in the medial pleural neurones, where the rapid ACh effect reverses at E_{Cl} (Kehoe, 1972*a*). Even when E_{Cl} was shifted by 15 mV there was no detectable change in the inversion potential of the DA response.

Reversal potentials identical to E_K

Medial pleural neurones

The DA inhibitory response of the medial pleural neurones inverts around -80 mV (-78 to -82 mV) which seems to be the potassium equilibrium potential, E_K (see Kehoe, 1972*a*). Thus, it appeared likely that the DA effect was exclusively due to an increase in potassium permeability.

The DA inversion potential was indeed observed to remain equal to E_K when $[K^+]_o$ was varied. Fig. 13 illustrates the dramatic effect of a reduction of $[K^+]_o$ from 10 to 2·5 mM, at which concentration the DA response became hyperpolarizing at all potential levels shown, even at -100 mV.

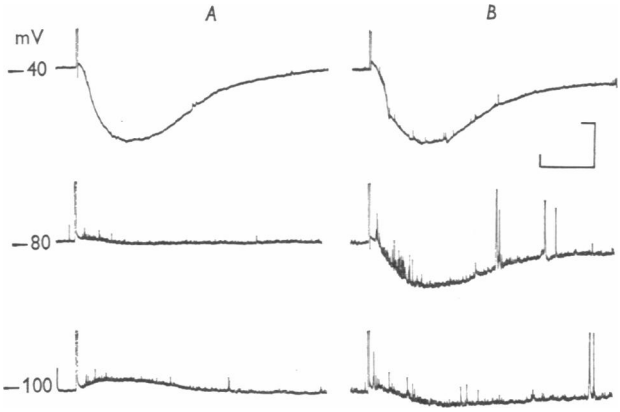

Fig. 13. Effects of variations of $[K^+]_o$ on the DA response of a medial pleural neurone, analysed at -40, -80 and -100 mV. *A*: $[K^+]_o$ = 10 mM. *B*: $[K^+]_o$ = 2·5 mM. Calibration: 5 mV, 40 sec.

A number of experiments were performed to obtain a quantitative evaluation of the inversion potential for various values of $[K^+]_o$. However, since the DA response is extremely slow, and since the injections were usually separated by 5 min, it was not possible to avoid changes in internal ionic concentrations (as a result of the alteration of external concentrations) during the measurement of the new inversion potential. In spite of these limitations, it is possible to state that the movement of the inversion potential of the DA response for a twofold change in $[K^+]_o$ (from 10 to 5 or to 20 mM) is approximately that predicted by the Nernst equation, i.e. 17 mV at 20° C. This result is identical to that obtained in the same neurones by Kehoe (1972*a*) for the slow component of the ACh potential. Indeed, in all experiments where ACh and DA were tested on a same medial pleural neurone, the DA response and the slow ACh response inverted at the same potential (cf. Kehoe, 1967).

Reduction of $[K^+]_o$ below 3 mM brought the inversion potential beyond -100 mV, in a potential region where observations are in general difficult. In the few cases where the inversion potential could be measured with some accuracy, its value seemed to depart from the prediction of the Nernst equation. This does not necessarily mean that DA had any other effect than to increase the potassium permeability. Low values of $[K^+]_o$ are likely to slow the sodium-potassium pump and to change the juxtacellular values of $[K^+]_o$ in the neuropile region (see Discussion).

RB visceral neurones

The inhibitory effects of DA on the RB visceral neurones are usually very striking since these neurones normally present a marked irregular spontaneous activity. Although at first glance the effect resembles that observed in the medial pleural neurones, differences appear when the responses are recorded on a background of artificial hyperpolarization. In most RB neurones the DA response breaks into early and late components, the earlier one inverting at more depolarized levels than the later ones, which occasionally cannot be inverted at all (see Fig. 16*B*). These complex responses will be analysed in detail below. However, it should be noted that, on a few occasions, the DA effects on the RB neurones behaved as those described in the pleural neurones: the response inverted at -80 mV in 10 mM potassium, did not show distinct components, and the inversion potential shifted by about 17 mV when $[K^+]_o$ was halved or doubled.

The ACh effect on the RB neurones is predominantly depolarizing, but sometimes involves an inhibitory component due to an increase in potassium permeability and triggered by activation of the same receptor as that controlling the potassium-dependent ACh response in the medial pleural neurones. When the excitatory effects of ACh are eliminated by hexamethonium (Tauc & Gerschenfeld, 1962) both ACh and DA induce hyperpolarizing effects which reverse around -80 mV. Thus it seems that the potassium equilibrium potentials of the medial pleural neurones and of RB visceral neurones are of the same order. Differences of a few mV, such as those reported by Kunze, Walker & Brown (1971) cannot, however, be excluded.

Effects of caesium and rubidium

The reversal potential of the DA responses was not modified by addition to the perfusion medium of caesium up to concentrations of 10 mM, whereas it was shifted in the depolarizing direction by addition of rubidium. The latter is illustrated in Fig. 14: addition of 10 mM rubidium shifted the inversion potential by approximately 10 mV, in a neurone where an increase of $[K^+]_o$ by 10 mM produced a shift of 17 mV. These results are

similar to those obtained by Kehoe (1972*a*) in her study of the potassium-dependent ACh potentials, and thus lead to a similar value (0·5) for the ratio of rubidium and potassium permeabilities of the 'potassium channels' opened by DA or ACh.

Fig. 14 also illustrates effects of rubidium on membrane resistance. The input resistance was increased at high membrane potentials, and decreased near the resting potential. These changes were also observed with caesium.

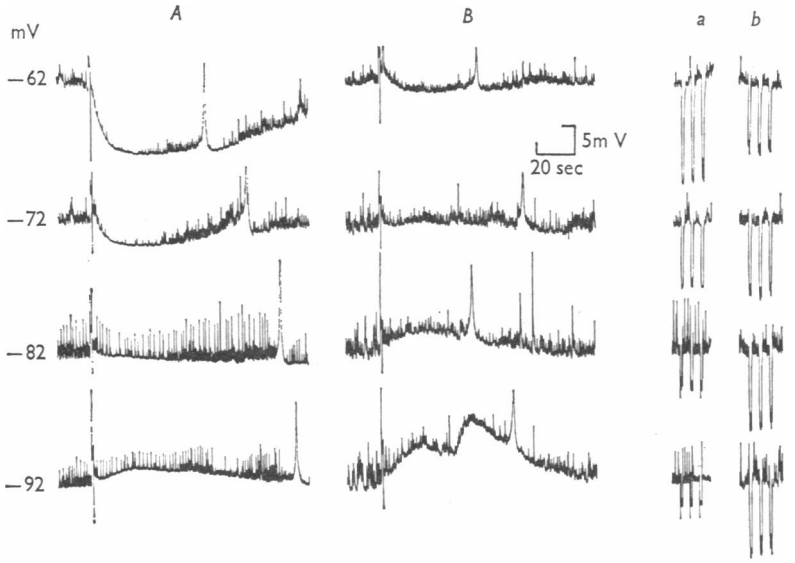

Fig. 14. Effects of rubidium on DA response and membrane resistance of a RB visceral neurone. *AB*: DA response before (*A*) and after (*B*) addition of 10 mM rubidium to normal sea water containing 10 mM potassium. *ab*: measurement of resistance before (*a*) and after (*b*) addition of rubidium. The anomalous rectification is abolished.

They occurred with variable delays. As a rule, they lead to an attenuation of the anomalous rectification (Ozeki, Freeman & Grundfest, 1966) or even to a situation where, after addition of caesium, the membrane resistance increased with hyperpolarization. However, the increased resistance under caesium does not result in an increased DA potential, which suggests that caesium also reduces the synaptic conductance.

Intracellular injection of TEA

TEA is known to interfere with a number of potassium permeability changes (cf. Hille, 1970). In the medial pleural neurones, as well as in the RB visceral neurones where the DA response inverted around −80 mV, intracellular TEA completely blocked the DA response. Fig. 15 illustrates

such an effect for a RB visceral neurone, in which the DA response was nearly abolished both at -45 mV (where it was hyperpolarizing) and at -95 mV (where it was depolarizing). TEA also reduced anomalous rectification, by increasing the membrane resistance of hyperpolarized neurones. On the other hand, external application of TEA (10^{-4} M) had no effect on the DA response, whereas at similar concentration it blocks certain ACh potentials (Kehoe, 1972*a*, *b*) and the excitatory DA effects (unpublished observations).

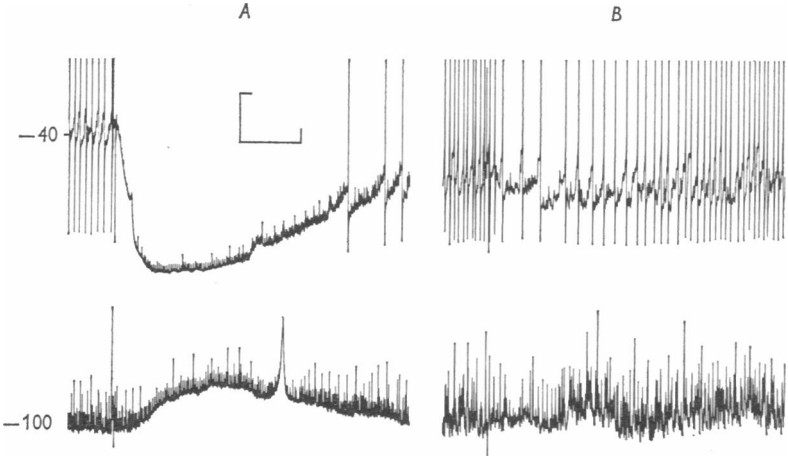

Fig. 15. Injection of TEA in a RB visceral neurone. DA response at -40 mV and -100 mV, before (*A*) and after (*B*) injection of TEA. Calibration: 5 mV, 20 sec.

The results presented above indicate that the DA responses which invert around -80 mV are due to a selective increase in potassium permeability.

Superposition of excitatory and inhibitory effects in the early phase of the DA responses

RB visceral neurones

As mentioned above, the DA response in RB neurones is always hyperpolarizing when recorded near the resting potential, but often takes a more complex shape at higher membrane potentials: earlier components of the response invert at levels where the later ones are still hyperpolarizing. For example, in Fig. 16*B*, the segments of the response starting 10 sec after the end of the injection appeared to invert around -72 mV whereas the latest components shown were still hyperpolarizing at -80 mV. The analysis of the late components will be deferred to the next section, and only the properties of the early components will be considered here.

These early components, which are not affected by changes in $[Cl^-]_o$, are modified by changes in $[K^+]_o$. However, the shifts in their inversion potential are often smaller than those of E_K. Fig. 16 illustrates a case where a reduction of $[K^+]_o$ from 10 to 5 mM produced only a shift of 10 mV, although an increase of $[K^+]_o$ from 10 to 20 mM produced a shift of about 17 mV.

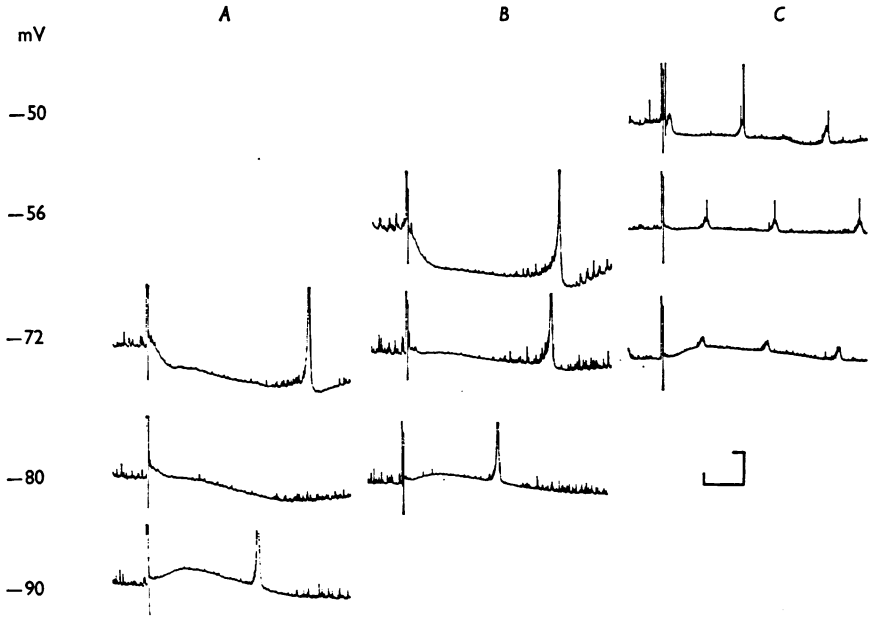

Fig. 16. Effect of changes in $[K^+]_o$ on DA response of a *RB* neurone. $[K^+]_o$ was changed from 5 mM (*A*) to 10 mM (*B*) and to 20 mM (*C*). Calibration: 5 mV, 20 sec. Note the frequently occurring depolarizations which are trains of synaptic potentials. DA injections were usually made in the middle of the interval between two trains.

The above experiments were then repeated in the presence of drugs that allow the pharmacological dissociation of excitatory and inhibitory effects. Methyl ergometrine unmasked a depolarizing component (Fig. 10) which was in turn eliminated by strychnine (10^{-4} M) or tubocurarine (10^{-3} M). Conversely, when strychnine (or tubocurarine) was applied in the absence of methyl ergometrine, the biphasic DA responses, observed around −75 mV, changed to a single-phased inhibition which usually inverted with additional hyperpolarization. In the case illustrated in Fig. 17, the 'new' reversal potential was −80 mV; on a few occasions, the value was even more hyperpolarized, an 'anomaly' probably similar to that described in the next section.

Strychnine blocks the excitatory effects of DA at lower doses than tubocurarine, but has more pronounced effects on the membrane resistance (Klee, Faber & Heiss, 1970). The fall in membrane resistance is probably the main cause of the reduction of DA responses in Fig. 17. On the other hand, the disappearance of the synaptic activity in this same figure is most probably due to the fact that strychnine is an antagonist of depolarizing ACh effects in *Aplysia* neurones (Kehoe, 1972*b*) and that most of the spontaneous EPSPs of RB neurones are cholinergic (Gerschenfeld *et al.* 1967, and unpublished observations).

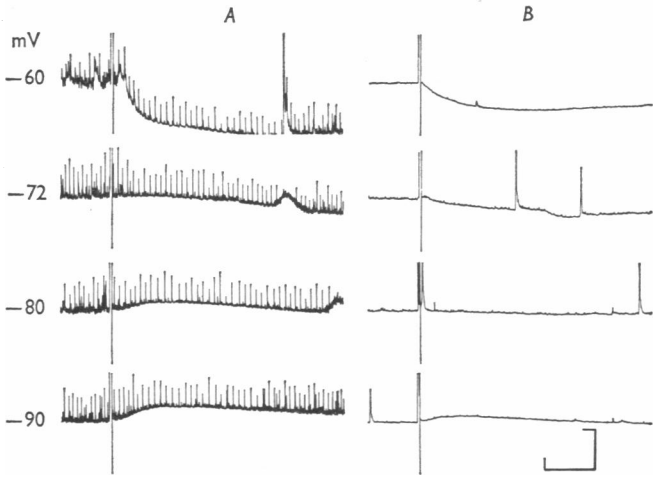

Fig. 17. Strychnine, RB neurone. *A*: control. *B*: under strychnine 5×10^{-4} M. Calibration: 5 mV, 20 sec. Note under strychnine the nearly complete disappearance of the spontaneous synaptic activity.

The above results strongly suggest that E_K is similar in RB and medial pleural neurones, and that the selective activation of the DA inhibitory receptors would in both groups of cells lead to a selective increase in potassium permeability. Responses of RB neurones which invert at less than −80 mV appear to be mixed responses involving the simultaneous activation of inhibitory and excitatory receptors.

Anterior pleural neurones

Although the DA response of anterior pleural neurones is predominantly excitatory, an inhibitory component can often be demonstrated, particularly if DA is applied on an artificially depolarized neurone. This is illustrated in Fig. 18, where the effects of DA were compared to those of ACh on a neurone maintained at −10 mV. At this potential the spike is inactivated and although the membrane shows some instability, the DA

response is clearly seen as an initial depolarizing component followed by a slow hyperpolarizing response. The ACh potential, on the other hand, which at resting level resembled the DA potential and was predominantly excitatory, is now inverted, which indicates a possible difference in the ionic mechanisms of DA and ACh excitatory effects.

Injection of TEA selectively blocks the inhibitory effects of DA. In Fig. 18, this is shown by the disappearance of the hyperpolarizing DA response, while the excitatory component persists.

Fig. 18. Intracellular injection of TEA. Anterior pleural neurone depolarized to −10 mV. ACh: injections of DA and ACh. *A*: control. *B*: after injection of TEA. Calibration: 5 mV, 1 sec.

Posterior pleural neurones

In these neurones the DA responses are usually biphasic. The inhibitory component is more marked than in anterior pleural neurones. On a few occasions, the inversion potential of this component was measured, and found to be, once again, at about −80 mV in normal sea water.

DA responses not inverted by hyperpolarization

The DA responses of RB neurones differ from those of the medial pleural neurones not only by the occasional superposition of excitatory effects, but also by the fact that often either the whole response, or its late components (Fig. 16), remained hyperpolarizing when the membrane potential was set at −80 mV or more. A fall in membrane resistance often occurs at higher polarization levels, which renders the analysis difficult.

This behaviour, which is occasional in RB neurones, is the rule for R15. Since this neurone presents the additional advantage that DA does not trigger any excitatory component, most of the analyses concerning the DA responses not inverted by hyperpolarization were performed on R15.

Changes in $[K^+]_o$

Changes in $[K^+]_o$ had surprisingly little effect on the DA response of R 15, at least in the absence of artificial hyperpolarization. The effects were more marked on hyperpolarized cells, but alterations of the membrane resistance complicated their interpretation.

In the absence of artificial hyperpolarization, R 15 emits bursts of action potentials separated by silent intervals of a few seconds. DA hyperpolarizes the neurone whether it is applied during the burst or during the silent period. As the maximum hyperpolarization reached is similar in both cases, it was found convenient to compare the responses obtained when DA was applied near the height of the inter-burst hyperpolarization. The inter-burst hyperpolarization was greater in higher potassium concentrations, which means that DA was applied at more hyperpolarized levels in high-potassium media. Nevertheless, this did not result in a marked reduction of the response, in contrast to that which would be observed in the medial pleural neurones if the DA response was measured at a higher membrane potential in high potassium solutions.

When the DA response was tested on hyperpolarized neurones, in which the membrane potential could be controlled after suppression of the burst pattern, the effects of changes in $[K^+]_o$ resembled those observed in medial pleural neurones: at a given membrane potential, the response was increased by reducing $[K^+]_o$, and decreased in high-potassium solutions. However, these variations were parallel to variations in membrane resistance, and thus may not be due to changes in the driving force of potassium currents.

These observations show that the DA responses of R 15 do not behave as could be expected if they were due to an increase in potassium permeability. However, additional observations on the undershoot of the action potential, on the one hand, and on the membrane resistance, on the other hand, suggest that the deviation from the behaviour observed in pleural neurones is not due to a different mechanism of DA action, but to the fact that the leakage of potassium during the action potentials buffers the variations of $[K^+]_o$ around the neurone.

As already observed by Strumwasser (1968), during each burst the undershoots of the successive action potentials shift toward more depolarized levels (at least at the beginning of the burst) but have returned to their original level at the start of the next burst. The peak hyperpolarization of the inter-burst period usually falls between the maximum and minimum levels of the action potential undershoot. When $[K^+]_o$ is changed, the effects on the whole pattern of activity are, once again, small. In most experiments where $[K^+]_o$ was varied from 2·5 to 20 mM, the only marked

change observed was an increase of the maximum hyperpolarization reached during the inter-burst period. But neither the spike frequency within each burst, nor the inter-burst intervals, were profoundly modified; and the range of undershoot values remained constant.

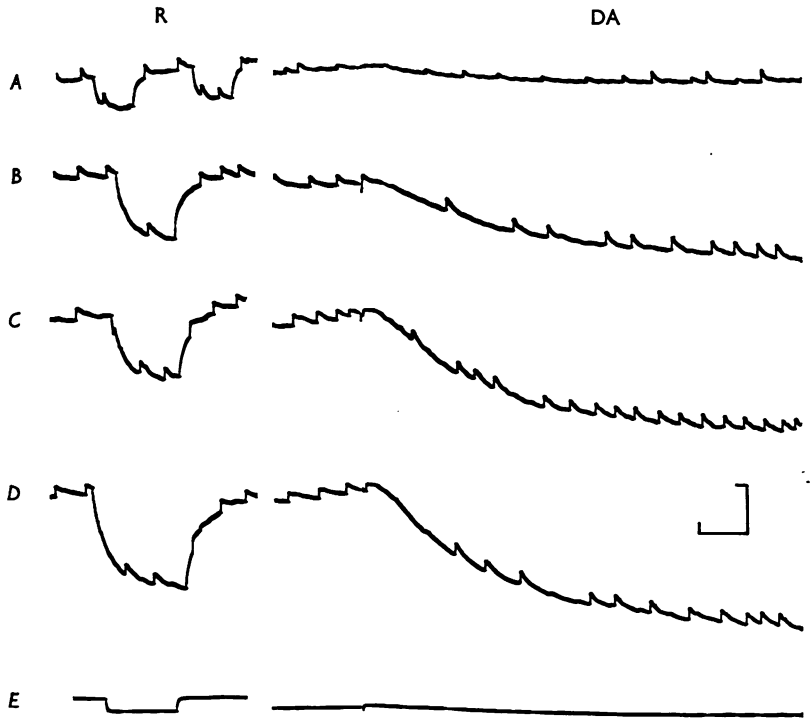

Fig. 19. Time dependent changes in resistance of neurone R 15. The resistance (*R*) and the DA response (DA) were analysed at various intervals (*A*: 30 sec; *B*: 7 min; *C*: 14 min; *D*, 21 min) after interrupting a train of action potentials and bringing the membrane potential to −90 mV. *ABCD* in potassium free sea water. *E* as in *D*, but in normal sea water. Calibration: 5 mV, 4 sec. The artifacts indicating the injection are barely visible, but all aligned with that seen in *E*. The duration of the injection was 0·4 sec.

That the undershoot of the action potential is little affected by changes in $[K^+]_o$ in the bath, while it is affected by the preceding spike emission, can be explained if the potassium concentration around the cell is assumed to be relatively independent of that of the bath. Two additional observations support this hypothesis. The first is that in extensively dissected preparations, changes in $[K^+]_o$ sometimes affected the undershoot of the spike as would be expected if it was due to an increase in potassium

permeability. The second is that the membrane resistance, which is usually markedly affected by external potassium ions, varied in time if measured at different intervals after a train of action potentials. This effect was observed in normal sea water, but was most marked in potassium-free sea water, as illustrated in Fig. 19. This Figure shows that after a train of spikes had been suddenly interrupted by hyperpolarizing R 15 to -90 mV, the membrane resistance (and the DA response) kept increasing for nearly 20 min. If the neurone was then allowed to fire a few spikes, then brought back to -90 mV, the resistance and the DA response were again reduced, and again slowly increased as time passed.

This effect can be compared to that observed by Kehoe (1972*c*) in neurones where the emission of some spikes induced a marked shift of the inversion potential of a potassium-dependent synaptic inhibition. The shift, which was attributed to an accumulation of potassium ions in the extracellular space surrounding the synaptic region, disappeared slowly after the end of the spike train. It is tempting to assume that the progressive increase in resistance observed after a burst has been interrupted, as in Fig. 19, is similarly due to the progressive diffusion of potassium accumulated during the burst in the extracellular space.

More generally, restriction of potassium diffusion around R 15 appears as the most parsimonious hypothesis explaining that the DA response, the action potential undershoot, and the membrane resistance all react in an atypical way to changes in $[K^+]_o$ (see also Alving, 1969).

Effects of various agents interfering with the sodium-potassium pump activity

The fact that the DA effects on R 15 are not inverted by hyperpolarization suggested an analogy with other hyperpolarizing responses presenting a similar property, many of which have been attributed to the activation of an electrogenic pump (see Discussion). The extent of the analogy was tested by analysing the effects of a variety of procedures known to interfere with the activity of the sodium–potassium pump: application of ouabain or strophantidin; substitution of sodium by lithium in the external medium; cooling.

The effects of *ouabain* on the spontaneous activity of R 15 had already been described by Strumwasser (1968) who had reported a progressive disappearance of the burst pattern in the presence of this drug. Ouabain first causes a regular spike discharge; a complete silence often follows, although spikes can still be evoked by artificial depolarization. When DA effects were tested under ouabain (10^{-4} M), they were found to decrease progressively if observed in the absence of artificial hyperpolarization. This decrease was slow, in contrast with the rapidity of ouabain effects on the sodium–potassium pump observed by Thomas (1969) in snail neurones.

When tested at -90 mV, not only did the hyperpolarizing DA effects diminish more rapidly, but they changed in sign, allowing the definition of a reversal potential. This definition, however, was only approximate, for as time passed, the reversal potential shifted progressively to more depolarized levels. Nevertheless, the rate of this shift was sufficiently slow to allow a test of the effects of external potassium, and to show that the inversion potential was indeed sensitive to variations of $[K^+]_o$ (see Fig. 20).

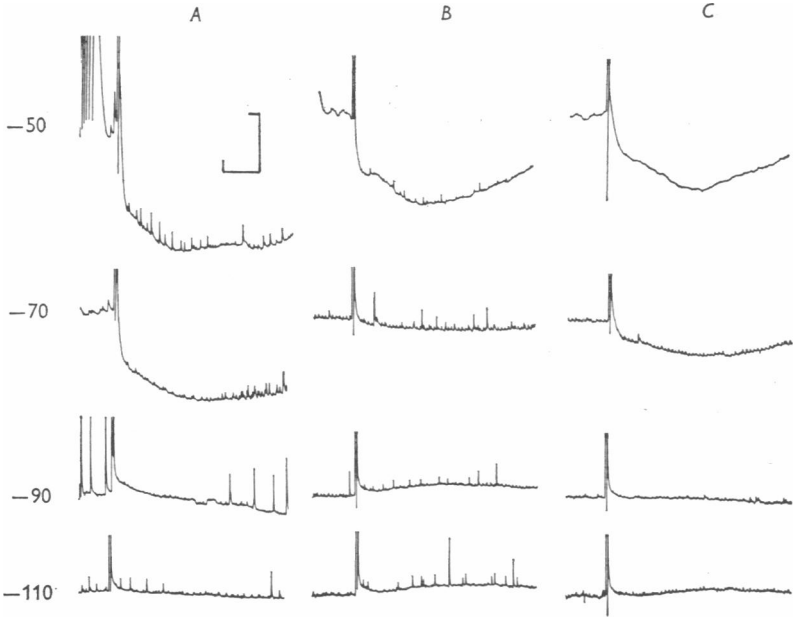

Fig. 20. Effects of ouabain on R 15. *A*: control in normal sea water. *B*: after addition of ouabain 3×10^{-5} M. *C*: under ouabain, $[K^+]_o$ has been changed from 10 to 5 mM. Calibration 5 mV, 20 sec.

Strophantidin (10^{-4} M) mimicked ouabain, and its effects were not found more reversible than those of ouabain over the 2–3 hr period of washing.

By contrast, *lithium sea water*, which also mimicked the effects of ouabain, presented the advantage of reversibility (Fig. 21).

Finally, the DA response was nearly completely blocked by *cooling* the preparation to about 4° C. Contrary to what was observed with ouabain or lithium, the response disappeared at all levels.

The effects of ouabain, lithium, and cooling were also tested on RB neurones, and the results were identical as far as the inhibitory component was concerned. But in addition, the analysis showed that the effects on the excitatory component were far less marked. An example of specificity is illustrated in Fig. 22 for the effects of cold, which unmasks the excitatory component of an initially inhibitory response.

If after ouabain application or replacement of external sodium by lithium the DA responses of R 15 or of RB neurones invert at a potential which varies with $[K]_o$, it appears likely that they involve an increase in potassium permeability. There is no reason to suppose that this effect is not present before the blockade of the sodium–potassium pump. What remains to be explained, however, is the change produced by blockade of the pump. The interpretation which will be discussed is that this change is due to potassium accumulation in the extracellular space.

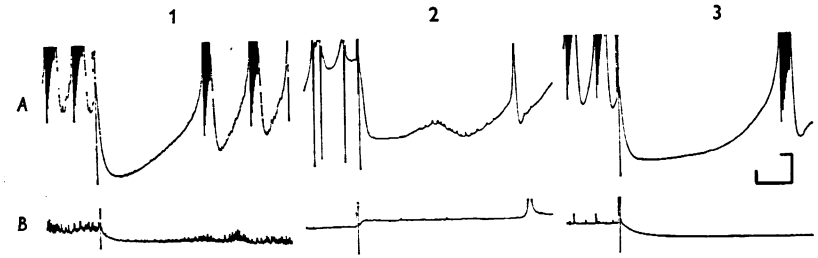

Fig. 21. Effect of replacing sodium ions by lithium ions on DA response of R 15. *A*: without artificial hyperpolarization. *B*: −90 mV. (1) Control; (2) 20 min after replacement of sodium sea water by lithium sea water. (3) One hour after returning to sodium sea water. Calibration: 5 mV, 20 sec.

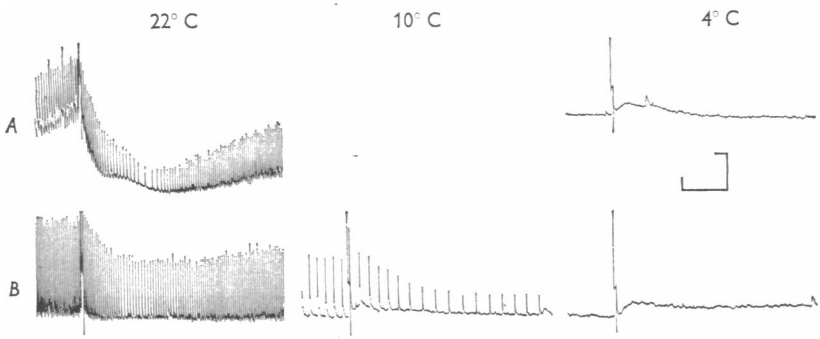

Fig. 22 Effects of cold on the DA response of a RB neurone. Calibration: 5 mV, 20 sec. *A*: −60 mV. *B*: −90 mV.

DISCUSSION

The results presented indicate that DA can have more than one effect on *Aplysia* neurones, a property it shares with other candidates for a role of transmitter in the molluscan nervous system e.g. ACh (Tauc & Gerschenfeld, 1962; Kehoe, 1972*b*), serotonin (Gerschenfeld & Stefani, 1966; Gerschenfeld, 1971), GABA, and glutamate (Gerschenfeld & Lasansky,

1964). As for these other compounds, the excitatory and inhibitory responses appear to be mediated by different receptors, the relative densities of which vary from cell to cell.

Lability of excitatory effects

A methodological observation of particular interest is that ionophoretic application of DA can reveal response types which would be difficult to detect if the drug was added to the perfusion medium. This was particularly clear for the neurones which showed a biphasic response to electrophoretically applied DA, but a purely inhibitory response to perfused DA. In this case, the data obtained suggest that a factor contributing heavily to the differences between the two types of responses is the greater susceptibility to desensitization of the receptors mediating excitation; however, other interpretations cannot be excluded (e.g. differences in the kinetics of DA-receptor interaction, in the kinetics of the permeability changes, or in the spatial distribution of receptors).

If ionophoretic injection appears superior to perfusion in revealing the heterogeneity of a population of receptors, it may not always be sufficient. The importance of excitatory effects will vary greatly with the position of the injection pipette. In the RB visceral neurones the excitatory component of the DA response is usually only revealed by quantitative analysis of the inversion potential, or by 'pharmacological dissection' of the predominantly inhibitory response. More generally, the excitatory effects appear often to require close positioning of the pipette, and long intervals between injection. The lability and the brevity of the excitatory effects, combined with the fact that in no cell DA seems devoid of long-lasting inhibitory effects, probably account for the predominance of observations on inhibitory responses by previous authors.

Polyphasic excitatory responses

An interesting characteristic of DA excitatory responses is their tendency to appear as a succession of discrete components. It seems unlikely that these components are due to different receptors, since they all showed similar sensitivity to tubocurarine and strychnine. They may be the result of the activation of discrete 'spots', similar to those described for the sensitivity of Arthropod muscles to glutamate and GABA (Takeuchi & Takeuchi, 1964, 1965; Beránek & Miller, 1968). Some of these spots may have a lower sensitivity than others. For example, in the experiment of Fig. 2, the shortest latency component (which would correspond, in the above interpretation, to the activation of the spot closest to the pipette) is that needing the highest injection current (and the most affected by desensitization).

It is possible that the DA receptors activated by ionophoretic injections constitute a heterogeneous population of 'extrajunctional' receptors (Miledi, 1960) with graded sensitivities as well as variable desensitization rates.

Characterization of dopaminergic synapses

This study was initiated with the hope that characterization of DA effects on *Aplysia* neurones would help in identifying dopaminergic synapses in the *Aplysia* nervous system. The results obtained indicate that this identification might prove difficult.

A first difficulty arises from the observation, already discussed, that DA effects depend critically on the position of the pipette, on the duration of the injection, etc. Thus, in a cell possessing both excitatory and inhibitory receptors, there is little chance that DA applied electrophoretically can mimic in location and concentration DA liberated by the synaptic terminals. In the example of the posterior pleural neurones, it is certainly true that a biphasic synaptic potential is *a priori* a good suggestion for a dopaminergic synapse, but one cannot truly exclude simple e.p.s.p.s or i.p.s.p.s from the search. Since the ratio of excitatory to inhibitory effects increases when the pipette is approached towards the neurone (Fig. 1, III), it is possible to imagine that the effects of 'synaptic' DA will be nearly purely excitatory. On the other hand, the low sensitivity of certain excitatory effects may be linked with the fact that they are due to 'extrajunctional' receptors, and that synaptic DA might reach only inhibitory receptors. Even if both electrophoretically applied and synaptic DA have a similar over-all effect, the inversion potentials of these effects are unlikely to be equal, contrary to a classical requirement in the identification of synaptic transmitters.

The relative proportion of excitatory and inhibitory effects does, however, show some stability for a given neurone in successive experiments. Thus, the fact that DA is predominantly excitatory for LB and LC neurones makes it seem unlikely that the inhibitory effects of 'interneurones I and II' (Waziri & Kandel, 1969) are due to DA. Similarly, as the hyperpolarizing effect of DA on the medial pleural neurones was never associated with an excitatory component, it is unlikely that DA could be the transmitter liberated by the excitatory interneurone described by Kehoe (1971). More generally, the data gathered enlarge the field for the search of dopaminergic synaptic potentials inasmuch as they extend the investigation, previously limited to inhibitory potentials, to excitatory and biphasic potentials.

A second difficulty in identifying dopaminergic synapses arises from the fact that the DA antagonists studied all present some serious drawbacks:

they show less specificity than the classical ACh antagonists, and most act on the membrane resistance.

Ergometrine was first shown by Walker *et al.* (1968) to antagonize the inhibitory effects of DA on snail neurones. This effect was fully confirmed, and is observed also with methyl ergometrine. The two drugs show some selectivity, since at 10^{-5} M they block neither DA excitatory receptors nor any of the three ACh receptors. Thus they can help in distinguishing ACh and DA mediated synaptic inhibitions, or in dissecting mixed dopaminergic potentials. However, they can only be used in a limited range of concentrations, for above 10^{-5} M they seem to hyperpolarize most neurones. As a consequence, the reduction of a synaptic potential by methyl ergometrine (Fig. 9) does not necessarily indicate dopaminergic transmission. This caution can be applied to the i.p.s.p. studied by Walker, Ralph, Woodruff & Kerkut (1971, Figs. 2 and 3), which was only partially reduced by ergometrine at a concentration 100 times higher than that needed (10^{-8} w/v) to block completely the powerful effects of a DA perfusion.

Tubocurarine and strychnine can help in differentiating excitatory components in dopaminergic synaptic potentials, even if their use is limited by the fact that they block other receptors (Kehoe, 1972*b*; Gerschenfeld & Stefani, 1966). Tubocurarine appears somewhat superior to strychnine as it has less effect on membrane conductance, even at high concentrations.

Noradrenaline antagonists are also DA antagonists, as already observed for DA inhibitory effects in many other neurones (see Phillis, 1970). However, in *Aplysia* neurones, the blockade of inhibitory responses by typical α- or β-antagonists occurs only at high concentrations of the antagonists, is only partial in conditions where the excitatory DA components are totally eliminated, and is not specific since all the ACh effects are also reduced. Thus noradrenaline antagonists appear of little potential help in identifying dopaminergic synapses in *Aplysia*.

The DA receptors of *Aplysia* neurones resemble in many respects the serotonin receptors described in snail neurones by Gerschenfeld & Stefani (1966), Stefani & Gerschenfeld (1969), and Gerschenfeld (1971). The resemblance is particularly marked for the excitatory receptors which are more localized than ACh ones, readily desensitized, and sensitive to tubocurarine. The similarities point out the need for a systematic comparison of the effects of DA and serotonin. Already, however, it appears that in *Aplysia* both drugs act in opposite ways on the medial pleural neurones (J. S. Kehoe, personal communication) and on R15 (unpublished observations) which suggests that they activate distinct receptors.

Effects of DA on the potassium permeability

There seems to be little doubt that in *medial pleural neurones* where the reversal potential of DA responses obeys the Nernst relationship for a potassium perm-selective membrane, the DA effect is entirely due to a selective increase in potassium permeability. Thus this effect appears comparable to the 'slow' effect of ACh on the same neurones (Kehoe, 1972*a*) as well as to the effect of ACh on the vertebrate heart (Burgen & Terroux, 1953; Trautwein & Dudel, 1958). The similarity between the potassium channels controlled by DA and those controlled by ACh (Kehoe, 1972*a*) is completed by the analysis of the effects of external caesium or rubidium, as well as those of intracellular TEA.

In the *visceral RB neurones*, it has been shown that when the reversal potential is at a more depolarized level than in medial pleural neurones, this is not due to differences in internal potassium concentrations (although such differences may exist; see Kunze *et al.* 1971), but rather to the simultaneous occurrence of an excitatory effect mediated by a different receptor. Thus, a difference between E_K and the reversal potential does not imply a less selective permeability change than in the preceding case. This conclusion might be of general significance, and one may wonder if, in cases where the reversal potential of an inhibitory effect did not seem to correspond to the equilibrium potential of a single ionic species, or did not vary as predicted by the Nernst equation, further pharmacological analysis will not show that independent, highly selective pathways are involved.

The DA response of R 15 differs from that of medial pleural neurones by the fact that it is usually not inverted by prior hyperpolarization of the neurone. This property has been observed in other 'non-invertible' hyperpolarizations (see Thomas, 1972) which are attributed to the activation of an electrogenic sodium–potassium pump on the basis of four additional properties: (1) they are not associated with a conductance change, (2) they are blocked by ouabain, (3) they are blocked by potassium-free media, and (4) they are blocked by cooling. The first three characteristics are not shared by the DA responses of R 15 which are: (1) associated with a change in conductance, (2) are only reduced by ouabain, and (3) often increase in low potassium media. Thus, if DA activated an electrogenic pump, this activation could only account for a part of the observed hyperpolarization, the other part being due to the increase in potassium permeability revealed after ouabain.

But even this hypothesis of a superposition of two effects, developed in an early presentation (Ascher, 1968), appears unlikely in view of the observations made in the study of synaptic cholinergic effects (Kehoe & Ascher, 1970; Kehoe, 1972*a*). This study showed that sensitivity to cooling

does not necessarily indicate the involvement of a metabolic process, as responses clearly due to an increase in potassium permeability were abolished by cooling to 4° C. Furthermore, anomalies of the late synaptic responses of certain visceral neurones (L 1–L 6) (Pinsker & Kandel, 1969), which resemble those of the DA responses of R 15 were satisfactorily explained either by an increase in potassium permeability occurring at a long distance from the cell soma, or by a hyperpolarization of the other side of an 'electrical' synapse (Kehoe, 1972*c*).

One of the decisive arguments in favour of the above interpretation was the difference between the behaviour of the synaptic responses and that of the response to ACh applied on the soma. The slow component of this ACh response behaves exactly like the ACh and synaptic potassium-dependent components of the medial pleural cells, and in particular inverts at −80 mV. A similar experiment could not be made in the analysis of DA responses, for, contrary to ACh receptors which are present both on the axon and on the soma in molluscan neurones (Tauc & Gerschenfeld, 1962; Stefani & Gerschenfeld, 1969) the DA receptors appear to be localized on the axon. An equivalent experiment would consist in recording, polarizing, and injecting DA in the synaptic region, but this has still to be done.

Even in the absence of this piece of evidence, it is tempting to adopt a common explanation for the fact that the DA responses of R 15 and the slow synaptic cholinergic inhibition of L 1–L 6 neurones deviate in similar ways from the 'standard' constituted by the DA response and the slow synaptic inhibition of the medial pleural neurones. This explanation (cf. Kehoe, 1972*c*) supposes mainly that the absence of inversion when the soma is hyperpolarized is due to the attenuation of the hyperpolarization between the soma (where the current is applied) and the axonal region (where the increase of potassium permeability occurs). The effects of ouabain and lithium are attributed to a change of E_K, and, more precisely, since ouabain does not modify appreciably $[K^+]_i$ in the first 30 min following its application (Russell & Brown, 1971), to potassium accumulation in the regions where diffusion is restricted. The accumulation is likely to be more important in the synaptic region (usually less exposed by dissection) but also more important in some neurones than in others, e.g. if membrane infoldings are particularly developed (Batham, 1961; Coggeshall, 1967; cf. Alving, 1969).

Variations of $[K^+]_o$ in the juxtacellular space may also give the clue to the peculiar behaviour of R 15 in media where the potassium concentration is varied. The changes of spike undershoots during the burst indicates an extracellular accumulation of potassium similar to that observed by Frankenhaeuser & Hodgkin (1956). On the other hand, the fact that the undershoot has returned to a more hyperpolarized value at the end of the

interburst period indicates that during this period the extracellular concentration of potassium has diminished, and this probably by the combined effects of diffusion and of increased activity of the sodium–potassium pump. It thus appears that, for a given potassium concentration of the bulk solution, the potassium concentration around the cell cannot be assumed to be identical to the former concentration: it may be higher (at the end of a train of spikes), or lower (if hyperactivity of the pump has depleted the extracellular space). A possible explanation of the stability of the DA response when $[K^+]_o$ was varied is that the variations of juxtacellular potassium concentrations during the burst buffer the variations of extracellular (bath) potassium concentrations. This is consistent with the effects observed when the DA responses were tested on a background of hyperpolarization which interrupted the burst pattern, the slow increase in membrane resistance being accounted for by a slow equilibration of the juxtacellular and bath potassium concentrations.

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