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DOPAMINERGIC MODULATION OF NEUROMUSCULAR TRANSMISSION IN THE PRAWN

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SUMMARY

1. The action of the putative crustacean neurohormone dopamine was examined in the fast extensor musculature of the prawn with intracellular and extracellular recording techniques.

2. Dopamine produced a concentration-dependent (10^{-7}–10^{-5} M) decrease in the size of the excitatory junctional potential (e.j.p.). It had no effect on the muscle fibre resting membrane potential or input resistance.

3. High concentrations (10^{-5} M) of dopamine had no effect on the amplitude distribution or decay time of quantal unit currents, indicating that the agent does not act by blocking post-synaptic receptors or channels.

4. Bath application of dopamine reduced the quantal content at single release sites with a similar time course and concentration dependence as that observed for the e.j.p.

5. Dopamine had no effect on histograms of synaptic delays determined over a 10 °C range, indicating that it does not modify the time course of phasic neurosecretion.

6. Twin-impulse facilitation experiments showed a marked decrease in the duration of facilitation in the presence of dopamine.

7. These results are interpreted according to recent theoretical and experimental findings as indicating that the dopamine-induced reduction in transmitter release is produced by a decrease in the entry of Ca during the nerve terminal action potential.

INTRODUCTION

Biogenic amines exert a number of effects which are considered to be neuromodulatory, i.e. they modify the efficacy of another process (reviewed by Kupfermann, 1979). Much evidence indicates that dopamine acts as a circulating neurohormone in crustaceans (Cooke & Sullivan, 1982). Among its modulatory actions, at neuromuscular junctions dopamine has been shown to increase nerve-evoked muscle tension (Kravitz, Battelle, Evans, Talamo & Wallace, 1976), increase excitatory

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junctional potential (e.j.p.) amplitudes, and increase muscle fibre input resistance (Lingle, 1981). Presynaptic actions have not been reported.

The predominant action of dopamine on electrophysiological activity in the mammalian central nervous system (C.N.S.) is inhibition (reviewed by Moore & Bloom, 1978). Biochemical measurements have shown a reduction of glutamate release from cortico-striatal fibres by dopamine agonists, suggesting an inhibitory presynaptic action of dopamine on glutamatergic neurotransmission (Mitchell, 1980; Rowlands & Roberts, 1980). However, the effects of dopamine on glutamate release from nerve terminals have not been examined directly. In our studies of transmitter release in the most extensively characterized glutamatergic synapse, the crustacean neuromuscular junction (Takeuchi, Onodera & Kawagoe, 1980; Kawagoe, Onodera & Takeuchi, 1981, 1982), we have observed a reduction of transmitter release in the presence of dopamine.

H. Parnas & Segel (1982) suggested a procedure for studying the mechanism of pharmacological actions upon transmitter release. With this approach, one can determine whether a drug acts by affecting Ca regulation or the synaptic release machinery, itself. Moreover, it is possible to resolve whether Ca-mediated effects are the result of actions on Ca channels, competition with Ca entry, or modifications in the intraterminal resting Ca level.

Recently, it was suggested that release of neurotransmitter depends not only on the increase in intracellular Ca ions after the action potential, but also on the activation by the membrane depolarization of a 'molecule or complex' that in the activated state can bind Ca. In other words, in order for synchronized release to occur both Ca and the activation of the molecule that binds Ca are required (Dudel, Parnas & Parnas, 1983; I. Parnas, Dudel & Parnas, 1984). Furthermore, the kinetics of quantal release, as determined from histograms of synaptic delays (Katz & Miledi, 1965*a*) is not altered either by a change in extracellular Ca concentration (Datyner & Gage, 1980) or after repetitive stimulation where large facilitation occurs presumably due to the accumulation of intracellular Ca (Datyner & Gage, 1980; H. Parnas, J. Dudel & I. Parnas, unpublished observations). The synaptic delay histograms are altered with level of membrane depolarization and it was concluded that the *kinetics* of quantal release depend mainly on the activation and inactivation of the molecule or complex, which is voltage-dependent (I. Parnas *et al.* 1984). Thus, a drug affecting neurotransmitter release can act at the level of entry of Ca into the terminal, or at any of the events that take place in parallel or after the entry of Ca. Our experimental data support the conclusion that dopamine reduces release at the glutamatergic synapse in crustaceans by inhibition of Ca entry into the terminal.

METHODS

Specimens of *Macrobrachium rosenbergii* (de Man) were generously provided by Kibbutz El Hama, Israel. They were housed in a fresh water system (22 °C) and fed fish fillet twice a week.

Experiments were made on abdominal segments 2 and 3 from animals 2–4 cm in length. The deep abdominal extensor musculature was dissected and pinned out, ventral side up, through pieces of the dorsal exoskeleton remaining near the points of insertion. The general morphological features are similar to those previously described in other decapods, including the crayfish (Pilgrim & Wiersma, 1963; I. Parnas & Atwood, 1966) and the prawn, *Palaemon elegans* (I. Parnas & Dagan,

1969). In each hemisegment there are three clearly defined muscles: a large medial bundle (M) with helically arranged muscle fibres and two smaller lateral muscles (L1 and L2) consisting of straight fibres. Each muscle receives its own excitatory axon and common excitatory and inhibitory axons which innervate the entire hemisegment (see I. Parnas & Atwood, 1966). All recordings in these experiments were made from the L1 muscle while selectively stimulating its private excitatory axon.

Standard electrophysiological techniques were used for stimulation and intracellular recording. Focal stimulation of the nerve near its first major bifurcation upon entering the musculature was found to consistently excite the L1 excitor axon at lowest intensities. This was always verified by the absence of e.j.p.s in intracellular recordings from fibres in the other muscles. Intracellular recording was made with 2 M-KCl-filled micro-electrodes (10–20 MΩ). For intracellular current injection, a second potassium acetate-filled micropipette was positioned within 50 μm of the recording electrode. Extracellular recordings were made with the patch-clamp method developed by Neher, Sakman & Steinbach (1978) and applied to the recording of synaptic currents by Dudel (1981). Pipettes with 10–20 μm outer diameters melted to 2–3 μm inner diameters were found to be most satisfactory. Patch-clamp electrodes were filled with saline containing the lowest concentration of Ca utilized in the particular experiment.

Normal saline contained (mM): NaCl, 220; KCl, 5·4; $CaCl_2$, 3·4; $MgCl_2$, 2·5; Tris HCl, 5. The pH was adjusted to 7·5. A constant flow (approximately one chamber volume, 3 ml, per minute) was used. Bath temperature was controlled by a Peltier unit. Washing for 30–60 min with this medium ($\frac{1}{4}$ normal Ca concentration) was found to eliminate contractions of the muscle effectively and to produce consistent release rates in L1 synapses. Individual quanta were clearly discerned (see Dudel, 1981 and Fig. 3) and the quantal content (m) could be estimated by the failure method (del Castillo & Katz, 1954). Quantal content ranged from 0·05 to 0·70 (at 21 °C) under the conditions utilized. At a given site, stationarity was confirmed by applying several stimulus series (128 sweeps, 1/s) prior to dopamine application. Recordings were averaged on a Nicolet (Model 1074) signal averager.

Experiments were recorded on magnetic tape (Hewlett-Packard 3964A) for further data analysis. Quantal unit current amplitude, time to peak, and decay times were measured on a Nicolet Explorer digital oscilloscope with 2–10 μs per address. The synaptic delay was measured from the negative peak of the terminal action potential (e.n.t.p.) to the onset of the synaptic current as defined by Katz & Miledi (1965*a*).

Dopamine (Sigma) stock solution (10^{-2} M) was prepared immediately prior to each experiment and diluted in saline for each bath application.

RESULTS

Dopamine-induced depression of the e.j.p.

Bath application of dopamine produced a concentration-dependent decrease in the amplitude of the e.j.p. (Figs. 1*A* and *B* and 4). Threshold concentrations ranged between 1 and 5×10^{-7} M). Maximal effects occurred around 1×10^{-5} M. The e.j.p. could rarely be reduced by more than 50%.

Dopamine had no effect on the resting membrane potential of muscle fibres. The e.j.p. remained depressed during prolonged application of dopamine (30 min), indicating a lack of desensitization of the response. Complete reversal usually required a 10–20 min wash with normal saline.

Dopaminergic modulatory actions on crustacean foregut preparations have been shown to result, at least in part, from an increase in muscle fibre input resistance (Lingle, 1981). It was therefore of interest to determine whether the decrease of e.j.p. magnitude observed in these experiments could be due to a dopamine-induced decrease in muscle fibre input resistance. The time constant of decay of synaptic potentials was noted to remain unchanged (Fig. 1*B*), indicating a lack of effect of dopamine on muscle fibre properties. In addition, the current–voltage relationship was determined and found to be unaffected by high dopamine concentrations (Fig. 1*C*).

Absence of dopamine effects on post-synaptic receptors and channels

Possible antagonistic effects of dopamine on glutamate receptors and the post-synaptic channels that they gate were tested with extracellular patch-clamp recordings (Fig. 2*A*). Peak amplitudes of quantal unit currents were measured and their distribution plotted as a histogram (Fig. 2*B*). In three such experiments, the

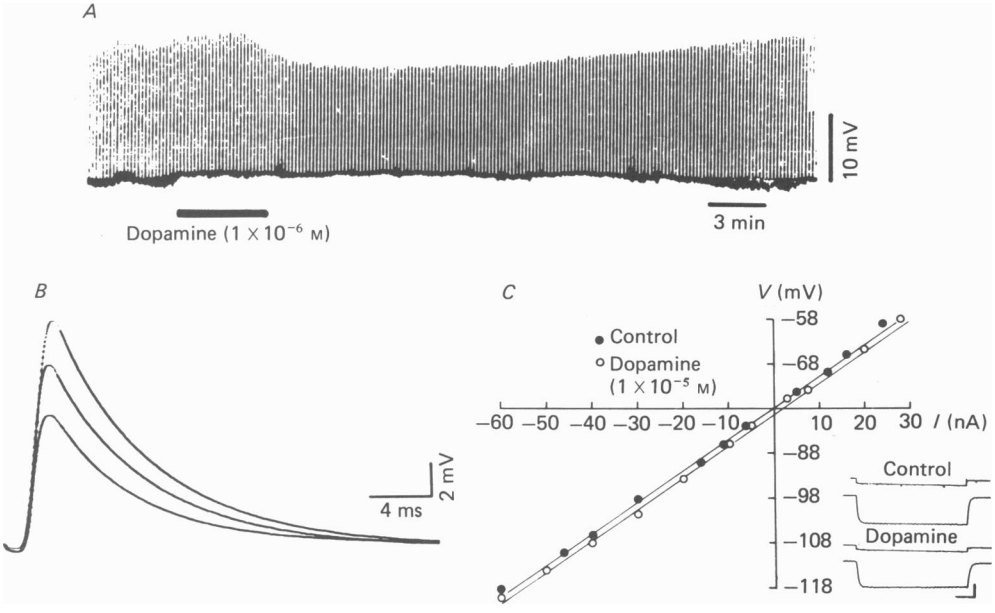

Fig. 1. Dopamine reduces e.j.p. without affecting muscle fibre properties. *A*, intracellular recording from L1 muscle fibre while stimulating L1 excitor axon (0·1 pulse/s). Bath application of dopamine (1×10^{-6} M) reduced the e.j.p. without affecting the muscle fibre membrane potential. Effect required approximately a 20 min wash with normal saline for complete reversal. *B*, concentration dependence of dopamine-induced reduction of e.j.p. Averaged recordings ($n = 32$) of e.j.p.s in L1 muscle fibre. Stimulation same as in *A*. Top, control; middle, dopamine (1×10^{-6} M); lower, dopamine (1×10^{-5} M). Note unchanged decay times ($t_{\frac{1}{2}}$ approximately 5 ms). *C*, muscle fibre current–voltage relationship is not affected by dopamine. Constant current pulses (600 ms) were injected through a second micro-electrode approximately 40 μm from the recording pipette. Input resistance (0·85 MΩ) was not affected by dopamine (1×10^{-5} M). Inset calibration: 100 ms; 10 mV and 90 nA.

quantum size ranged from 150 pA to 2 nA. Application of high concentrations of dopamine had little or no effect on the quantum size, indicating the absence of an antagonism at post-synaptic glutamate receptors.

Since some agents have been shown to act by blocking open synaptic channels resulting in altered current decays (Adams, 1976; Lingle, Eisen & Marder, 1981), we compared decay rates in the presence and absence of dopamine (Fig. 2*C*). The time constant of decay, which is thought to reflect the average channel open time (Anderson & Stevens, 1973; Crawford & McBurney, 1977) ranged from 350 to 1220 μs

and was unaffected by high (1×10^{-5} M) concentrations of dopamine (three experiments).

Dopaminergic reduction of transmitter release

The above experiments indicated that the reduction of the e.j.p. by dopamine is not the result of post-synaptic actions and that presynaptic effects should be tested. Patch-clamp recordings at single release sites indeed revealed a decrease in the

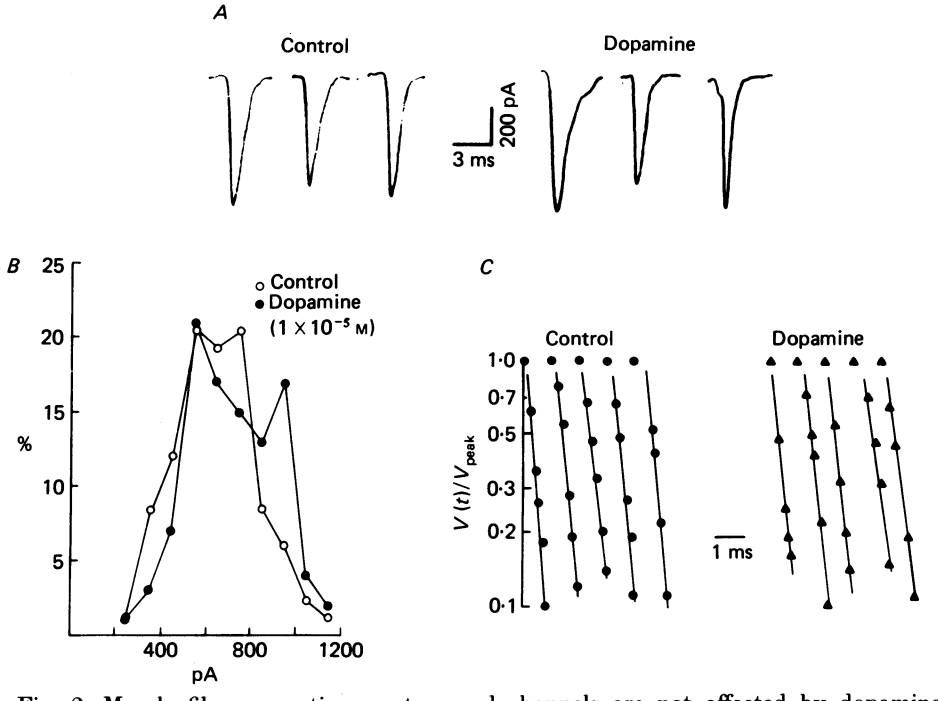

Fig. 2. Muscle fibre synaptic receptors and channels are not affected by dopamine *A*, spontaneous unitary quantal currents recorded at single release site on L1 fibre. *B*, histogram of amplitudes determined before and during dopamine application. Bin width: 100 pA. *C*, plot of decay times of quanta prior to and during bath application of dopamine (1×10^{-5} M).

quantal content in the presence of dopamine. At the Ca concentration used in these experiments (3·4 mM), quantal content in controls ranged between 0·08 and 0·70 (mean = $0{\cdot}29 \pm 0{\cdot}14$, $n = 33$). After addition of dopamine the quantal content was always reduced (Fig. 3, right panel). At many synaptic sites the e.n.t.p. was clearly visible (arrow, Fig. 3). The reduction in release was not associated with failure of action potential invasion into the nerve terminals, as indicated by the unfailing e.n.t.p. The time course of the dopamine-induced inhibition of transmitter release was similar to that noted for the reduction of the e.j.p., exhibiting an immediate onset and requiring 10–20 min wash-out for complete recovery.

All synapses tested in a given preparation responded similarly to dopamine application. It became apparent that the degree of inhibition of release from terminals

paralleled the observed inhibition of the e.j.p. (Fig. 4). Given the dense multiterminal innervation of these muscle fibres (M. W. Miller, unpublished observations), such a relationship would be expected if dopamine produced a somewhat uniform reduction of release. In Fig. 4*A* the extracellular recording was made from a single release site on the same muscle fibre as the intracellular recording. Averaged signals were

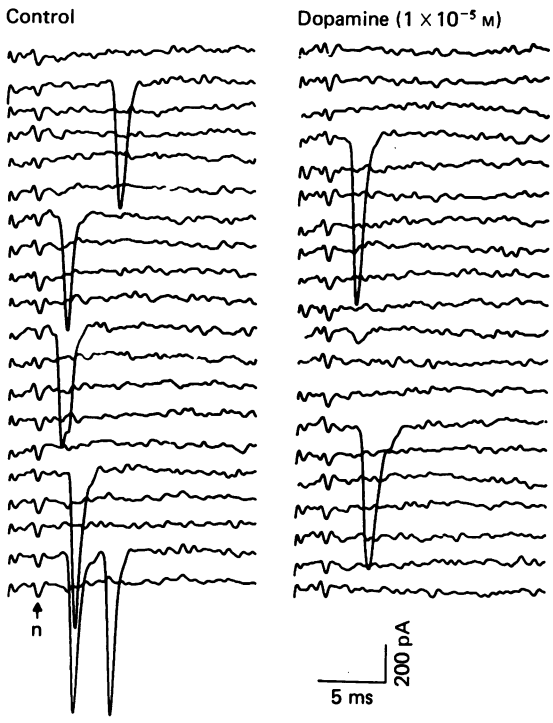

Fig. 3. Dopamine reduces quantal transmitter release. Patch-clamp recording from single L1 release site (15 °C). Quantal content, determined by counting failures was 0·21 for control (525 trials) and 0·12 in dopamine (980 trials). Observe unfailing e.n.t.p. (n).

similarly reduced by dopamine, and this reduction was similar to that of the quantal content at this release site determined by counting failures. Dose–response curves constructed from such experiments showed the similar concentration dependencies of the dopamine-induced e.j.p. reduction and the decrease in quantal content from individual release sites (Fig. 4*B*). Dopamine did not appear to preferentially affect synaptic sites either regionally or according to control release sites.

Dopamine does not alter release kinetics

The reduction in transmitter release in the presence of dopamine could be the result of a reduction of Ca entry into the nerve terminal, inhibition of one or more of the steps in the release process, or both. Histograms of synaptic latencies have been used to describe the time course of the process underlying impulse-evoked transmitter release (Katz & Miledi, 1965*a*; Barrett & Stevens, 1972). Recently, it has been shown

that treatments which affect the release process, for example pre- or post-pulse hyperpolarization, modify the kinetics of release (I. Parnas, H. Parnas & J. Dudel, unpublished). On the other hand, changes in either extracellular Ca concentration (Datyner & Gage, 1980), or intracellular Ca did not affect delay histograms (H. Parnas *et al.*, unpublished). The effect of dopamine on histograms of synaptic delays was therefore examined in order to distinguish between its possible effect upon Ca level and other release steps.

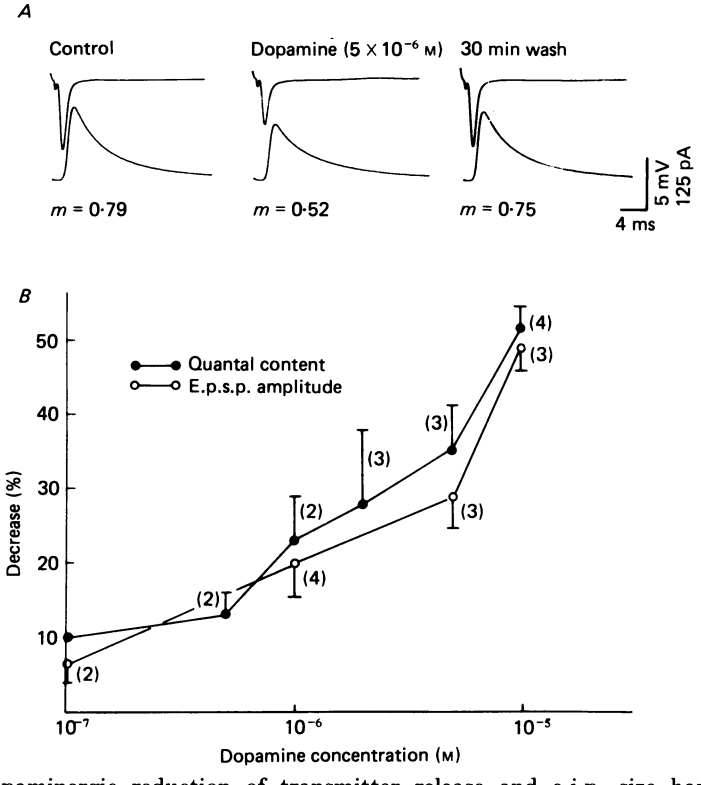

Fig. 4. Dopaminergic reduction of transmitter release and e.j.p. size have similar concentration dependence. *A*, extracellular patch-clamp recording and intracellular recording from L1 muscle fibre. Average of 128 responses (0·5/s). Quantal content (m) estimated by counting failures is shown below each record. *B*, dose–response relationship of dopamine effects on quantal content and e.j.p. amplitude determined from experiments as in *A*. Standard deviations drawn in one direction only for clarity.

Synaptic latencies were measured (see Methods) for stimulation series at five temperatures ranging from 11 to 22 °C. Examples at the two extreme temperatures are shown in Fig. 5. As the temperature was changed, the minimal latency and peak release occurred at different times (note different abscissa scales in Fig. 5*A* and *B*), conditions which were expected to enhance possible dopamine-induced shifts either toward earlier or later values (Katz & Miledi, 1965*b*). Addition of dopamine (up to 1×10^{-5} M), while reducing the absolute amplitude of the release histogram by decreasing quantal release (Fig. 5*A*), never had an appreciable effect on the release kinetics.

Effect of dopamine on facilitation

The lack of effect of dopamine on the kinetics of release suggested that its effect is in reducing Ca entry rather than inhibiting other steps in the release process. There are several ways to further investigate its mode of action. These include: (1) measurement of quantal release while varying the extracellular Ca concentration or (2) determining drug effects on twin-pulse facilitation (H. Parnas & Segel, 1982). The

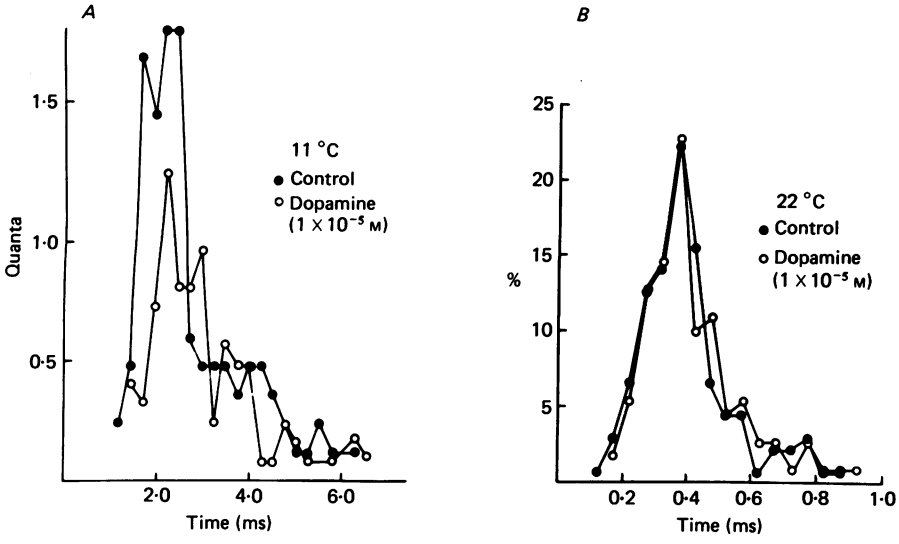

Fig. 5. Dopamine does not modify time course of phasic release. Histograms of synaptic latencies at 11 °C (*A*) and 22 °C (*B*). Note different abscissa time scales. Bin width: *A*, 250 μs; *B*, 50 μs. *A* shows reduction in release (quanta) without much effect on time course. In *B* release in another preparation is shown as a percentage of the total, but was actually reduced by 42 %.

first approach proved difficult, as the times required to change the Ca precluded obtaining more than two or three release measurements at a single release site. Moreover, it has been shown that determination of drug effects on the duration of twin-pulse facilitation (*Tf*) provides a straightforward way to test for effects on Ca entry (H. Parnas & Segel, 1980; H. Parnas, Dudel & Parnas, 1982).

According to this model, the duration of facilitation is given by

$$Tf = Y/R, \tag{1}$$

where: Y is the amount of Ca that enters during the impulse and R is the maximal rate of lumped intraterminal Ca removal processes. Equation (1) shows that *Tf* should be lowered by a substance which decreases Ca entry (Y). Recordings from a release site while testing for *Tf* are shown in Fig. 6*A* and pooled data from a number of sites are plotted in Fig. 6*B*. Dopamine reduced *Tf* to less than half of control values, indicating that it acts by decreasing Ca entry during the terminal action potential.

DISCUSSION

Two aspects of the present results should be considered. One is the modulatory role of dopamine in neurotransmitter release at glutamatergic synapses, and the second is the analysis of the mechanism of presynaptic action of the drug.

An increasing number of diverse peripheral and central actions indicates that circulating amines may modulate complex behavioural responses in crustaceans and

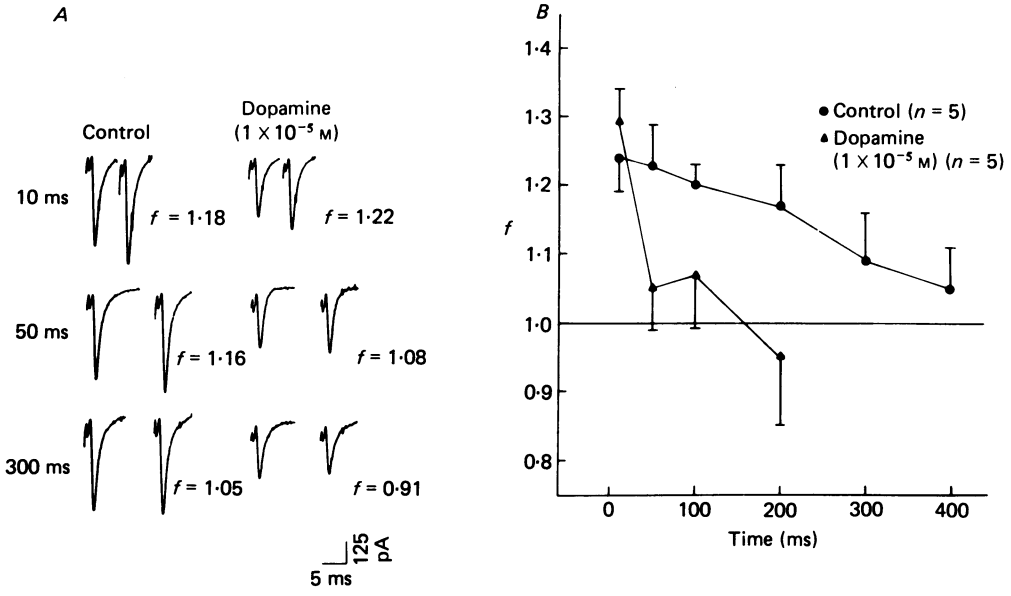

Fig. 6. Dopamine reduces the time course of twin impulse facilitation. *A*, averaged ($n = 128$) patch-clamp recordings prior to and during bath application of dopamine. Facilitation values (f) are ratios of peak-to-peak measurements. Control facilitation lasted for more than 300 ms. *B*, pooled data for experiments such as that shown in *A*. Five sites in a discrete region of the L1 muscle were tested prior to and in the presence of dopamine. Standard deviation bars drawn in one direction only for clarity.

other invertebrates (Kravitz, Glusman, Harris-Warrick, Livingstone, Schwarz & Goy, 1980; Kandel, Klein, Bailey, Hawkins, Castellucci, Lubit & Schwartz, 1981; Willard, 1981). Dopamine is synthesized in central and peripheral crustacean ganglia (Barker, Molinoff & Kravitz, 1972; Barker, Kushner & Hooper, 1979) and is also present in the neurosecretory pericardial organs (Cooke & Goldstone, 1970; Sullivan, Friend & Barker, 1977). Cardioexcitatory responses to dopamine have been observed in a number of species, suggesting that the agent may facilitate its own transport to distant target tissues (Berlind, Cooke & Goldstone, 1970; Florey & Rathmayer, 1978; Cooke & Sullivan, 1982).

In the present study, we have demonstrated a presynaptic action of dopamine reducing the release of the neurotransmitter glutamate. In the mammalian C.N.S. a similar action of dopamine has been suggested (Mitchell, 1980; Rowlands & Roberts, 1980) although, there, the system is less accessible to the experimental procedures

applicable to the neuromuscular junction. Interestingly, some data indicate the presence of presynaptic dopamine receptors on mammalian motor nerve terminals, raising the possibility that these effects may not be restricted to glutamatergic systems (Ganguly & Das, 1979).

The anatomical organization of the abdominal extensor muscles and their pattern of innervation have been extensively studied (I. Parnas & Atwood, 1966; Atwood & Parnas, 1968). These muscles are innervated by only five axons, four of which are excitatory and one inhibitory. No presynaptic innervation has been noted in electrophysiological (I. Parnas & Atwood, 1966) or ultrastructural (A. Dorman & I. Parnas, unpublished) studies. We may therefore conclude that the glutamatergic terminals in this system possess dopamine receptors which are not associated with any specific presynaptic innervation. We did not test whether inhibitory terminals are affected by dopamine.

The threshold concentrations noted in these experiments ($1–5 \times 10^{-7}$ M) are indicative of specific presynaptic dopamine receptors but are at least two orders of magnitude higher than those reported for modulatory actions of 5-hydroxytryptamine on molluscan and crustacean muscles (Weiss, Cohe & Kupfermann, 1978; Glusman & Kravitz, 1982; Fischer & Florey, 1983), and octopamine on insect and crustacean muscles (Evans & O'Shea, 1978; Breen & Atwood, 1983). However, this concentration range is consistent with dopamine actions on crustacean peripheral ganglia (Anderson & Barker, 1981; Miller, Benson & Berlind, 1984) and molluscan buccal ganglia (Wieland & Gelperin, 1983; Trimble & Barker, 1984). The possibility that dopamine is released locally must be considered. Indeed, preliminary histofluorescence observations indicate the presence of one or two catecholamine-containing fibres in peripheral nerves of *Macrobrachium* (R. Goldstein, personal communication).

Until recently, modulation of neurotransmitter release was solely attributed to changes in the intracellular Ca concentration, whether by affecting its entry (Klein & Kandel, 1978; Dunlap & Fischbach, 1978) or by modulation of intracellular stores (Glusman & Kravitz, 1982). I. Parnas *et al.* (1984) have shown that release of neurotransmitter depends, in addition to intracellular Ca, upon the depolarization–activation of the release machinery itself. The action potential thus has at least two roles in inducing release, one to increase membrane conductance to Ca, and the second to activate the release machinery, for example to activate a molecular complex rendering it sensitive to Ca. Therefore modulation of release can be achieved by more than one mechanism, and not only by affecting entry of Ca during the action potential. Recent experiments in our laboratory on the presynaptic effects of the neurotransmitter glutamate on these synapses (I. Cohen *et al.* unpublished) and *d*-tubocurarine on frog neuromuscular synapses (H. Matzner, unpublished) have shown a reduction in quantal content and a marked shift of the delay histogram to the right, without effects on the duration of facilitation. These agents appear to act directly on the release machinery.

Dopamine had no effect on the form of the axonal action potential recorded extracellularly from the nerve at its entry to the muscle. The unchanging form of the axonal action potential leads us to conclude that the depolarization of the terminal is not affected by dopamine. The reduced duration of facilitation in the presence of dopamine (Fig. 6) indicates, according to the residual Ca theory of

facilitation (Katz & Miledi, 1968), that dopamine reduces the entry of Ca into the terminal. It may be argued that dopamine accelerates the compensatory mechanisms to remove Ca, as the duration of the facilitation has been shown to depend on such processes (I. Parnas, Parnas & Dudel, 1982). However, acceleration of the Ca extrusion or sequestration mechanisms should not cause a reduction of the quantal content of the first pulse in a pair. The reduction in quantal content and reduction in duration of facilitation are consistent with inhibition of entry of Ca by dopamine during the action potential. Although the direct measurement of Ca current, as accomplished in the squid stellate synapse (Llinas, Steinberg & Walton, 1976), is not possible at present in the crustacean terminals (about 1 μm), these less direct methods still provide considerable insight into mechanisms of modulation.

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