Proc. R. Soc. Lond. B **211**, 143–150 (1980)
Printed in Great Britain

Blocking of acetylcholine-induced channels by extracellular or intracellular application of D600

By R. Miledi, F.R.S., and I. Parker
Department of Biophysics, University College London, Gower Street, London WC1E 6BT, U.K.

(*Received* 29 *September* 1980)

D600 antagonizes the action of acetylcholine (ACh) at frog endplates, and can be applied iontophoretically. A pulse of D600 released during a prolonged application of ACh caused a rapid and long-lasting depression of the ACh current. Currents induced by ACh were depressed even when a pulse of D600 preceded a test dose of ACh by tens of seconds. A similar blocking action was also observed with intracellular application of D600. It seems possible that uncharged molecules of D600 enter the lipid phase of the membrane and bind to a site on the active ACh–receptor–channel complex, causing it to close rapidly.

Introduction

The combination of acetylcholine (ACh), or related agonist drugs with receptors, embedded in the muscle fibre membrane, leads to the transient opening of membrane channels through which various cations can flow (Katz & Miledi 1970, 1972; Anderson & Stevens 1973; Neher & Sakmann 1976). Antagonist drugs can block this action of ACh in various ways. For instance some antagonists, like α-bungarotoxin and low doses of curare, reduce the number of functional ACh receptors; but those that remain functional open with their characteristic lifetime and conductance (Katz & Miledi 1972, 1973*a*, 1978). This is presumably because such antagonists prevent the binding of ACh to the 'active site' in the receptor (Miledi & Potter 1971; Eldefrawi & Eldefrawi 1977). In contrast, other antagonists still allow ACh to combine with the receptor and open the channel, albeit with altered kinetics. For example, atropine causes the lifetime of the channel to be greatly reduced (Katz & Miledi 1973*b*; Adler & Albuquerque 1976; Feltz *et al.* 1977).

Local anaesthetics, and a number of neuromuscular blocking drugs, antagonize the action of ACh by blocking open channels (Steinbach 1968*a*, *b*); and it has been suggested that they simply plug the open channel (Adams 1976; Neher & Steinbach 1978). However, it has been shown that procaine is able to block the action of ACh even when applied intracellularly, probably because it binds to the receptor–channel complex at a site accessible through the lipid phase of the membrane (Katz & Miledi 1980). The present experiments were made to see if

D600, a 'calcium channel blocker', which has recently been shown to antagonize ACh action at the neuromuscular junction (Bregestovski *et al.* 1979, 1980), could be applied iontophoretically to the endplate, as this may help to understand its mode of action better.

Methods

Experiments were made on frog sartorius muscles by using techniques described previously (Katz & Miledi 1965, 1980). The muscles were kept at 5–7 °C, usually in normal Ringer solution. For some experiments calcium was replaced by 5 mM Mg^{2+} and 1 mM EGTA was used to keep free calcium at a very low level to prevent the contraction which normally results even after small doses of ACh (Miledi 1980); and also to see if D600 was active in the absence of external Ca^{2+}.

Endplates were voltage clamped with two microelectrodes. ACh and D600 (Knoll pharmaceuticals) were applied iontophoretically either from two separate pipettes or from the two lateral barrels of a triple-barrelled micropipette. The drugs were usually released at some distance from the muscle membrane to minimize possible complications arising from spurious movements.

Results

It was shown previously that, when D600 is present in the bathing solution, the current elicited by moderately long (100 ms) pulses of ACh applied to an endplate was not maintained, but decayed rapidly during the pulse. Furthermore, this block was markedly dependent on activity, for if the same ACh pulse was repeated a few seconds later the response was then considerably reduced, and recovered with a time constant of 12–20 s. These results, and others, suggested that D600 blocked active ACh-induced channels (Bregestovski *et al.* 1980.) To examine how rapidly D600 could block active channels, we tried to apply D600 iontophoretically.

Extracellular iontophoretic application of D600

As illustrated in figure 1*a*, an iontophoretic pulse of D600 blocked rapidly the current induced by ACh. The degree of block depended on the intensity of the pulse of D600 and in all cases the block persisted for a very long time (cf. figure 1*a*). It might be thought that such a long block is simply due to slow diffusion of D600. This, however, does not seem to be so, because D600 would be expected to diffuse freely in the medium at a rate comparable to that of ACh; as illustrated in figure 1*b*, when the pulse of ACh is turned off the ACh current quickly disappears, indicating that the ACh diffuses rapidly away from the receptors. Thus, it appears that D600 binds to the active ACh–receptor–channel complex quite rapidly, but dissociates from it much more slowly.

In the experiments described above, D600 was shown to block active channels. To see if D600 applied in the absence of ACh could antagonize the response to a subsequent pulse of ACh, pulses of D600 were followed at various intervals by

test pulses of ACh. Figure 2 shows sample records from one such experiment, which demonstrates that the response to ACh was depressed for many seconds after a pulse of D600. The recovery in two endplates is shown in figure 3. At both endplates the early part of the recovery fits moderately well an exponential (time constant *ca.* 45 s), but at longer intervals the rate of recovery seems to be slower. More detailed experiments are required to see if two time courses of recovery are consistently observed.

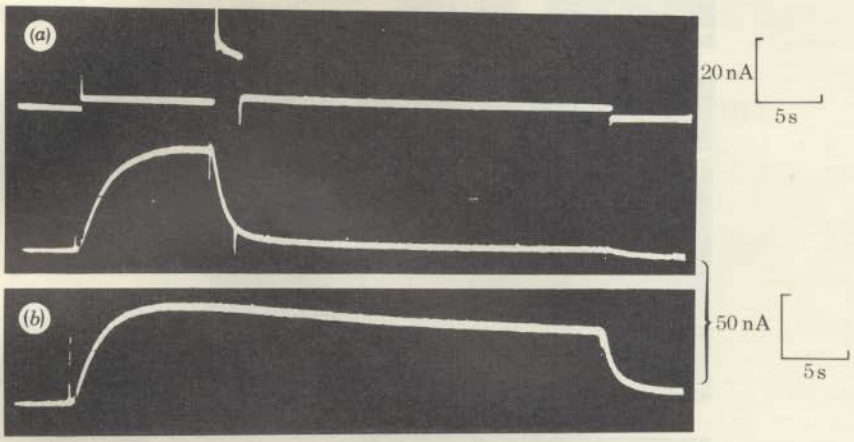

FIGURE 1. Extracellular iontophoretic application of D600 during the response produced by a prolonged pulse of ACh. Upper trace monitors iontophoretic current through both the ACh and D600 barrels of a triple-barrelled pipette, and the lower traces show clamp current. The endplate was voltage-clamped at -90 mV. (*a*) D600 pulse given during the ACh application; (*b*) Control ACh current obtained 2 min later, with the use of the same ACh pulse, but without D600 pulse. EGTA Ringer solution without Ca^{2+} and with Mg^{2+} was used, at a temperature of 6 °C.

Thus, a pulse of D600 applied to the resting membrane antagonizes the action of ACh at a time when most of the drug would have diffused away from the endplate. Since the pK value of D600 is approximately 8.5 (Dörrscheidt-Käfer 1977), at the neutral pH used in these experiments about 5 % of the drug would have been in an uncharged form. These molecules might then be able to enter the lipid phase of the membrane and exit from it slowly, thus contributing to the slow recovery after a pulse of D600.

Intracellular application of D600

D600 might enter the membrane, and act there on the ACh–receptor–channel complex, to inhibit its response to ACh. If that were the case, D600 would be expected to act also if injected inside the muscle fibre. That this is so is illustrated in figure 4*a*, where three pulses of D600 were *intracellularly* applied to an endplate during a train of *extracellularly* applied ACh pulses. The response to ACh was markedly depressed by the intracellular injection of D600, and several minutes were required for the response to recover.

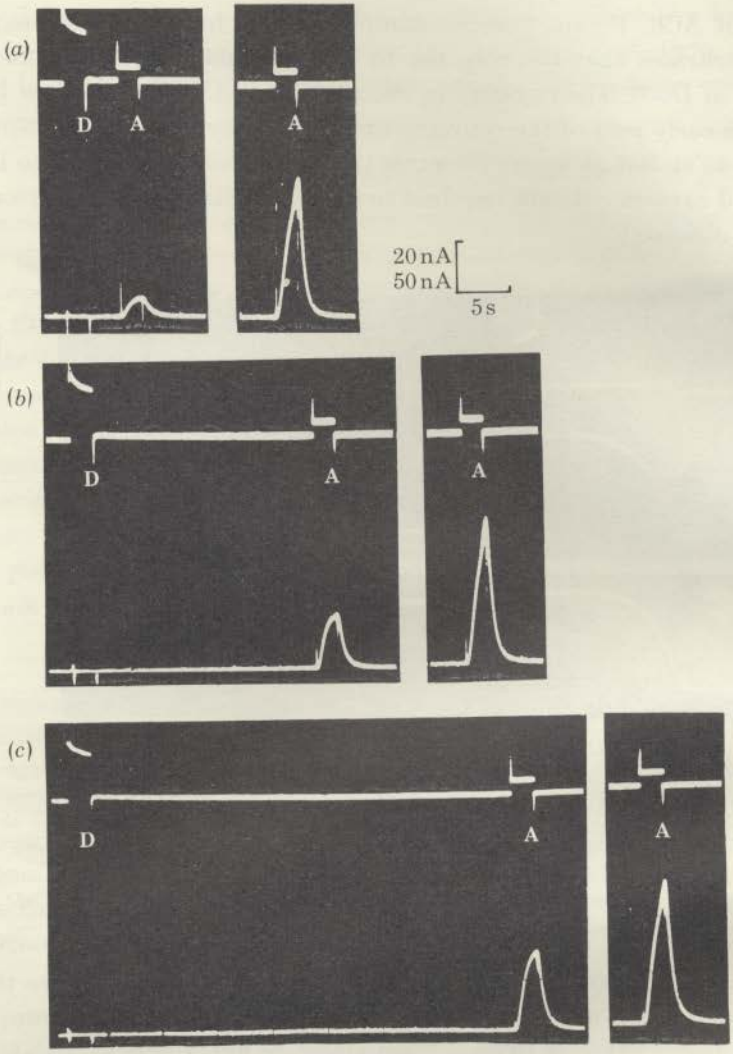

FIGURE 2. Depression of ACh currents by a preceding extracellular pulse of D600. Endplate voltage was clamped at −90 mV; Ringer without Ca^{2+} was used at a temperature of 6 °C. Records show the effect of various intervals between the D600 pulse (D) and the ACh pulse (A). The upper trace in each frame (vertical scale bar = 20 nA) monitors iontophoretic current through both the ACh and D600 barrels of the triple-barrelled pipette, and the lower trace (vertical scale bar = 50 nA) shows clamp current. In the left-hand frames, a pulse of D600, applied just after the beginning of each record, was followed after different intervals by an ACh test pulse. The right-hand frames show control responses, elicited by an ACh pulse alone, delivered at least 2 min after the preceding record.

During inactivation by internally applied D600, the ACh currents had characteristics similar to those seen after external application. For instance, the striking depression of the response to the second of a pair of ACh pulses, seen with bath applied D600 (Bregestovski *et al.* 1980) was also seen after internal application (figure 4*b*, *c*), as was also the failure of the ACh current to increase progressively during relatively short ACh pulses (figure 4*c*).

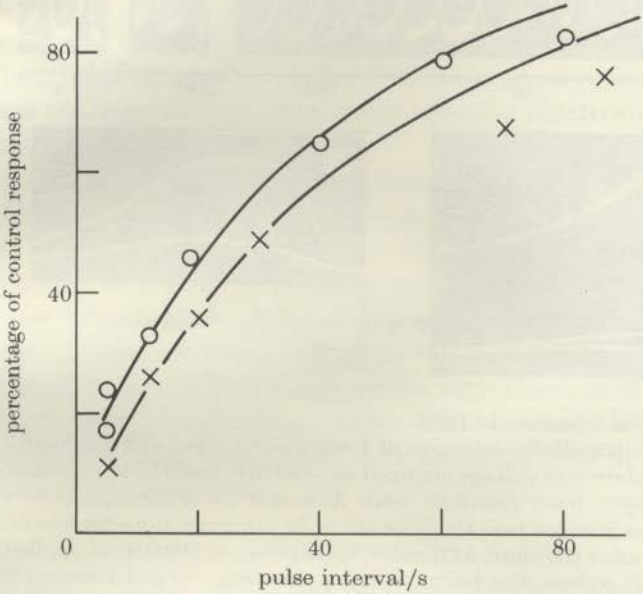

FIGURE 3. Depression of ACh-induced current caused by a preceding pulse of D600, plotted against the interval between the end of the D600 pulse and the start of the ACh pulse. Peak ACh responses were measured from traces similar to those shown in figure 2, and are expressed as a percentage of the control response obtained at least 2 min after the last D600 pulse. Data from two endplates are shown, and have been fitted by exponentials with time constants of 42 s (○) and 46 s (×).

DISCUSSION

The present experiments confirm our previous observation (Bregestovski *et al.* 1980) that D600 antagonizes the action of ACh at the neuromuscular junction. They show further that D600 is effective regardless of whether it is applied extracellularly or injected into the muscle fibre. In this respect D600 is unlike other antagonists such as curare, gallamine and tetraethylammonium, which are ineffective when applied intracellularly, but resembles procaine, which has been shown to have its characteristic blocking action irrespective of the route of application (Katz & Miledi 1980).

Another interesting feature is that the blocking action of a brief extracellular

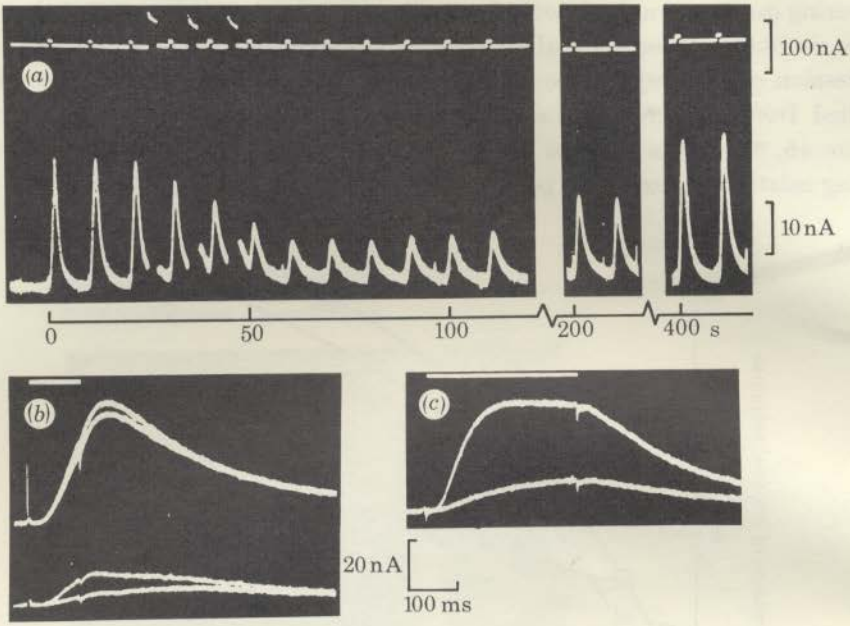

FIGURE 4. Intracellular injection of D600.

(*a*) Effect of intracellular injection of D600 during a train of externally applied ACh pulses. The endplate was voltage clamped at -90 mV, and the lower trace shows clamp current. The upper trace monitors both ACh and D600 iontophoretic currents. The D600 pipette was inserted into the fibre about 50 μm away from the site of ACh application. Beginning after the third ACh pulse, three pulses of D600 were applied, interspersed between the ACh pulses. The train of ACh pulses was stopped between 250 and 380 s.

(*b*)–(*c*) Time course of a pair of ACh-induced endplate currents following intracellular injection of D600. Each set shows two superimposed responses to ACh pulses applied with a 5 s interval. In all cases the smaller response resulted from the second pulse of the pair. The bars indicate the duration of the ACh pulses. Calibrations apply to both (*b*) and (*c*).

The upper set in (*b*) shows control responses before intracellular injection of D600, while the lower pair was obtained by using the same ACh test pulse, but after injection of about 100 pulses of D600. A small amount of D600 had been injected into the fibre about 10 min before the control record was obtained, and the second response is slightly depressed, probably because of the presence of residual D600 in the fibre.

The record in (*c*) was obtained after further injection of about 300 pulses of D600, and the duration of the ACh pulse was increased to demonstrate the flattening of the ACh response during the duration of the pulse.

All records in this figure were obtained from the same endplate, which was bathed in normal Ringer solution, at a temperature of 5 °C.

pulse of D600 is long-lasting, so that a pulse of ACh applied tens of seconds later is still depressed, even though D600 would have been expected to have diffused away from the endplate. Moreover, if repeated ACh pulses are given at this time, the amplitude of the currents induced by successive pulses declines rapidly. Thus, after a transient application of D600 there is an activity dependent block similar to that previously observed in the continued presence of D600 (Bregestovski *et al.*

1980); but since the amplitude of miniature endplate currents is decreased and even the response to the first pulse of ACh is depressed, D600 may also antagonize inactive channels.

Perhaps the simplest explanation of our results is that, like procaine (cf. Katz & Miledi 1980), the uncharged molecules of D600 penetrate the lipid phase of the membrane and bind to a site in the ACh receptor (different from its ACh binding site), and prevent ACh from opening the channels, or if they open the D600 causes them to close rapidly. To explain the increased block of activated channels it may be postulated that ACh induces a conformational change of the ACh receptor complex, which leads to more binding of D600 from a reservoir of drug present in the lipid phase of the membrane, or to a more effective inactivating action of D600 already bound to the receptor.

Other non-competitive antagonists appear to alter channel kinetics through binding to the ACh–receptor–channel complex at a site different from the ACh binding site (Katz & Miledi 1973; Weber & Changeux 1974; Tsai *et al.* 1978). One of these agents, a derivative of procaine, has been shown to bind to the 66000 molecular mass subunit of the ACh receptor complex (Saitoh *et al.* 1980). It will be interesting to know to which subunit(s) the many different non-competitive antagonists bind. In the meantime our results with D600, as those with procaine (Katz & Miledi 1980), suggest that rather than binding through the aqueous phase of the channel these antagonists have access to their binding site through the lipid phase of the membrane.

References

Adams, P. R. 1976 Drug blockade at open endplate channels. *J. Physiol., Lond.* **260**, 531–552.

Adler, M. & Albuquerque, E. X. 1976 An analysis of the action of atropine and scopolamine on the end-plate current of frog sartorius muscle. *J. Pharmac. exp. Ther.* **196**, 360–372.

Anderson, C. R. & Stevens, C. F. 1973 Voltage clamp analysis of acetylcholine produced end-plate current fluctuations at frog neuromuscular junction. *J. Physiol. Lond.* **235**, 655–691.

Bregestovski, P. D., Miledi, R. & Parker, I. 1979 Calcium conductance of acetylcholine-induced endplate channels. *Nature, Lond.* **279**, 638–639.

Bregestovski, P. D., Miledi, R. & Parker, I. 1980 Blocking of frog endplate channels by the organic calcium antagonist D600. *Proc. R. Soc. Lond.* B **211**, 15–24. (This volume.)

Cohen, J. B., Weber, M. & Changeux, J. P. 1974 Effects of local anaesthetics and calcium on the interaction of cholinergic ligands with the nicotinic receptor protein from *Torpedo marmorata*. *Molec. Pharmac.* **10**, 904–832.

Dörrscheidt-Käfer, M. 1977 The action of D600 on frog skeletal muscle: facilitation of excitation–contraction coupling. *Pflügers Arch. ges. Physiol.* **369**, 259–267.

Eldefrawi, M. E. & Eldefrawi, A. T. 1977 In *Receptors and recognition* (ed. P. Cuatrecasas & M. F. Greaves), vol. 4, pp. 197–258. London: Chapman & Hall.

Feltz, A., Large, W. A. & Trautmann, A. 1977 Analysis of atropine action at the frog neuromuscular junction. *J. Physiol., Lond.* **269**, 109–130.

Katz, R. & Miledi, B. 1965 Propagation of electrical activity in motor nerve terminals. *Proc. R. Soc. Lond.* B **161**, 453–482.

Katz, B. & Miledi, E. 1970 Membrane noise produced by acetylcholine. *Nature, Lond.* **226**, 962–963.

Katz, B. & Miledi, R. 1972 The statistical nature of the acetylcholine potential and its molecular components. *J. Physiol., Lond.* **224**, 665–699.

Katz, B. & Miledi, R. 1973*a* The effect of α-bungarotoxin on acetylcholine receptors. *Br. J. Pharmac.* **49**, 138–139.

Katz, B. & Miledi, R. 1973*b* The effect of atropine on the action of acetylcholine at the neuromuscular junction. *Proc. R. Soc. Lond.* B **184**, 221–226.

Katz, B. & Miledi, R. 1978 A re-examination of curare action at the motor endplate. *Proc. R. Soc. Lond.* B **203**, 119–133.

Katz, B. & Miledi, R. 1980 Blockade of endplate responses by intracellular application of procaine. In *Ontogenesis and functional mechanisms of peripheral synapses* (ed. J. Taxi), pp. 171–178. Amsterdam: Elsevier–North Holland.

Miledi, R. 1980 Intracellular calcium and desensitization of acetylcholine receptors. *Proc. R. Soc. Lond.* B **209**, 447–452.

Miledi, R. & Potter, L. T. 1971 Acetylcholine receptors in muscle fibres. *Nature, Lond.* **233**, 599–603.

Neher, E. & Sakmann, B. 1976 Single channel currents recorded from membrane of denervated frog muscle fibres. *Nature, Lond.* **260**, 779–802.

Neher, E. & Steinbach, J. H. 1978 Local anaesthetic transiently blocked currents through single acetylcholine-receptor channels. *J. Physiol., Lond.* **277**, 153–176.

Saitoh, T., Oswald, R., Wennagle, L. P. & Changeux, J. P. 1980 Conditions for the selective labelling of the 66000 dalton chain of the acetylcholine receptor by the covalent non-competitive blocker 5-azido-[^{3}H]trimethisoquin. *FEBS Lett.* **116**, 30–36.

Steinbach, A. B. 1968*a* Alteration by xylocaine (lidocaine and its derivatives) of the time course of the end-plate potential. *J. gen. Physiol.* **52**, 144–161.

Steinbach, A. G. 1968*b* A kinetic model for the action of xylocaine on receptors for acetylcholine. *J. gen. Physiol.* **52**, 162–180.

Tsai, M.-C., Mansour, N. A., Eldefrawi, A. T., Eldefrawi, M. E. & Albuquerque, E. X. 1978 Mechanism of action of amantadine on neuromuscular transmission. *Molec. Pharmac.* **14**, 787–803.