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PRESYNAPTIC ACTIONS OF CURARE AND ATROPINE ON QUANTAL ACETYLCHOLINE RELEASE AT A CENTRAL SYNAPSE OF *APLYSIA*

BY G. BAUX AND L. TAUC
From the Laboratoire de Neurobiologie cellulaire et moléculaire, C.N.R.S., 91190 Gif sur Yvette, France

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SUMMARY

1. In a cholinergic synaptic couple in the buccal ganglion of *Aplysia californica*, where the synaptic areas are situated close to the somata (500 μm), we were able to control transmitter release by stimulating the cell body of the presynaptic neurone with long depolarizing pulses in the presence of tetrodotoxin (TTX).

2. Statistical analysis of noise occurring at the peak of the long-depolarization-induced post-synaptic current (p.s.c.) responses allowed us to calculate the amplitude and the decay time of the miniature post-synaptic currents (m.p.s.c.s). These data were used to calculate the quantal content of the responses.

3. Bath-applied tubocurarine reduced the amplitude of the long-depolarization-induced p.s.c. more than that of the m.p.s.c.s, indicating that tubocurarine exerts a depressive presynaptic action on the quantal content of the post-synaptic responses.

3. Tubocurarine injected into the presynaptic neurone blocked synaptic transmission without decreasing the size of the m.p.s.c.s probably by acting on the mechanism of transmitter release.

5. Bath-applied atropine (10^{-6} and 10^{-5} M) caused a slight decrease of the m.p.s.c.s but the long-depolarization-induced p.s.c.s increased, as did the quantal content. Higher concentrations of atropine depressed strongly both the m.p.s.c. and the quantal content.

6. Injection of atropine into the presynaptic neurone had the same effect as its bath application, probably due to the leakage of the drug into the synaptic cleft; the effect depended on the concentration reached in the cleft, i.e. on the quantity of injected drug. The synapses of the neighbouring cholinergic neurone were also affected by this leak of atropine.

7. The presence of nicotinic presynaptic receptors blocked by tubocurarine, and muscarinic presynaptic receptors blocked by atropine, which regulate synaptic transmission by facilitating and depressing the ACh release respectively, is discussed.

INTRODUCTION

Tubocurarine and atropine are widely used as tools to study the mechanism of cholinergic synaptic transmission. The post-synaptic effects of these drugs are relatively well known. Since the work of Dale, Feldberg & Vogt (1936), the blocking

action of tubocurarine has been attributed to its ability to bind to the post-synaptic nicotinic acetylcholine receptors and to prevent the binding of acetylcholine (ACh). Later it was shown that, besides exerting a competitive antagonism, tubocurarine can also act as a non-competitive antagonist by binding to allosteric sites on the ionic channel of the ACh receptor, thus occluding the channel once it has opened (Ascher, Marty & Neild, 1978; Katz & Miledi, 1978; Colquhoun, Dreyer & Sheridan, 1979; Lambert, Volle & Henderson, 1980). Furthermore, tubocurarine is a weak agonist in opening, at low concentrations, the ionic channels associated with ACh receptors (Trautmann, 1982).

Atropine antagonizes the actions of ACh mediated by muscarinic receptors, and is especially effective in autonomic effectors innervated by the post-ganglionic cholinergic nerves. However, at higher concentrations, it can also act on the nicotinic or nicotinic-like ACh receptors of *Aplysia* (Tauc & Gerschenfeld, 1962), probably by shortening the mean open time of the post-synaptic ionic channel as shown at the neuromuscular junction (Katz & Miledi, 1973; Feltz, Large & Trautmann, 1977).

Both tubocurarine and atropine seem also to interfere with a presynaptic mechanism. In vertebrate brain preparations (Molenaar & Polak, 1970; Rospars, Lefresne, Beaujouan & Glowinski, 1977; Szerb, 1979; Nordstrom & Bartfai, 1980; Raiteri, Leardi & Marchi, 1984; Meyer & Otero, 1985), in *Torpedo* electric organ preparations (Michaelson, Avissar, Kloog & Sokolovsky, 1979; Dunant & Walker, 1982; Pinchasi, Burstein & Michaelson, 1984) or in the guinea-pig myenteric plexus (Kilbinger & Wessler, 1980), the presence of exogenous ACh, carbachol or oxotremorine in the bathing medium reduces the release of endogenous ACh, an effect that is blocked by the muscarinic antagonist, atropine. Atropine enhances ACh release in the vertebrate brain preparations *in vitro* or *in vivo* (Mitchell, 1963; Molenaar & Polak, 1970; Jones, Guyenet, Cheramy, Gauchy & Glowinski, 1973; Bourdois, Mitchell, Somogyi & Szerb, 1974; Hadhazy & Szerb, 1977; Nordstrom & Bartfai, 1980; Weiler, Misgeld & Cheong, 1984). These results were interpreted as indicating the presence of presynaptic regulation mediated by muscarinic autoreceptors localized on the cholinergic nerve ending itself.

A presynaptic effect of tubocurarine at the vertebrate neuromuscular junction was also deduced from experiments which showed that the decline in amplitude of successive end-plate currents or potentials during a train of impulses was faster when tubocurarine was added to the preparation (Lilleheil & Naess, 1961; Hubbard, Wilson & Miyamoto, 1969; Galindo, 1971; Glavinovic, 1979; Magleby, Pallotta & Terrar, 1981). Although the mechanism of this depressive action on quantal release could not be ascertained, these observations suggested the presence of pre-junctional nicotinic receptors which mediate positive feed-back control of ACh release and are blocked by tubocurarine (Briggs & Cooper, 1982; Rowell & Winkler, 1984; Bowman, Marshall & Gibb, 1984).

In the present study we have obtained evidence that both tubocurarine and atropine act on quantal transmitter release in a central cholinergic synapse of *Aplysia*. Extracellularly applied tubocurarine leads to a decrease of the number of quanta released during a long-lasting depolarization imposed on the presynaptic endings. On the contrary, atropine applied in the bath at low concentration enhances the quantal content of the post-synaptic responses. We propose that the effects of

tubocurarine and atropine could be explained by the presence on the nerve ending of the presynaptic neurone of both nicotinic and muscarinic autoreceptors.

METHODS

Preparation

Dissected and desheathed buccal ganglia of *Aplysia californica* obtained from the Pacific Biomarine Supply Co. (Venice, CA, U.S.A.) were used. Each buccal ganglion contains two large cells (250 μm diameter) which are presynaptic to a group of post-synaptic cells (300 μm) situated close by (Gardner, 1971). After removing the connective tissue, the resting potentials recorded from both pre- and post-synaptic cells were between -50 and -60 mV. The post-synaptic cells produced Cl^--dependent inhibitory post-synaptic potentials when spikes were generated in the presynaptic neurone. Because 3 M-KCl-filled micropipettes were used, the Cl^- equilibrium potential had a tendency to shift towards less negative values; therefore it was continuously controlled throughout the experiment by establishing the holding potential for which the evoked or ACh ionophoretic responses gave zero current.

The monosynaptic nature of the connection between the pre- and the post-synaptic cells is well established (Gardner, 1971; Tauc, Hoffmann, Tsuji, Hinzen & Faille, 1974) as well as its cholinergic nature (Tauc *et al.* 1974; Baux, Simonneau & Tauc, 1979; Tauc & Baux, 1982; Baux & Tauc, 1983).

Solutions

The ganglia were bathed with artificial sea water (ASW) of composition (mM): NaCl, 460; KCl, 10; $CaCl_2$, 11; $MgCl_2$, 25; $MgSO_4$, 28; Tris HCl buffer, 10; pH 7·8. When it was necessary to block Na^+ channels, tetrodoxin (TTX, Sigma) was added to the normal ASW at a final concentration of 10^{-4} M. Bath-applied tubocurarine chloride (Sigma) and atropine sulphate (Merck) were used at 10^{-6}–10^{-4} M. Tubocurarine (10^{-3} M in ASW) was injected intracellularly by an air-pressure system. Injection of ASW alone was without any effect. Atropine sulphate (1 M) in distilled water was injected into the soma of the presynaptic cell by ionophoresis using a constant-current device. For ionophoretic application of ACh on the somatic ACh receptors (Tauc & Gerschenfeld, 1961) of the post-synaptic cell, the micropipette was filled with a solution of ACh (1 M) in distilled water.

Electrical recordings and calculation of quantal parameters

Glass micro-electrodes were pulled on a De Fonbrune microforge and filled with 3 M-KCl; their resistance was 1–5 MΩ. Measurements of post-synaptic current (p.s.c.) responses evoked by a presynaptic spike or an ionophoretic application of agonist were performed using the classical voltage-clamp method.

Because of the great number of neurones afferent to the post-synaptic cell in this preparation it was not possible to record the spontaneous individual miniature post-synaptic currents (m.p.s.c.s) resulting from the release of quanta by a given presynaptic neurone. Hence, the quantal aspects of synaptic transmission in the buccal ganglion were studied by an indirect method previously described by Simonneau, Tauc & Baux (1980). Briefly, pre- and post-synaptic cells were simultaneously voltage-clamped to holding potentials of -50 and -80 mV respectively. When the voltage-dependent Na^+ conductance was blocked by TTX (10^{-4} M), a 3 s step depolarization of the presynaptic neurone induced a post-synaptic response (long-depolarization-induced p.s.c.) at the peak of which appeared fluctuations (noise) resulting from the summation of discrete events which could be identified as m.p.s.c.s. The amplitude of the m.p.s.c. was calculated using the Campbell's theorem, in which the size of the unitary element or individual m.p.s.c. (i_{min}) is related to the variance of the noise (E^2) and the mean observed current change (I) by the equation: $i_{min} = 2E^2/I$ (Katz & Miledi, 1972). Because the Cl^- reversal potential could shift during the course of experiments, the results were expressed as conductances by the following equation $g_{min} = 2E^2/I(V-V_{eq})$ where V is the holding potential and V_{eq} the Cl^- reversal potential. This method also permitted calculations of the relative number of quanta released during a 3 s long-depolarization-induced p.s.c., using the ratio: $(I.\ 3)/(i_{min}.T_{min})$ where T_{min} (0·013 s) is the decay time of the m.p.s.c. calculated from the noise by using a Fast Fourier Transform. Such a calculation of the quantal content is independent of the post-synaptic modifications, since the latter affect i_{min} and I to the same proportion.

RESULTS

Action of tubocurarine on ACh receptors

Tauc & Gerschenfeld (1961) showed that, in *Aplysia* central neurones, pharmacologically identical ACh receptors are present not only on the synapse but also on the cell bodies, and that these H-type receptors that activate a Cl^- permeability are blocked by turbocurarine. In order to obtain information on the potency of tubocurarine on the receptors of the post-synaptic cell used in the present study, ACh

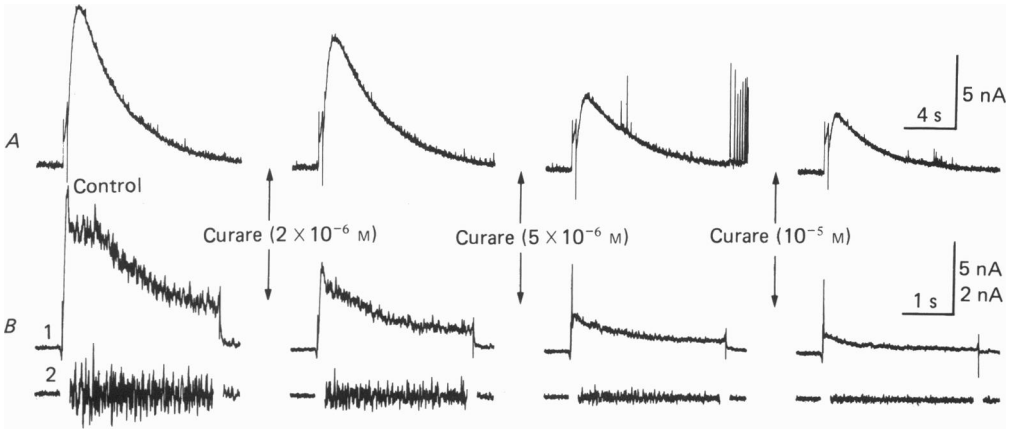

Fig. 1. Effects of tubocurarine applied in the bath at different concentrations, on post-synaptic and ACh responses recorded in a post-synaptic cell of the buccal ganglion. *A*, post-synaptic responses to ionophoretically applied ACh on somatic receptors were depressed by indicated concentrations of tubocurarine to 81, 48 and 35 %, respectively, of the control. *B*, long-depolarization-induced p.s.c.s induced by presynaptic depolarizations to 0 mV were depressed by the same concentrations of curare to 50 %, 25 % and 10 % of the control. Quantal content calculated by the relation: $(I/2I^2/I)(3/T_{min}) = (I^2/2E^2)(3/0{\cdot}013)$ was 12883 for the control, 9030 in curare (2×10^{-6} M) and 6787 in curare (5×10^{-6} M). The size of the m.p.s.c.s calculated from the same responses were depressed to a similar proportion as the ionophoretic responses (control: 1·63 nS; curare (2×10^{-6} M): 1·15 nS; curare (5×10^{-6} M): 0·68 nS; incalculable for curare (10^{-5} M)). The post-synaptic cells were voltage clamped at −80 mV. *B*1: d.c. recordings; *B*2: a.c. recordings. 5 nA represent 90 nS.

was applied ionophoretically to the somatic ACh receptors in the presence of different concentrations of tubocurarine chloride (Fig. 1). Concentrations which were effective without abolishing the responses ranged from 10^{-6} to 10^{-5} M, depressing the ACh currents by about 30–75 % of the control response in a few minutes. Wash-out of the drug led to a slow recovery of the ACh responses to about 80 % of its control size within 30 min.

Action of tubocurarine on the evoked post-synaptic response

The responses to depolarizations of the presynaptic neurone to 0 mV in the presence of different concentrations of tubocurarine are shown in Fig. 1. Both the long-depolarization-induced p.s.c. and the m.p.s.c. decreased as the concentration of tubocurarine was increased over a range similar to that which reduced the response

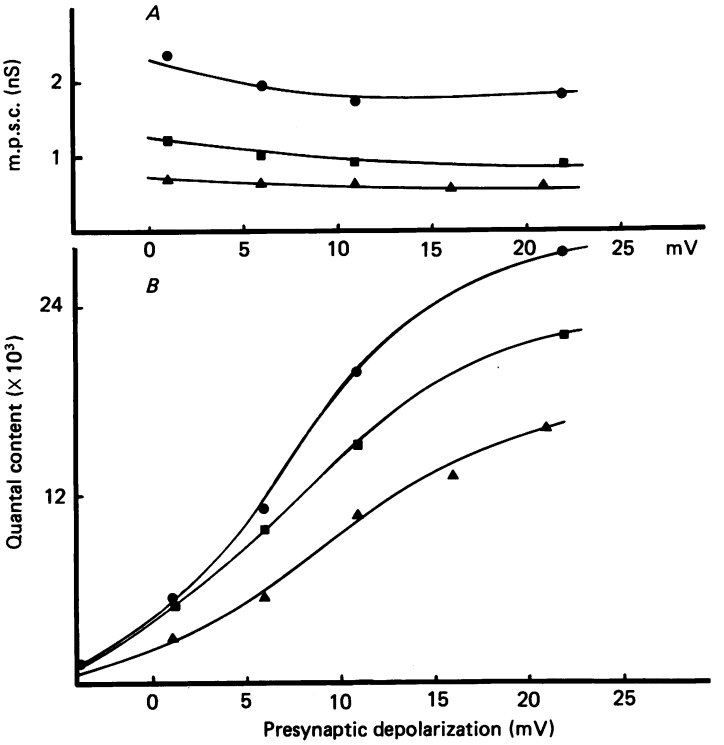

Fig. 2. Reduction by two concentrations of bath-applied tubocurarine of the calculated miniature post-synaptic current (m.p.s.c.) expressed as a conductance (*A*) and of the quantal content of long-depolarization-induced p.s.c. (*B*) for increasing presynaptic depolarizations. Preparation different from Fig. 1. ●: control; ■: tubocurarine (2×10^{-6} M); ▲: tubocurarine (5×10^{-6} M).

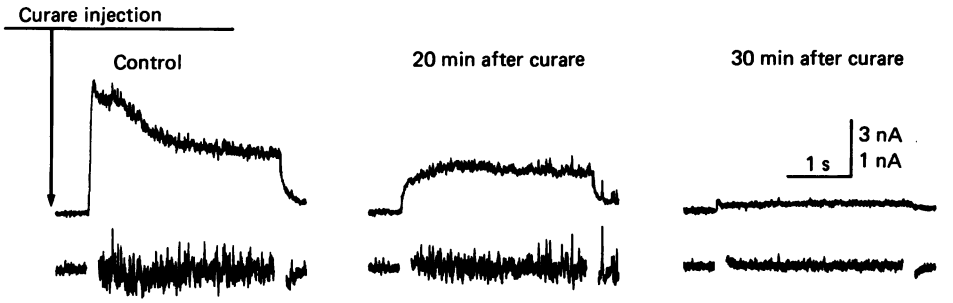

Fig. 3. Decrease by tubocurarine of the p.s.c. induced by depolarizations of the presynaptic neurone to +10 mV. Upper traces: d.c. recordings; lower traces: a.c. recordings. Tubocurarine was injected into the presynaptic neurone just before the recording that we used as the control. The post-synaptic response began to decrease 10 min after injection of the drug. 3 nA represent 150 nS.

to ionophoretic application of ACh. The decrease of the size of the m.p.s.c. was, therefore, attributed to the action of the drug on the post-synaptic receptor–channel complex. However, the decrease in amplitude of the long-depolarization-induced p.s.c.s was more pronounced than that of the underlying m.p.s.c.s. The ratio mean long-depolarization-induced p.s.c./m.p.s.c. decreased, while T_{min} (0·013 s) was unchanged, indicating that the quantal content of the response was diminished. A 'dynamic' representation of the changes of the calculated m.p.s.c. amplitude and of the quantal content of the responses with different presynaptic depolarizations is shown in Fig. 2.

It seems reasonable to suppose that the voltage-sensitive Ca^{2+} permeability in the somatic membrane is identical to that in the synaptic terminal membrane (Stinnakre & Tauc, 1973), to which we have no access. Therefore, we tested the effect of tubocurarine on the Ca^{2+} conductance of neurones of the group L1–L6 of the abdominal ganglion, which generate spikes in Na^+-free saline or in the presence of TTX (Baux *et al.* 1979). Tubocurarine did not alter the Ca^{2+} spikes.

Action of tubocurarine injected intracellularly into the presynaptic neurone

The large size of the cell body of the presynaptic cell and the short distance separating the soma and the nerve endings make it possible to study the effects on transmitter release of substances which, following their introduction into the soma, migrate to the presynaptic terminal (Tauc *et al.* 1974; Baux & Tauc, 1983). When tubocurarine was injected, it was found to depress the synaptic transmission (Fig. 3, and Davies & Hinzen, 1979). This depression was attributed by Davies & Hinzen (1979) to a leakage of tubocurarine from the terminal, a conclusion not in agreement with our present findings. The method using the long-depolarization-induced p.s.c. and the synaptic noise analysis showed that the depression of the synaptic transmission after pressure injection of tubocurarine is not due to a diminution of the m.p.s.c.s but to a decrease in the number of quanta released (Fig. 4). This excludes the existence of any significant leakage of the injected drug at the synapse and points to a presynaptic mechanism for the depression, by which intracellular tubocurarine would decrease the number of quanta liberated per given stimulus.

Action of atropine on the post-synaptic response

Atropine also acts as an antagonist on the H-type receptors, but higher concentrations of the drug are needed to produce depressions of the post-synaptic responses identical to those produced by tubocurarine (Tauc & Gerschenfeld, 1982, and compare Fig. 2 with Fig. 5).

Quantal analysis revealed that both long-depolarization-induced p.s.c. and m.p.s.c.s were depressed when the concentration of atropine was 10^{-4} M or greater. But at 10^{-6} and 10^{-5} M, the size of the m.p.s.c. was only slightly decreased and the quantal content of the post-synaptic response was increased, since the amplitude of the long-depolarization-induced p.s.c. was enhanced (Fig. 6). Moreover, it appeared that atropine, besides being more efficient at 10^{-5} M than at 10^{-6} M, also had a proportionally more pronounced facilitatory effect on long-depolarization-induced p.s.c.s of higher amplitude produced by greater release of ACh (Figs. 5 and 6). To

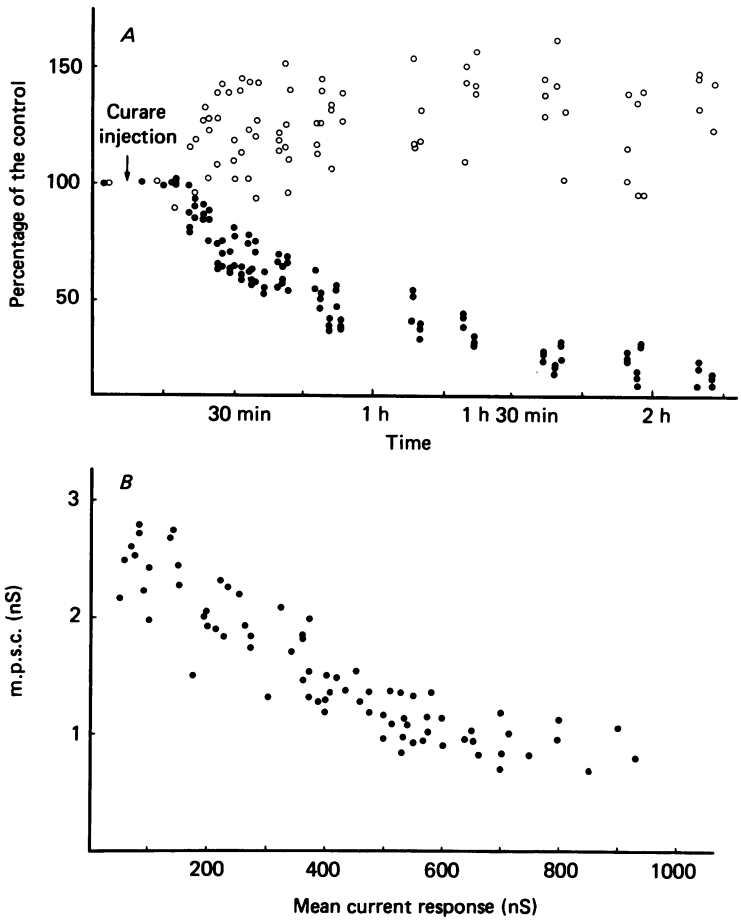

Fig. 4. *A*, effect of tubocurarine pressure-injected into a presynaptic neurone of the buccal ganglion, on the amplitude of the p.s.c. induced by a presynaptic depolarization to 0 mV membrane potential. ●: mean amplitude of the long-depolarization-induced p.s.c. expressed as percentage of the control response. ○: calculated conductance of the m.p.s.c. The increase of the m.p.s.c. after tubocurarine injection was due to a dependence of the size of the calculated m.p.s.c. on the amplitude of the long-depolarization-induced p.s.c., i.e. the presynaptic depolarization as shown in *B* for another untreated preparation. As previously shown (Simonneau, Tauc & Baux, 1980), the size of the calculated m.p.s.c. was higher for low presynaptic depolarizations. No satisfactory explanation was found for this phenomenon.

analyse further the site of action of atropine we have injected the drug into the presynaptic neurone.

Action of intracellularly injected atropine

The long-depolarization-induced p.s.c. technique was used to analyse the effects of atropine injected into the presynaptic neurone. Ionophoretic injection of atropine for 15 min or more (50 nA constant current) decreased both the long-depolarization-induced p.s.c. and the calculated m.p.s.c. (Fig. 7*A*). By reducing the time of injection to 10 or 5 min, thereby reducing the quantity of atropine injected, it was possible

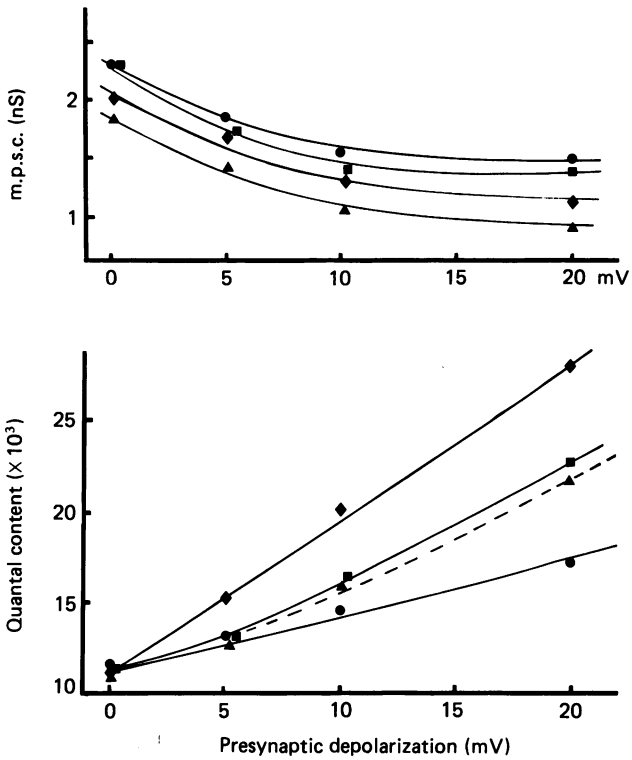

Fig. 5. Effect of bath-applied atropine on the m.p.s.c. and on the quantal content of the post-synaptic response measured in the same preparation. *A*, the m.p.s.c. was slightly and progressively decreased as the concentration of atropine in the bath was raised from 10^{-6} M to 10^{-4} M. ●: control; ■: 10^{-6} M-atropine; ◆: 10^{-5} M-atropine; ▲: 10^{-4} M-atropine. *B*, the quantal content of the long-depolarization-induced p.s.c. was increased by atropine at concentrations 10^{-6} and 10^{-5} M, but a recovery towards the control value began with 10^{-4} M concentration (dashed line). The increase in quantal content was proportionally greater for higher presynaptic depolarizations: at +5 mV, from 13125 to 15184 (increase by 16%); at +20 mV, from 17264 to 28080 (increase by 62%), when atropine (10^{-5} M) was bath-applied.

to induce an increase of the quantal content of the responses (Figs. 7*B* and 8) as reflected by the simultaneous enhancement of the long-depolarization-induced p.s.c. and the slight reduction of the m.p.s.c. (Fig. 8).

The effects of atropine were reversible, no matter what quantity was injected (Fig. 7*A*). This suggests that the intracellular concentration of the drug decreased with time. A plausible mechanism is that atropine leaks across the cytoplasmic membrane, which would explain the decrease of the m.p.s.c. as resulting from an accumulation of atropine in the synaptic cleft. The cellular configuration of this preparation allowed us to examine whether such a leak occurs, as detailed below.

Intracellularly injected atropine affects the neighbouring neurone

The buccal ganglion contains, in addition to the presynaptic neuorone used in our experiments, a second 'equivalent' large cholinergic cell. The two neurones have their

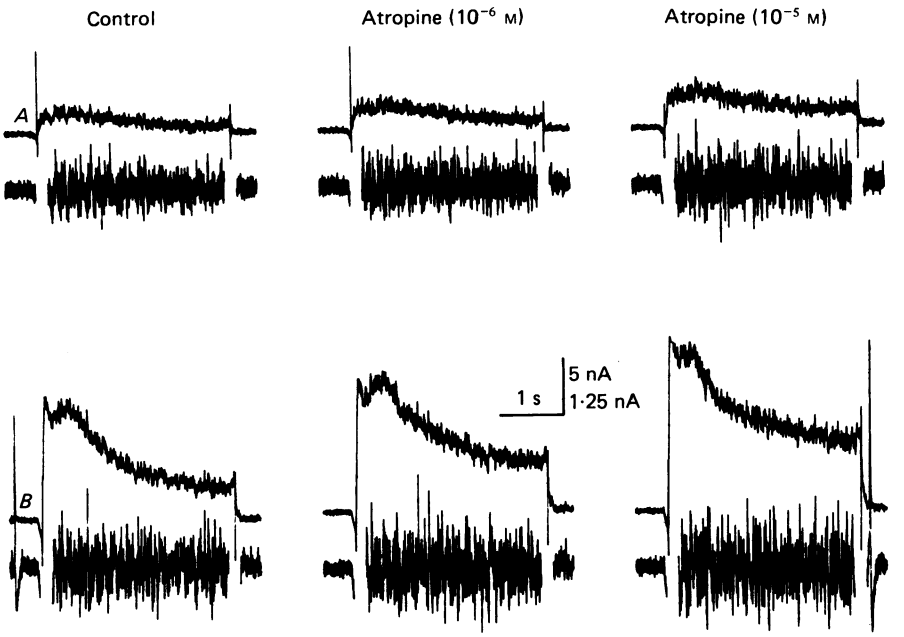

Fig. 6. Effect of bath-applied atropine on the amplitude of the long-depolarization-induced p.s.c.s which were induced by depolarizations of the presynaptic neurone to 0 mV (*A*) or +20 mV (*B*). Upper traces: d.c. recordings; lower traces: a.c. recordings. In both cases, atropine enhanced the long-depolarization-induced p.s.c., proportionately more in *B* when a greater quantity of ACh was released into the synaptic cleft by the presynaptic neurone than in *A*. As the bath concentration of atropine was increased, the quantal content increased in *A* from 2990 to 3500 (increase by 17%) and to 4830 (increase by 61%), and in *B* from 14280 to 24480 (increase by 60%) and to 34500 (increase by 141%). 5 nA represent 130 nS.

cell bodies side by side and make afferent contacts on the same post-synaptic cells (Fig. 9). The nerve endings of these two neurones are most probably intermingled and it is possible that a substance leaking from one of these neurones would contact the synaptic sites of its neighbour.

To test this possibility the two presynaptic neurones, as well as the common post-synaptic nerve cell, were penetrated by two micro-electrodes each and the directly evoked p.s.c.s were monitored in the voltage-clamped post-synaptic cell. The response to one of the presynaptic neurones was considered as the control; the other presynaptic neurone was injected with atropine. It is clear from Fig. 9 that following the injection of atropine the decrease of the post-synaptic current is not limited to the response initiated by the injected neurone; the response to activation of the control neurone is equally depressed. This depression most probably results from the leakage of atropine from the injected neurone (as suggested by the above recovery of the long-depolarization-induced p.s.c. response) and its penetration into the synaptic regions of the neighbouring neurone.

Because of the existence of an electrotonic coupling between the two presynaptic

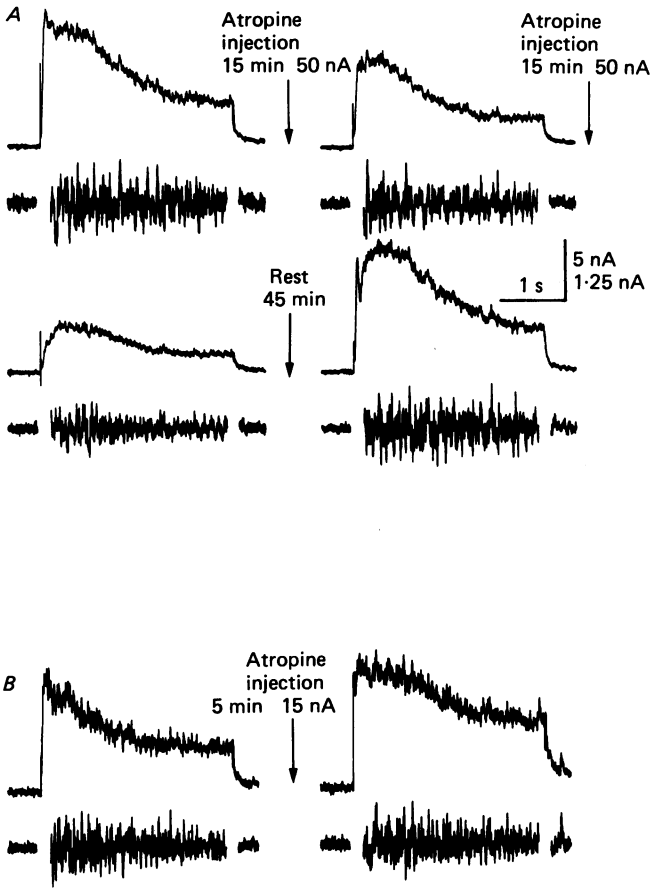

Fig. 7. Effects of intracellular injection of atropine into the presynaptic neurone. Long-depolarization-induced p.s.c.s were induced by depolarizations of the presynaptic neurone to +10 mV (*A* and *B*). Upper traces: d.c. recordings; lower traces: a.c. recordings. In *A*, large injections of atropine by ionophoresis using a constant current apparatus led to a decrease in the size of the long-depolarization-induced p.s.c., which recovered after 45 min. In *B*, in another preparation, when less atropine was introduced into the presynaptic neurone, the size of the long-depolarization-induced p.s.c. increased.

neurones we cannot exclude the possibility that the injected atropine penetrated directly into the other interneurone. Nevertheless, this is unlikely because the electrotonic coupling is small (coupling ratio 0·05) and we never observed such molecular interchange for molecules of an equivalent molecular weight, like curare (in this paper), ACh (Poulain, Baux & Tauc, 1986) or Ruthenium Red (Baux *et al.* 1979).

By comparing the degree of depression of the p.s.c.s in the experiments illustrated in Fig. 7*A* and Fig. 9 with that obtained when atropine was applied extracellularly (Fig. 5), it can be estimated that the concentration of atropine in the synaptic cleft reached a final value near 10^{-4} M as a result of leakage from the injected neurone.

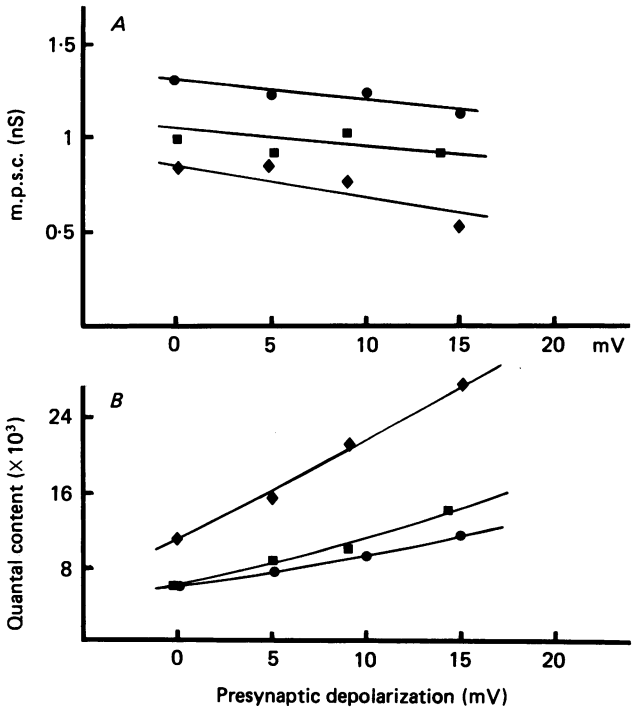

Fig. 8. Effects, in the same preparation as in Fig. 7, of ionophoretic injection of atropine into the presynaptic neurone of the buccal ganglion on the size of the m.p.s.c. (*A*) and the quantal content of long-depolarization-induced p.s.c. (*B*) for different presynaptic depolarizations. ●: control; ■: after 5 min injection with 50 nA current. ◆: after additional 5 min injection.

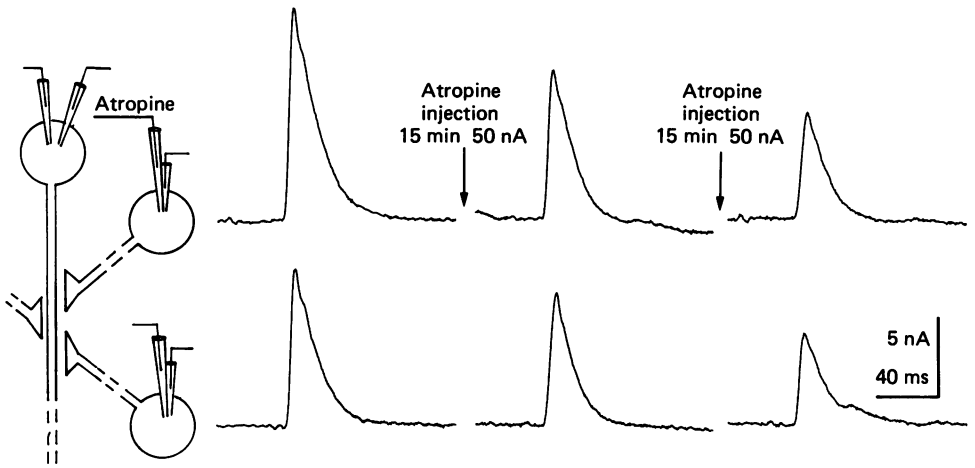

Fig. 9. Effect of ionophoretic injections of atropine into one of the two presynaptic cholinergic neurones of the buccal ganglion, on the post-synaptic response evoked by a spike. The post-synaptic cell was voltage clamped at −80 mV and the two presynaptic neurones left at their membrane resting potential (near −50 mV) were stimulated to produce a spike, which is not shown in the Figure. With increasing durations of ionophoretic injection (arrows) the post-synaptic responses were progressively depressed, even that evoked by a spike in the untreated presynaptic neurone.

Shorter intracellular injections loaded the neurone with less atropine (Fig. 7*B*); consequently less atropine leaked and accumulated in the synaptic cleft and the size of the calculated m.p.s.c.s was only slightly affected. Comparison of this result with the action on m.p.s.c. of atropine applied extracellularly showed that the atropine concentration in the cleft due to the leak was approximately 10^{-5} M. It is highly significant that the quantal content of the post-synaptic response was equally increased whether atropine was administrated extra- or intracellularly.

DISCUSSION

The results presented here show that tubocurarine and atropine have opposite effects on the release of ACh at a neuro-neuronal synapse: tubocurarine decreases while atropine increases the number of quanta liberated. Our experiments give further indications concerning the mechanisms which may be involved in this control of transmitter release.

Presynaptic action of extracellularly applied tubocurarine

Hubbard, Schmidt & Yokota (1965) tentatively explained the presynaptic action of tubocurarine at the neuromuscular junction by a fall of presynaptic conductance and subsequent decrease in the amplitude of the spike. However, the long-depolarization-induced p.s.c. method gave us the possibility of calculating simultaneously the sizes of the evoked response and m.p.s.c., and the presynaptic voltage clamp allowed us to eliminate the possible action of tubocurarine on the presynaptic action potential.

It also seems unlikely that tubocurarine acted presynaptically by altering the Ca^{2+} influx, since tubocurarine applied extracellularly or intracellularly did not change the voltage-dependent Ca^{2+} current in *Aplysia* cells displaying Ca^{2+} spikes.

Other hypotheses have been proposed to explain the presynaptic action of tubocurarine, some of which supposed an intervention in ACh metabolism. Bhatnagar & MacIntosh (1967) working on mouse brain, found that the ACh synthesis was limited by tubocurarine, and Martin (1968) showed that the choline uptake by human erythrocytes is inhibited. However, in our preparation we have not observed any relation between the tubocurarine-induced change in the quantal content of the responses and previous neuronal activity: the effect of tubocurarine on the quantal content of the responses was observed even if the preparation was not previously stimulated in the presence of the drug. One would expect the contrary if choline uptake was involved.

It is more likely that the presynaptic action of extracellularly applied tubocurarine is mediated by nicotinic or nicotinic-like receptors present on the presynaptic membrane. The activation of these receptors by released ACh would activate a process which facilitates more or less directly the release of the neurotransmitter. By blocking this positive feed-back, tubocurarine would reduce ACh release. The existence of presynaptic nicotinic receptors has been advanced for cholinergic transmission in the rat neuromuscular junction (Bowman *et al.* 1984), in synaptosomes of the myenteric plexus of the guinea-pig (Briggs & Cooper, 1982) and in mouse cerebral cortical synaptosomes (Rowell & Winkler, 1984).

Via presynaptic nicotinic receptors, ACh released by a terminal could affect the

ionic conductances at that terminal and facilitate the release of ACh. However, this possibility was not supported by our voltage-clamp experiments in which conductance changes of the presynaptic membrane did not alter ACh release (Poulain *et al.* 1986). Rather, presynaptic nicotinic receptors would appear to act via intracellular second messengers.

Presynaptic action of intracellularly injected tubocurarine

Tubocurarine introduced into the presynaptic neurone depressed the quantal content of the post-synaptic responses without affecting the size of the m.p.s.c. Thus the injected tubocurarine did not leak from the terminal, and acted only presynaptically.

Ca^{2+} channels were not affected by intracellular tubocurarine which can therefore be reasonably assumed to act on the transmitter release process. One possibility would be the interference with the uptake of ACh by synaptic vesicles or with their fusion with the presynaptic membrane. On the other hand, considering the non-vesicular hypothesis for ACh release, according to which the quantal release is performed by the binding of ACh to specific intrasynaptic receptors which form a part of the releasing structure or 'vesigate' (Tauc, 1982; Tauc & Baux, 1982), tubocurarine could also bind to some of these receptors. Because only 'vesigates' which are fully occupied with releasable transmitter can liberate ACh into the synaptic cleft, tubocurarine would block the release of the whole of the quanta.

Combining the results obtained with extracellularly and intracellularly applied tubocurarine, the possibility of an entry of the bath-applied tubocurarine into the presynaptic terminal, thereby depressing the quantal release, cannot be excluded. Such a possibility has already been suggested (Riker & Okamoto, 1969; Kuriyama, Roberts & Vos, 1968).

Presynaptic action of atropine

Atropine is a highly potent muscarinic antagonist. It can also act, although far less effectively, on nicotinic receptors; it is about 100 and 2000 times less effective on the frog and rat motor end-plates respectively (Beranek & Vyskocil, 1967). In these preparations it was shown that atropine shortens the mean open time of the post-synaptic ionic channel (Katz & Miledi, 1973; Feltz *et al.* 1977). However, in our preparation the concentrations of atropine used did not alter the decay of the evoked p.s.c. (Fig. 9), a clear indication that the time constant of the m.p.s.c. (and thus the channel open time) was not modified, a factor which has simplified our calculation of the quantal content.

At low concentrations (10^{-6}–10^{-5} M), atropine had a facilitatory action on ACh release. This increase may be interpreted as resulting from an interference of applied atropine with presynaptic muscarinic or muscarinic-like receptors implied in a negative feed-back control of ACh release. This hypothesis is supported by the results of Murray, Mpitsos, Siebenaller & Barker (1985) on *Aplysia* ganglia, where quinuclidinyl benzilate binding sites suggest the presence of muscarinic receptors. Increase in the amount of ACh liberated in the presence of atropine was described in vertebrate brain (for a review see Chesselet, 1984). The observed enhancement varied from 25% (Hadhazy & Szerb, 1977) to 120% (Jones *et al.* 1973).

Atropine at a concentration of 10^{-4} M reduced the quantal content (Fig. 5) and

acted like tubocurarine in this respect. If we accept the existence of presynaptic nicotinic receptors, the above-mentioned result may be due to a tubocurarine-like action of atropine at high concentration, as seen for post-synaptic receptors (Tauc & Gerschenfeld, 1962).

Conclusion

In conclusion, we have shown that at a molluscan cholinergic synapse, atropine and tubocurarine have opposing effects on the evoked quantal release of ACh. Unless tubocurarine enters the terminal, our evidence suggests the presence on the presynaptic terminal of two types of ACh receptors, one blocked by tubocurarine (presumably a nicotinic or nicotinic-like receptor), the other blocked by atropine (presumably a muscarinic or muscarinic-like receptor). Such an arrangement has already been proposed by Briggs & Cooper (1982) working on the synaptosomes prepared from myenteric plexus. Our study has the advantage that the observations were made on an identifiable, physiologically active, central synapse, which is fully accessible to several methods of investigation. Thus we hope to test our hypothesis in the near future, analyse the mechanism by which these two presynaptic receptors exert their opposite actions on transmitter release, and explore the implications of such receptors in the physiology of this synapse.

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