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ACTIONS OF DENDROTOXIN ON K^+ CHANNELS AND NEUROMUSCULAR TRANSMISSION IN *DROSOPHILA MELANOGASTER*, AND ITS EFFECTS IN SYNERGY WITH K^+ CHANNEL-SPECIFIC DRUGS AND MUTATIONS

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Summary

The blockade of K^+ channels and enhancement of neuromuscular transmission by dendrotoxin (DTX), a convulsant peptide from mamba snake venom, were examined in normal and mutant larval preparations of *Drosophila*. Two-microelectrode voltage-clamp experiments showed that DTX reduced the transient K^+ current, I_A, in muscle membrane. This effect was suppressed by raising the Mg^{2+} concentration or by lowering the temperature. The interaction of DTX with Mg^{2+} was further analyzed at a low cation concentration, at which DTX reduced both I_A and the delayed rectifier I_K. These results were correlated with the action of DTX on the neuromuscular junction. Its facilitatory effect on excitatory junctional potentials (EJPs) was relatively mild but the effect was drastically enhanced when combined with certain mutations and K^+ channel blocking drugs, leading to repetitive or prolonged giant EJPs. Only the mutations or drugs that reduced I_K or the Ca^{2+}-dependent K^+ current, I_{CF}, could yield these synergistic effects with DTX. In contrast, the abnormal EJPs caused by the mutation or drug that blocked I_A were not further enhanced by DTX, indicating that DTX also affects I_A at the neuromuscular junction. Thus, the A-type K^+ channels in muscle and nerve terminals appeared very similar in their sensitivity to the specific toxin, drugs and mutations examined here.

Introduction

A diversity of K^+ channels gives rise to a large repertoire of firing patterns in nervous systems, and a definitive classification of different K^+ channels is not yet possible (Latorre & Miller, 1983; Wu & Ganetzky, 1986; Rudy, 1988). Specific toxins, drugs and mutations thus provide useful tools to compare the types and properties of the channels in different excitable membranes.

Key words: dendrotoxin, *Drosophila* mutants, presynaptic terminals, muscle, K^+ channel diversity, quinidine, 4-aminopyridine.

Dendrotoxin (DTX), a non-enzymatic venom protein from the mamba snake *Dendroaspis angusticeps*, is a potent convulsant neurotoxin when injected into the brain. DTX is also the first snake toxin found to facilitate neurotransmitter release at vertebrate neuromuscular junctions (Harvey & Karlsson, 1980; Harvey *et al.* 1984; Harvey, 1987) and central synapses (Dolly *et al.* 1984). Its action has been characterized in several vertebrate species by voltage-clamp experiments and a reduction in K^+ conductances is correlated with the facilitatory effect of the toxin (Dolly *et al.* 1984; Weller *et al.* 1985; Benoit & Dubois, 1986; Halliwell *et al.* 1986). However, the specific types of K^+ conductances that are affected by this toxin vary in different preparations. In frog peripheral nerve nodes of Ranvier, a component of slowly inactivating K^+ current is decreased by DTX (Weller *et al.* 1985; Benoit & Dubois, 1986), whereas in the cell body of central hippocampal neurons a transient K^+ current, I_A, is suppressed (Dolly *et al.* 1984; Halliwell *et al.* 1986). However, the transient K^+ current in rat sympathetic ganglion neurons is reported to be insensitive to DTX (Dolly *et al.* 1987). The endplate potential (EPP) in the frog neuromuscular junction is enhanced by increased quantal content and by multiple firing of the motor axon, but the type of K^+ channels affected by DTX is unknown (Harvey & Anderson, 1985; Harvey, 1987). Although these contrasting results may be attributed to species differences, a more intriguing possibility is that they reflect distinctions and similarities among different K^+ channels.

The generality of a DTX blocking action on K^+ channels has not been well established since only vertebrate species have been studied. In the present study, we examined the effects of DTX on neuromuscular transmission and on muscle K^+ channels of an arthropod species, *Drosophila melanogaster*. Both the wild-type and K^+ channel mutants were studied to examine whether DTX can be used as a probe for characterization of various K^+ channels. The genetics of membrane excitability in *Drosophila* has been well studied. Several mutations that eliminate or alter specific types of K^+ currents are available (Ganetzky & Wu, 1986; Wu & Ganetzky, 1986).

The larval neuromuscular preparation of *Drosophila* is suitable for electrophysiological studies because the properties of neuromuscular transmission and of various ion channels in the muscle membrane have been characterized in detail (Jan & Jan, 1976*a*,*b*; Jan *et al.* 1977; Wu *et al.* 1978, 1983; Ganetzky & Wu, 1982, 1983, 1985; Wu & Haugland, 1985; Gho & Mallart, 1986; Haugland, 1987; Singh & Wu, 1989; F. N. Haugland & C.-F. Wu, in preparation). Voltage-clamp studies of these larval muscle fibers have demonstrated that mutations of three genes separately affect different types of K^+ channels. The transient I_A is missing in *Shaker* (*Sh*) mutants (Wu *et al.* 1983; Wu & Haugland, 1985); the delayed rectifier, I_K, is reduced to about 50 % in an *ether a go-go* (*eag*) mutant (Wu *et al.* 1983); and a Ca^{2+}-dependent K^+ current, I_{CF}, is eliminated in the *slowpoke* (*slo*) mutant (Elkins *et al.* 1986; Singh & Wu, 1987, 1989). We used these mutants in conjunction with other K^+ channel blocking agents to characterize the effect of DTX on neuromuscular transmission. The synergism between the effects of DTX and various mutations was studied to infer the types of presynaptic K^+ channels

affected by DTX. The action of DTX on muscle membrane currents was also directly monitored by the voltage-clamp technique. These experiments using *Drosophila* mutants not only extend our understanding of the action of DTX on invertebrate K^+ channels but also offer a unique opportunity for comparing the types and properties of the K^+ channels present in nerve terminals and muscle fibers.

Materials and methods

Mutants

The wild-type strain, Canton-S, of *Drosophila melanogaster* was used for characterization of normal physiology. Among available mutant alleles of the *Sh* and *eag* loci (Ganetzky & Wu, 1986), Sh^{KS133} and eag^1 were chosen for this study because they have been described in the most detail. They are also the most extreme alleles of these two loci; Sh^{KS133} completely blocks I_A and eag^1 removes about 50 % of I_K (Wu *et al.* 1983; Wu & Haugland, 1985; Y. Zhong & C.-F. Wu, unpublished results). The *slo* locus is represented by a single allele (Elkins *et al.* 1986), which was used in this study.

Electrophysiological recording

For intracellular recording of EJPs, the larval neuromuscular preparation (Jan & Jan, 1976*a*; Wu *et al.* 1978) was used. Briefly, a late third-instar larva was pinned out in saline, cut along the dorsal midline and eviscerated. The nerves innervating the body wall muscles in the abdominal segments were severed near the ventral ganglion. The cut end of a segmental nerve was stimulated *via* a suction electrode and EJPs were recorded in the ventral longitudinal muscle fibers. Experiments were carried out in *Drosophila* saline containing 128 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 35 mmol l^{-1} sucrose, and buffered at pH 7·1 with 5 mmol l^{-1} Hepes. The concentrations of Ca^{2+} (0·1, 0·2 or 1·8 mmol l^{-1}) and Mg^{2+} (0·5, 2, 4 or 14 mmol l^{-1}) were varied as specified in each experiment. Other details of the experimental protocol have been described elsewhere (Jan & Jan, 1976*a*; Wu *et al.* 1978).

An identical preparation was used for conventional two-microelectrode voltage-clamp measurements of muscle membrane currents. We followed the established paradigms and protocols (Wu & Haugland, 1985; Haugland, 1987; F. N. Haugland & C.-F. Wu, in preparation) and only a brief description is given here. Measurements of I_A and I_K were made in Ca^{2+}-free saline containing 0·5 mmol l^{-1} EGTA. The concentration of $MgCl_2$ varied in each experiment, as specified. The composition of the Ca^{2+}-free saline was otherwise identical to that used for EJP recordings (see above). The large differences in inactivation and recovery kinetics between I_A and I_K were utilized to separate these two currents. At a holding potential (V_H) of −80 mV, step depolarizations elicited I_A, which peaked rapidly and then inactivated, and I_K, which rose to a sustained plateau with a delay (Figs 6, 8 and 10). When a depolarizing prepulse (to −20 mV)

preceded the test pulse, I_A was inactivated, leaving I_K alone. Digital subtraction of the two measurements (with or without the prepulse) yielded the inactivating I_A in isolation from I_K (Figs 7 and 9). The duration of the prepulse (2 s) and the interval between the prepulse and test pulse at 5°C (10 ms) and 16°C (5 ms) were chosen from previously established values to maximize the separation of these two currents (Haugland, 1987; F. N. Haugland & C.-F. Wu, in preparation; F. N. Haugland, Y. Zhong & C.-F. Wu, unpublished data).

When Ca^{2+} currents and Ca^{2+}-dependent K^+ currents were examined, 20 mmol l^{-1} $CaCl_2$, 4 mmol l^{-1} $MgCl_2$ and no EGTA were present in saline. All voltage-clamp measurements were stored digitally and analyzed by a microcomputer (Macintosh, Apple Computer, Cupertino, CA). After subtraction of linear leakage currents, the density of active currents was determined by adjustment for the cell membrane area of the fiber and expressed in $\mu A\,\mu F^{-1}$ using membrane capacitance as an indicator. For each experiment, data collected from a number of fibers were averaged as shown in the traces and current–voltage (I–V) relationships in Figs 6–10. The numbers of fibers and larvae used in each experiment are specified in the legend of the figure.

Measured amounts of DTX from *D. angusticeps*, 4-aminopyridine (4-AP) and quinidine stock solutions were added to the bathing chamber (volume, 1 ml) to achieve the final concentrations. Solutions were carefully mixed and enough time was allowed for equilibration. Quinidine and 4-AP were acquired from Sigma Chemical Co. (St Louis, MO) and DTX was a gift from Drs F. J. Joubert and F. F. H. Carlsson.

Intracellular recordings of EJPs were performed at 25–29°C, except where stated otherwise. The results in each figure (Figs 1–5) were based on observations on 5–11 fibers in 3–8 larvae. In voltage-clamp experiments, preparations were cooled to either 5 or 16°C. At higher temperatures, the rapid kinetics of the membrane currents made it difficult to achieve satisfactory control of voltage jumps (Wu & Haugland, 1985).

Results

DTX effects on transmitter release at normal neuromuscular junctions

The ventral longitudinal body-wall muscle fibers in the abdominal segments of *Drosophila* larvae are each innervated by two motor neurons; and only excitatory junctional potentials (EJPs), but not inhibitory potentials, can be evoked by nerve stimuli (Jan & Jan, 1976*a*). As in other arthropods, glutamate is the putative transmitter at the neuromuscular junction (Jan & Jan, 1976*b*; Johansen *et al.* 1989) and there are Ca^{2+} channels and various types of K^+ channels, but no Na^+ channels, in the muscle membrane (Wu & Haugland, 1985; Haugland, 1987; Singh & Wu, 1989). Normally, only graded potentials are evoked in these muscle fibers, although all-or-none Ca^{2+} action potentials may be generated when the K^+ channels are blocked (Suzuki & Kano, 1977).

It has been reported that the activity, but not binding, of DTX is suppressed by increased Mg^{2+} concentrations (3 mmol l^{-1}) in isolated vertebrate nerve–muscle preparations (Harvey & Anderson, 1985). When this phenomenon was investigated in larval muscles, we found that the effects of DTX on neuromuscular transmission in *Drosophila* depended on Ca^{2+} as well as Mg^{2+} concentrations in the saline. Even at the normal Ca^{2+} concentration (1·8 mmol l^{-1}), the action of DTX was apparently inhibited since EJPs were not significantly altered in the presence of 4·2 μmol l^{-1} (30 μg ml^{-1}) DTX. This was true for preparations bathed in salines containing either normal (4 mmol l^{-1}, data not shown) or reduced (2 mmol l^{-1}, Fig. 1A) concentrations of Mg^{2+}.

The action of DTX became apparent when the external Ca^{2+} concentration was lowered (Fig. 1B,C). At 0·1 mmol l^{-1} Ca^{2+}, EJPs showed quantal fluctuations in size and miniature EJPs (mEJPs) were the only spontaneous activity observed, consistent with previous reports (Jan *et al.* 1977; Ganetzky & Wu, 1982). Upon addition of DTX, the amplitude of EJPs increased appreciably (on average to 164 % of the control value in two fibers) and repetitive EJPs were occasionally evoked by a single nerve stimulus (Fig. 1B). The DTX effects were further enhanced if the Mg^{2+} concentration was also reduced from 4 to 2 mmol l^{-1}. At low Mg^{2+} and Ca^{2+} concentrations the neuromuscular junction became more excitable, often leading to more than one, and sometimes up to five, EJPs in response to a single stimulus (Fig. 1C). In the presence of DTX, prolonged trains of repetitive EJPs (up to 30) of increased size occurred spontaneously or in response to stimulation (Fig. 1C).

Further experiments showed that at very low cation concentrations (0·2 mmol l^{-1} Ca^{2+} and 0·5 mmol l^{-1} Mg^{2+}), DTX caused clearly enhanced neuromuscular transmission at either high (20°C) or low (8°C) temperature. This effect was completely suppressed by 14 mmol l^{-1} Mg^{2+} (data not shown). The suppressive effect of Mg^{2+} is apparently on the activity rather than on the binding of DTX to its target site, as previously shown in vertebrate preparations (Harvey & Anderson, 1985). Application of DTX (2·8 μmol l^{-1}) in high-Mg^{2+} saline (4 or 14 mmol l^{-1} Mg^{2+} and 0·2 mmol l^{-1} Ca^{2+}) did not change larval EJPs at 20°C. Nevertheless, following a rapid wash with low-Mg^{2+} saline (containing 0·5 or 1 mmol l^{-1} Mg^{2+} and 0·2 mmol l^{-1} Ca^{2+}, but no DTX), a single nerve stimulus evoked repetitive EJPs with increased amplitude. These facilitated EJPs were suppressed again by high-Mg^{2+} saline (without DTX) and reappeared in low-Mg^{2+} saline (data not shown).

Calcium ions may suppress the effect of DTX on EJPs by a mechanism similar to that of magnesium ions. But at least part of the suppression at high external Ca^{2+} concentrations can be attributed to the activation of a Ca^{2+}-dependent K^+ conductance in the nerve terminals (Jan *et al.* 1977; Ganetzky & Wu, 1982, 1983). This notion was supported by the results obtained in *slo* mutants, in which a Ca^{2+}-dependent K^+ current, I_{CF}, is eliminated (Elkins *et al.* 1986; Singh & Wu, 1987, 1989). In saline containing 1·8 mmol l^{-1} Ca^{2+} and 4·0 mmol l^{-1} Mg^{2+}, EJPs in *slo* larvae were nearly normal. Following treatment with 4·2 μmol l^{-1} DTX, the

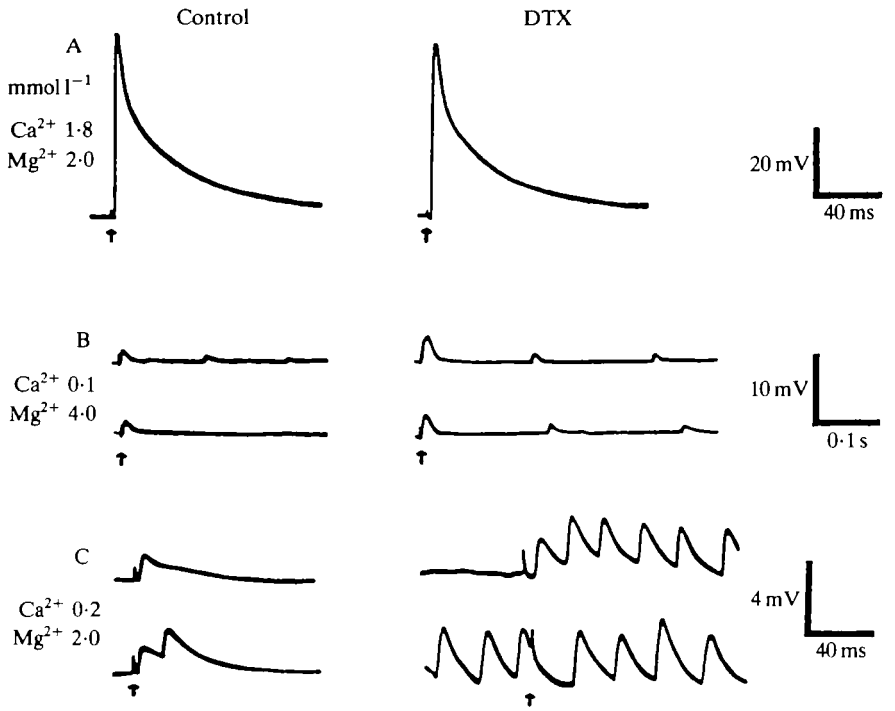

Fig. 1. Effects of dendrotoxin (DTX) on excitatory junctional potentials (EJPs) recorded intracellularly from ventral longitudinal muscle fibers of normal larvae at the Ca^{2+} and Mg^{2+} concentrations indicated. (A) Addition of 4·2 μmol l^{-1} (30 μg ml^{-1}) DTX did not result in any apparent changes in standard saline containing 1·8 mmol l^{-1} Ca^{2+} and 4 mmol l^{-1} Mg^{2+}. Even when the Mg^{2+} concentration was lowered to 2 mmol l^{-1}, no differences in EJPs were observed 30 min after the application of DTX. (B) At a low concentration (0·1 mmol l^{-1}) of external Ca^{2+}, only small EJPs and some spontaneous miniature EJPs were seen. Treatment with DTX (4·2 μmol l^{-1}, 3 min) caused an increase in EJP amplitude and repetitive discharges of EJPs. (C) When the external Mg^{2+} concentration was lowered from 4 to 2 mmol l^{-1}, the motor nerve terminals became more excitable at 0·2 mmol l^{-1} Ca^{2+}, leading to occasional multiple EJPs. After adding 4·2 μmol l^{-1} DTX to saline, long trains of repetitive EJPs of increased size were elicited by nerve stimuli or occurred spontaneously. A nerve stimulus failed to elicit additional EJPs during a spontaneous discharge (lower trace). In this and the following four figures, stimulus artifacts are indicated by arrows. Recordings were performed at 25–29 °C.

duration of EJPs showed an approximately twofold increase (three fibers in different larvae, data not shown). Thus, DTX might affect some conductances other than I_{CF} and its effects become more obvious when I_{CF}, and preferably all other K^+ currents that are not affected by DTX, are removed. In the following experiments, EJP data were collected at low Ca^{2+} concentrations (0·1 or 0·2 mmol l^{-1}) to minimize the complications introduced by the Ca^{2+}-dependent K^+ currents.

Combined effects of DTX with a drug and a mutation that block I_A

The effects of DTX on larval neuromuscular junctions were relatively minor in comparison with the changes induced by the K^+ channel blocker 4-AP or the *Sh* mutation. Voltage-clamp measurements in larval muscle have shown that the *Sh* mutation eliminates I_A and that 4-AP at 0·1 mmol l^{-1} blocks nearly all I_A with a minimal reduction in I_K (Haugland, 1987; Haugland & Wu, 1987). In addition, quinidine at 0·1 mmol l^{-1} suppresses I_K without affecting other K^+ currents (Singh & Wu, 1987, 1989).

As previously described (Jan *et al.* 1977), treatment with 0·1 mmol l^{-1} 4-AP drastically increased both the amplitude and the duration of EJPs (Fig. 2A,B), reflecting enhanced presynaptic Ca^{2+} influx and repetitive firing (Ganetzky & Wu, 1982). Addition of 4·2 μmol l^{-1} DTX to the bath did not further enhance the transmitter release (Fig. 2C). In contrast, further application of just 50 μmol l^{-1} quinidine prolonged the duration of EJPs from tens to hundreds of milliseconds (Fig. 2D). The prolonged EJPs were similar to those previously reported in drug-treated or double-mutant preparations in which both I_A and I_K are reduced (Wu *et al.* 1983; Ganetzky & Wu, 1983, 1985). These phenomena parallel the

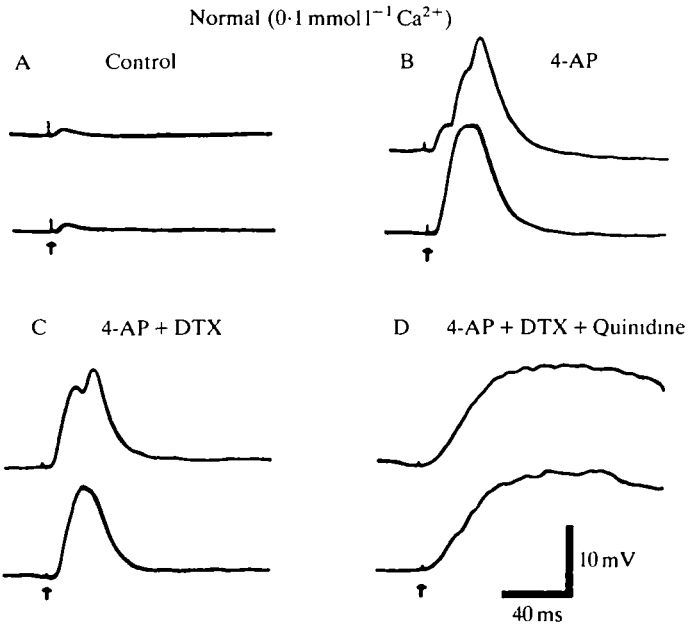

Fig. 2. Combined effect of DTX and 4-aminopyridine (4-AP) on normal neuromuscular junctions in saline containing 0·1 mmol l^{-1} Ca^{2+} and 4 mmol l^{-1} Mg^{2+}. Treatment with 0·1 mmol l^{-1} 4-AP transformed small responses (A) into giant EJPs (B) as a result of increased transmitter release and repetitive firing in the motor axon (Jan *et al.* 1977; Ganetzky & Wu, 1982). No further enhancement of transmitter release was produced by addition of DTX (4·2 μmol l^{-1}, 20 min) to the 4-AP-treated preparation (C). In contrast, 50 μmol l^{-1} quinidine immediately augmented the effect of 4-AP, resulting in greatly prolonged EJPs. Sequential data from the same cell.

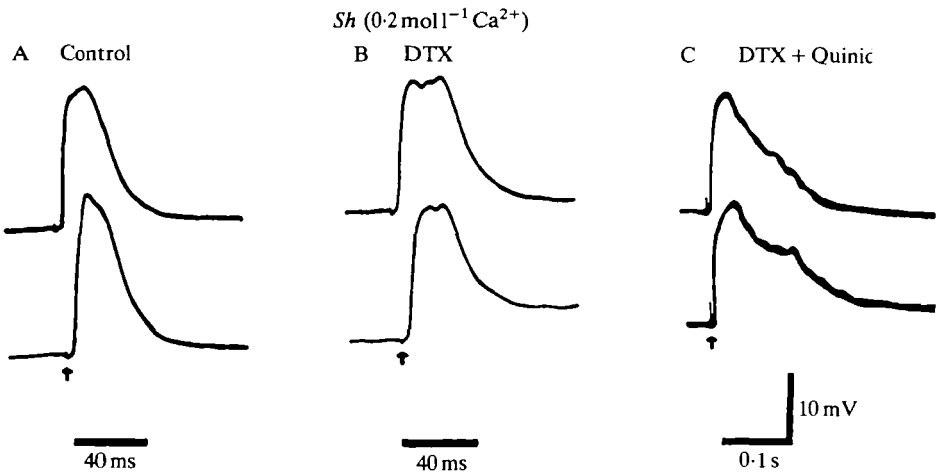

Fig. 3. Combined effects of DTX and a mutation eliminating I_A in saline containing 0·2 mmol l^{-1} Ca^{2+} and 4 mmol l^{-1} Mg^{2+}. (A) EJPs in *Sh* larvae resembled those in 4-AP-treated normal larvae. (B) The presence of 4·2 μmol l^{-1} DTX did not further enhance the transmitter release in *Sh* larvae. (C) Quinidine (50 μmol l^{-1}) generated greatly prolonged EJPs in the same preparation. Note the different time scale in C. Sequential recordings from the same cell. The resting potential had decreased from −47 mV in A and B to −35 mV in C owing to muscle contractions, resulting in a smaller amplitude of EJPs in C.

observations in *Sh* larvae. Identical results were obtained by adding quinidine (50 μmol l^{-1}) alone to *Sh* larvae with or without DTX pre-treatment. The *Sh* phenotype, i.e. raised and prolonged transmitter release (Fig. 3A, also see Jan *et al.* 1977), was further enhanced by quinidine but not by DTX (Fig. 3B,C).

Synergistic effects of DTX with a drug and a mutation that reduce I_K

The above results raised the possibility that DTX acted mainly to reduce I_A in nerve terminals and thus was not able to exert additional effects when I_A was blocked by 4-AP or the *Sh* mutation. This interpretation was confirmed in a different set of experiments employing quinidine and the *eag* mutation, which blocks about 50 % of I_K in larval muscle (Wu *et al.* 1983).

Although the effects of DTX on transmitter release are relatively mild in normal larvae (Fig. 1), it produced strong enhancement of neuromuscular transmission in *eag* larvae or in normal preparations pre-treated with quinidine. A low dose (50 μmol l^{-1}) of quinidine alone only slightly increased the amplitude of EJPs (Fig. 4A,B) and sometimes produced repetitive discharges of motor axons (data not shown). Application of 4·2 μmol l^{-1} DTX qualitatively altered the EJPs, increasing their amplitude and duration several times (Fig. 4C). A further increase in the concentration of DTX (8·4 μmol l^{-1}) enhanced transmitter release to an even greater extent. As shown in Fig. 4D, this resulted in large, prolonged EJPs similar to those caused by diminishing both I_A and I_K (see Figs 2D and 3C).

Consistent with the above finding, our experiments on *eag* larvae further

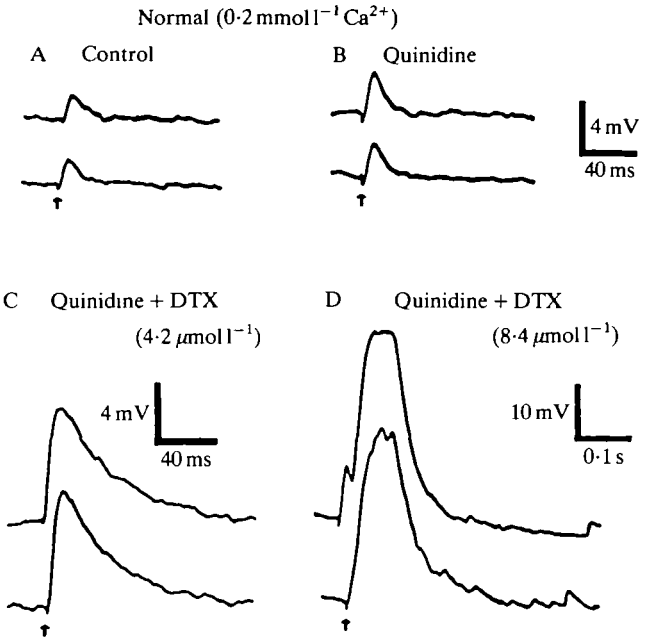

Fig. 4. Synergistic interaction between DTX and quinidine in normal larvae. The bath solution contained 0·2 mmol l^{-1} Ca^{2+} and 4 mmol l^{-1} Mg^{2+}. Treatments with 50 μmol l^{-1} quinidine only slightly facilitated neuromuscular transmission (compare A and B). Adding 4·2 μmol l^{-1} DTX to the preparation clearly enhanced the quinidine effect (C). Adding another 4·2 μmol l^{-1} DTX produced prolonged giant EJPs (D). Sequential recordings from the same cell. Note the different voltage and time calibrations in D.

demonstrated the facilitatory effect derived from the synergistic interaction of DTX with a defect in I_K. The EJPs in *eag* muscles were not substantially greater than normal (Fig. 5A, also see Ganetzky & Wu, 1983, 1985; Wu *et al.* 1983). Application of 4·2 μmol l^{-1} DTX immediately increased the size of EJPs from 2–4 mV to 5–10 mV (Fig. 5B) and in a few minutes caused repetitive discharges of EJPs of varying sizes (Fig. 5C), some reaching 30 mV. Adding 0·1 mmol l^{-1} 4-AP to the preparation transformed these repetitive EJPs into the typical prolonged giant EJPs (data not shown) observed when both I_A and I_K are blocked (see Figs 2D and 3C). In sum, our findings indicate that DTX has an effect on the neuromuscular junction similar to, but smaller than, those caused by the *Sh* mutation or 4-AP treatment.

Voltage-clamp analysis of DTX actions on I_A and I_K in muscle membrane

Direct evidence for alterations in K^+ currents caused by DTX requires measurements by voltage-clamp experiments. The *Drosophila* presynaptic nerve terminals are not easily accessible to this type of analysis and such measurements were performed in larval body-wall muscles. These large, identifiable muscle fibers meet isopotential conditions, are enveloped by very little connective tissue

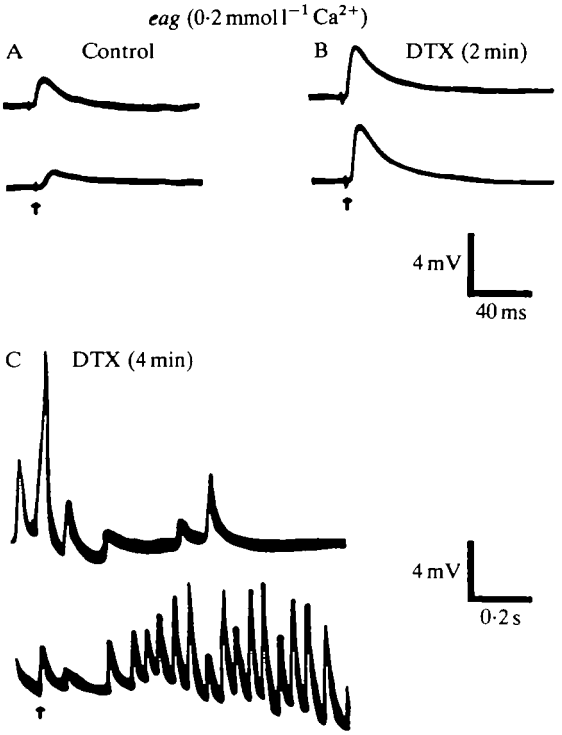

Fig. 5. Synergistic interaction between DTX and a mutation reducing I_K. (A) In saline containing 0·2 mmol l^{-1} Ca^{2+} and 4 mmol l^{-1} Mg^{2+}, the size of EJPs in *eag* larvae was not substantially greater than normal. (B) Within 2 min of the addition of 4·2 μmol l^{-1} DTX, EJPs increased in size. (C) As time progressed (another 2 min elapsed), repetitive discharges of giant EJPs appeared. Sequential data from the same cell.

and are directly exposed to the external medium (Wu & Haugland, 1985). Experiments were first carried out in Ca^{2+}-free saline to eliminate Ca^{2+} and Ca^{2+}-dependent K^+ currents. Under this condition only I_A and I_K remain, and they can be physiologically separated by their differences in inactivation properties (Wu & Haugland, 1985; Haugland, 1987; N. H. Haugland & C.-F. Wu, in preparation). The current–voltage (I–V) relationships in Figs 6–9 illustrate I_A and I_K, extracted as described in Materials and methods, and the effects of DTX at different Mg^{2+} concentrations and temperatures. The time courses of the total active currents (I_A plus I_K) are shown in Figs 6 and 8 and the extracted I_A currents are shown in Figs 7 and 9.

Previous studies of larval muscle currents were conducted at low temperature (4–15 °C) for the ease of voltage-clamp control and, to improve membrane stability, a high concentration of Mg^{2+} (10–15 mmol l^{-1}) was usually present in the Ca^{2+}-free saline (Salkoff & Wyman, 1981; Wu & Haugland, 1985). The EJP experiments (Fig. 1) suggest that the DTX effect is suppressed by high Mg^{2+} concentration. We found that, in the presence of 14 mmol l^{-1} Mg^{2+}, DTX

($1{\cdot}4\,\mu mol\,l^{-1}$) did not exert any significant effect on either I_A or I_K at 5°C (Figs 6 and 7, $14\,mmol\,l^{-1}$ Mg^{2+} data). Subsequently, the concentration of Mg^{2+} was lowered to examine the possible inhibitory effect of Mg^{2+} on DTX activity. At low temperature (5°C), we still could not detect a significant effect of DTX at the physiological concentration ($4\,mmol\,l^{-1}$) of Mg^{2+} (Figs 8 and 9, 5°C data). Not until the Mg^{2+} concentration was further lowered to a non-physiological level ($0{\cdot}5\,mmol\,l^{-1}$) could we observe a clear effect of DTX at 5°C. Long-term treatment (30 min) with DTX reduced both I_A and I_K (Figs 6 and 7, $0{\cdot}5\,mmol\,l^{-1}$ Mg^{2+} data). The effect of DTX at low temperature and low Mg^{2+} concentration

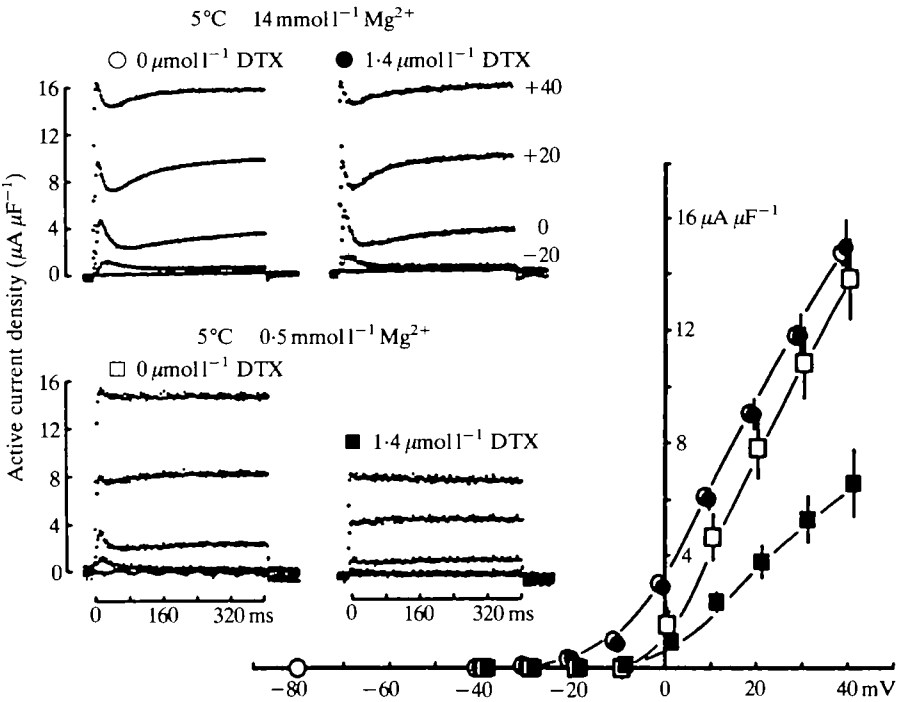

Fig. 6. Action of DTX on K^+ currents in normal larval muscle fibers measured by the two-microelectrode voltage-clamp technique at 5°C. Inward Ca^{2+} current and Ca^{2+}-dependent outward currents were abolished by the use of Ca^{2+}-free saline in this and the following figures. The effects of $1{\cdot}4\,\mu mol\,l^{-1}$ DTX at 14 and $0{\cdot}5\,mmol\,l^{-1}$ Mg^{2+} were determined from 6–14 fibers in 3–7 larvae for each experiment. Superimposed traces averaged from the fibers show the outward K^+ currents elicited by voltage steps (not shown) to −40, −20, 0, +20 and +40 mV from a holding potential (V_H) of −80 mV. The outward currents contain the transient I_A, which peaks rapidly and then inactivates, and the delayed I_K, which rises gradually to a plateau. The active current density was determined by subtraction of leakage currents and adjustment for membrane area (expressed in $\mu A\,\mu F^{-1}$ by using membrane capacitance as an indicator). The current–voltage (I–V) relationships show the mean ± S.E.M. of the amplitude of the remaining I_K after subtraction of the inactivating I_A (measured at the end of the 400 ms pulses to different voltages). There was a decrease in I_K in low-Mg^{2+} saline.

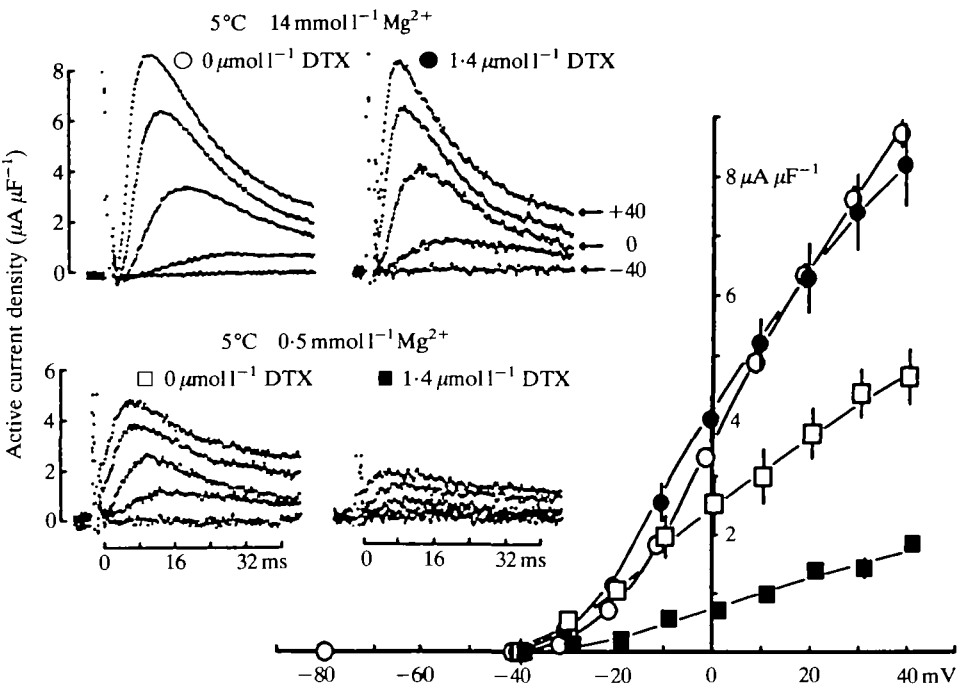

Fig. 7. The effect of DTX on I_A extracted from the data shown in Fig. 6. Traces averaged from different fibers represent the inactivating I_A determined by subtracting from the total current (Fig. 6 traces) the remaining non-inactivating current attainable following a prepulse to inactivate I_A (see Materials and methods). The I–V relationships show the mean ± S.E.M. of the peak current density of I_A. At 5°C, the effect of DTX was apparent in saline containing 0·5 mmol l^{-1} Mg^{2+} but was suppressed by 14 mmol l^{-1} Mg^{2+}.

was not reversed by washing and could not be suppressed again if the Mg^{2+} concentration was raised (data not shown).

It should be noted that at 0 mmol l^{-1} Ca^{2+} and 0·5 mmol l^{-1} Mg^{2+} the membrane underwent a characteristic sequence of changes even in the absence of DTX (see Discussion and Fig. 10 for details). At 5°C, the changes in membrane currents took about 50–70 min to reach a final steady state. Addition of DTX to saline at this time again initiated a similar sequence of events (see Discussion and Fig. 10) before reaching a new steady state 30 min later. The above results (Figs 6 and 7, 0·5 mmol l^{-1} Mg^{2+} data) include only steady-state data.

The activity of DTX is known to be enhanced at high temperature in chick biventer cervicis and mouse diaphragm preparations (Harvey & Anderson, 1985). The effect of temperature on the action of DTX was therefore examined in saline containing a physiological concentration (4 mmol l^{-1}) of Mg^{2+}. Raising the temperature to 16°C revealed a readily demonstrable effect of DTX. As previously reported (Wu & Haugland, 1985; Haugland, 1987), both I_A and I_K approximately doubled in size for an increase in temperature of about 10°C (see

5°C and 16°C data in Figs 8 and 9). At 16°C, DTX reduced the amplitude of I_A without any obvious change in its kinetics (Fig. 9). In contrast, I_K was largely unaltered (Fig. 8). The same experiment was performed at lower DTX concentrations. We found that at 470 nmol l^{-1} DTX had nearly reached a plateau level in its inhibitory effect on I_A, with a reduction in I_A almost identical to that caused by 1·4 μmol l^{-1} DTX. By extrapolation, the blockade of I_A by DTX might be larger at room temperature and could account for the observed changes in EJPs (Figs 1–5) at the physiological concentration of Mg^{2+} (4 mmol l^{-1}).

The same type of voltage-clamp analysis was carried out in the presence of 20 mmol l^{-1} Ca^{2+} at both 5 and 16°C. Under these conditions, the inward Ca^{2+} current and outward Ca^{2+}-dependent K^+ currents could be examined (Singh & Wu, 1987, 1989) but, in more than 10 fibers, no apparent effects of DTX (1·4 μmol l^{-1}) on these currents could be demonstrated (data not shown). Even though this result is consistent with previous reports on several vertebrate preparations (Halliwell *et al.* 1986; Harvey, 1987), we cannot rule out the

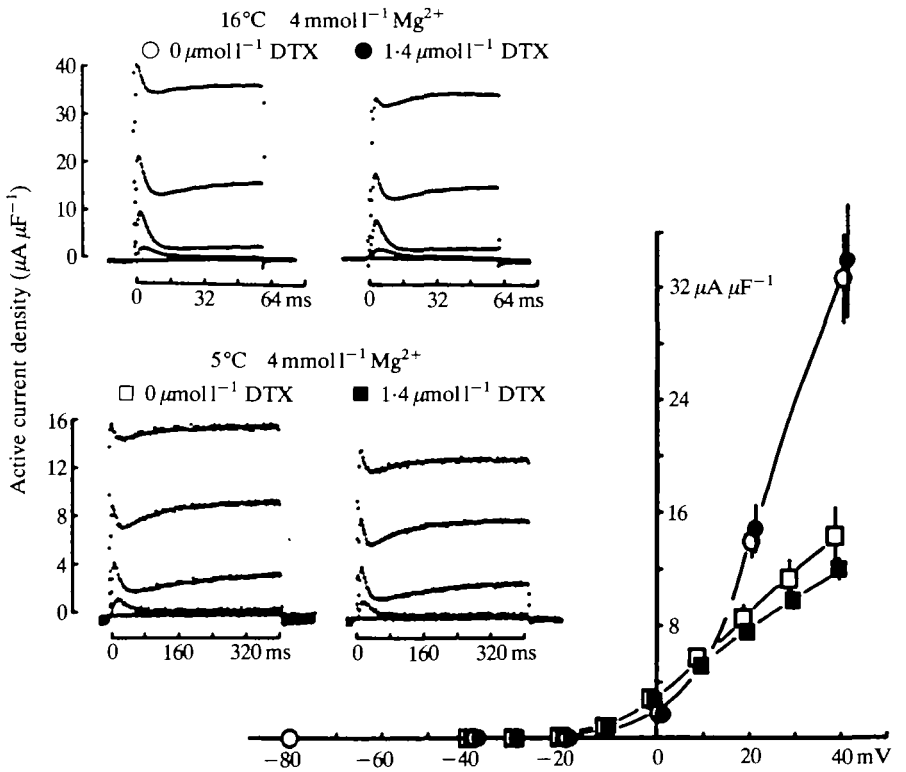

Fig. 8. Temperature dependence of the effect of DTX at a physiological concentration (4 mmol l^{-1}) of Mg^{2+}. Same experimental protocol as in Fig. 6. In each experiment 5–8 fibers from three larvae were used. Superimposed traces show the total active current density from the mean of different fibers. The I–V relationships show the mean ± S.E.M. of I_K measured at the end of voltage pulses. DTX did not significantly affect I_K in saline containing 4 mmol l^{-1} Mg^{2+} at either 5 or 16°C.

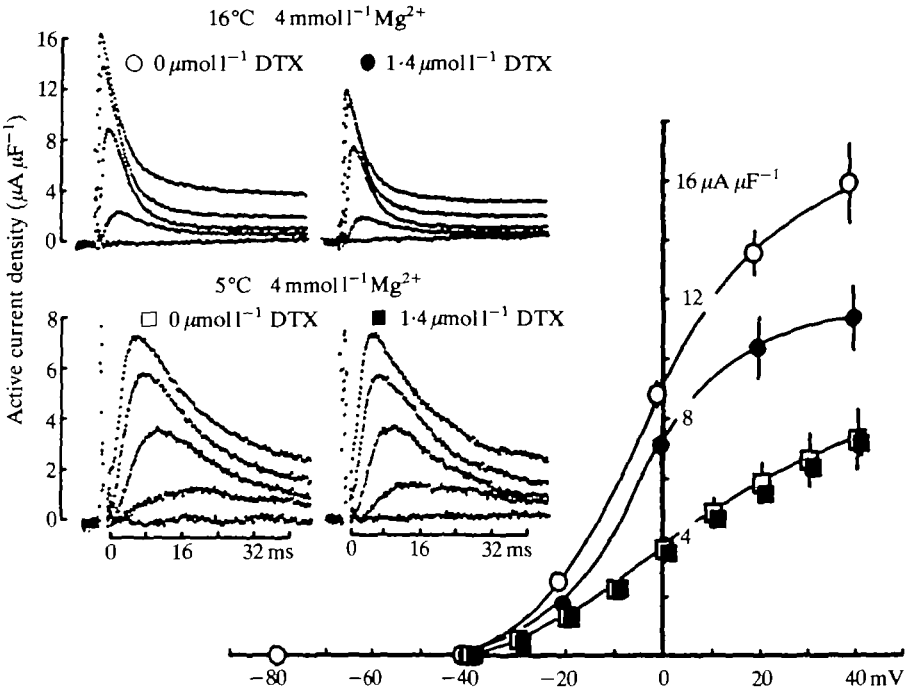

Fig. 9. Temperature dependence of the effect of DTX on I_A. Traces represent the mean density of I_A extracted from the data shown in Fig. 8. The I–V relationship shows the mean ± S.E.M. of the peak I_A. In saline containing 4 mmol l^{-1} Mg^{2+}, a significant reduction in I_A was observed at 16°C but not at 5°C.

possibility of a minor effect on these channels in *Drosophila*. Since the Ca^{2+}-dependent currents are intrinsically more variable than I_A and I_K among *Drosophila* muscle fibers (Singh & Wu, 1989), a small quantitative change in their amplitude would be difficult to detect.

Discussion

Effects of DTX on K^+ channels

On the basis of electrophysiological and pharmacological criteria, a variety of functional types of K^+ channels have been identified in a large number of vertebrate and invertebrate species, but there is no information about their molecular distinctions and similarities (Latorre & Miller, 1983; Rudy, 1988). Mutations that alter K^+ currents can provide insight into this problem (Wu & Ganetzky, 1986). A combination of genetic and pharmacological techniques may prove to be a powerful approach to the study of the functioning and mechanisms of this class of membrane channels.

So far, only a few specific, high-affinity toxins for K^+ channels are known. Thus DTX represents an especially valuable probe for functional and biochemical

analyses of these channels (Dolly *et al.* 1984, 1987; Rehm & Lazdunski, 1988). Previous voltage-clamp studies on vertebrate preparations have indicated that the sensitivity of K^+ channels to DTX in different excitable cells does not necessarily reflect similarity in channel function. DTX suppresses the non-inactivating or slowly inactivating K^+ currents in the node of Ranvier (Weller *et al.* 1985; Benoit & Dubois, 1986), in nodose ganglion neurons (Dolly *et al.* 1987), in dorsal root ganglion neurons (Penner *et al.* 1986) and in visceral sensory neurons (Stansfeld *et al.* 1986) but acts on the rapidly inactivating transient I_A in hippocampal neurons (Halliwell *et al.* 1986). However, a rapidly inactivating variant of the transient K^+ current in superior cervical ganglion neurons is reportedly not sensitive to DTX (Dolly *et al.* 1987). Recent toxin binding studies using DTX_I (Rehm & Lazdunski, 1988) and molecular genetic analyses of the *Sh* gene in *Drosophila* (Kamb *et al.* 1988; Pongs *et al.* 1988; Schwarz *et al.* 1988) have suggested the possibility of multiple subtypes of closely related K^+ channel proteins.

Our experiments extended the study of DTX to an invertebrate preparation. The action of toxin on muscle membrane was analyzed by voltage-clamp experiments. The results showed that, at physiological concentrations of Mg^{2+}, DTX affects mainly I_A without apparent effects on other currents. Although direct voltage-clamp measurements of the DTX effects at higher temperatures are not available for technical reasons (see Materials and methods), our data at 16°C and 4 mmol l^{-1} Mg^{2+} (Figs 8 and 9) could be extrapolated to infer a similar, or a greater, DTX effect on I_A at 25–29°C to account for the observed changes in EJPs (Figs 1–5). At low divalent cation concentrations I_K, in addition to I_A, was reduced (Fig. 6).

The exact mechanisms of DTX actions on membrane channels remain unknown. It has been previously reported that the toxin potency of enhancing neuromuscular transmission decreases drastically when temperature is lowered or when Mg^{2+} concentration is raised (Harvey & Anderson, 1985). In *Drosophila*, the effect of DTX also critically depends on temperature and the concentration of Mg^{2+}, and possibly Ca^{2+}, in the saline. It is worth pointing out the intriguing interactions between DTX and Mg^{2+}, as suggested by the experiments in Ca^{2+}-free saline at 5°C (Fig. 10). Decreasing the Mg^{2+} concentration to 0·5 mmol l^{-1} initiated a sequence of changes in the amplitude and kinetics of membrane currents. Before reaching a steady state in 70 min, the voltage dependence of the total current was strikingly different (Fig. 10A). Outward currents larger than normal were elicited by strong depolarizing steps but the current turned inwards at some intermediate levels of depolarization (Fig. 10Aii, arrow). At the steady state, the extracted I_A was reduced in amplitude whereas I_K retained nearly normal amplitude but seemed to rise more rapidly (Figs 6 and 7, 0 μmol l^{-1} DTX data, and Fig. 10Bi). Interestingly, when DTX was added at this time, the membrane currents again underwent characteristic changes similar to the above sequence of events (Fig. 10Bi,ii). At the new steady state there was a decrease in both I_A and I_K (Figs 6 and 7, 0·5 mmol l^{-1} Mg^{2+} data, and Fig. 10Biii). These non-physiological changes induced by low Mg^{2+} concentration were prevented by the

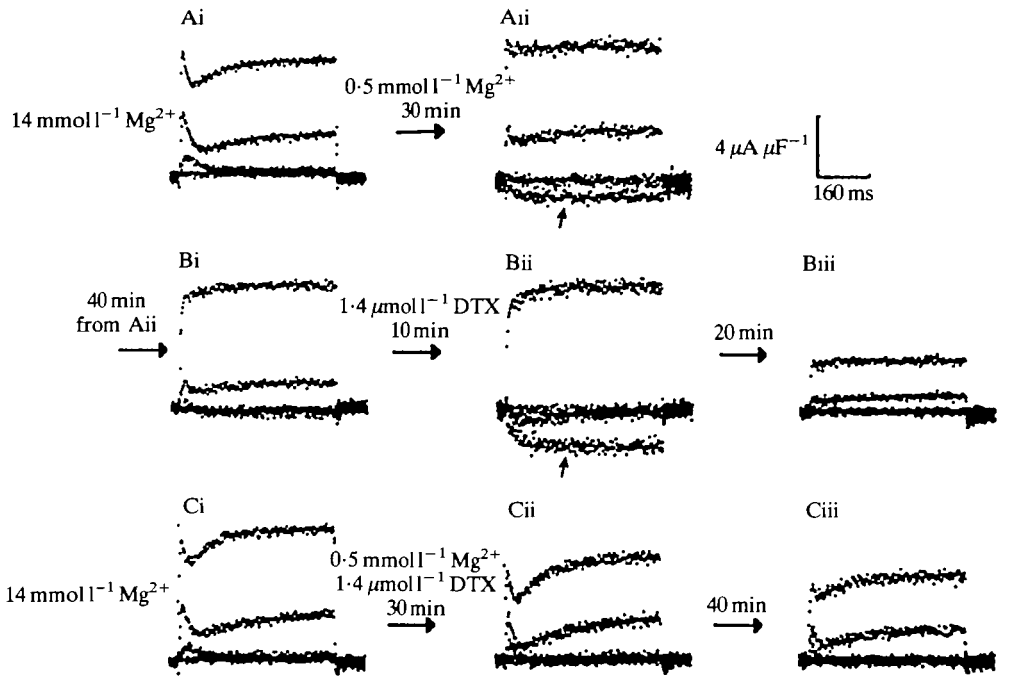

Fig. 10. Interaction of DTX and Mg^{2+} on membrane currents in Ca^{2+}-free saline. Superimposed traces represent active currents elicited by voltage steps to −40, −20, 0 and +20 mV from V_H (−80 mV) at 5°C. Lowering the Mg^{2+} concentration from 14 mmol l^{-1} to 0·5 mmol l^{-1} initiated a transient change in the voltage dependence of the membrane currents (Ai and Aii). Note that, at an intermediate level of depolarization, the total current was inward (arrow in Aii). At the steady state (Bi), both I_A and I_K regained nearly normal voltage dependence, in spite of the reduced amplitude of I_A and apparently more rapid kinetics of I_K (compare Ai and Bi, also see Figs 6 and 7, 0 μmol l^{-1} DTX data). Addition of 1·4 μmol l^{-1} DTX at this time again induced a similar transient change (Bii, arrow). Eventually, both I_A and I_K were decreased and the membrane reached a new steady state (Biii). If DTX (1·4 μmol l^{-1}) was also present in the low-Mg^{2+} (0·5 mmol l^{-1}) saline during the solution replacement, no transient changes in the voltage dependence or kinetics of I_A and I_K were induced (Ci and Cii). I_A and I_K gradually decreased and no alterations in kinetics were apparent (Ciii). Sequential data from the same cell are shown in A and B with leakage current subtracted. Data from a different cell are shown in C.

presence of DTX. Replacing 14 mmol l^{-1} Mg^{2+} saline by 0·5 mmol l^{-1} Mg^{2+} saline containing DTX caused only a gradual reduction in the amplitude of I_A and I_K without evident alterations in their kinetics (Fig. 10C).

The basis of the non-physiological events initiated by reducing Mg^{2+} concentration (Fig. 10A) is not understood. Divalent cations can change the local electric potential profile in the membrane by interaction with fixed negative charges on its outer surface (Hille, 1984). Divalent cations may also directly affect the gating of certain ion channels (Dingledine, 1986; Armstrong & Lopez-Barneo, 1987; Stern *et al.* 1987). One possibility is that an inward current was induced transiently after

reducing the Mg^{2+} concentration in the Ca^{2+}-free saline (Fig. 10Aii, Bii, arrows). Permeation of Na^+ through muscle Ca^{2+} channels in Ca^{2+}-free saline has been observed in one insect species (Yamamoto, 1987) and some vertebrate species (McCleskey *et al.* 1986) but not in another insect species (Ashcroft & Stanfield, 1982). Whether permeation of Na^+ is responsible for the phenomenon in *Drosophila* muscle at very low cation concentrations must await further investigation. No matter what exact mechanism underlies these changes, the surprising effect of DTX, preventing their initiation (Fig. 10C) and reiterating the sequence of changes once they are induced by Mg^{2+} reduction (Fig. 10B), must be considered in future physicochemical approaches to the mechanism of DTX actions.

The effects of several presynaptic snake toxins, such as β-bungarotoxin (β-BTX) and related toxins, are known to show a similar temperature and cation dependence (Chang, 1985). More interestingly, DTX, β-BTX and another toxin, the mast cell degranulating peptide, have been shown to bind to common protein targets and inhibit K^+ currents (Petersen *et al.* 1986; Bidard *et al.* 1987; Stansfeld *et al.* 1987). The structures of these toxins and DTX have been completely or partially determined (Lee, 1979; Joubert & Taljaard, 1980; Bidard *et al.* 1987). Such structural information may be important in designing further biophysical and biochemical experiments to study the nature of the observed temperature and cation dependence, which may in turn give clues to the mechanism of interaction of toxins with relevant membrane sites.

Effects of DTX on neuromuscular transmission

Direct measurements of the various ion currents in presynaptic nerve terminals by voltage-clamp are technically difficult. Therefore, *Drosophila* mutants of the genes that encode specific types of ion channels provide a unique opportunity to infer a defective current underlying the mutant phenotypes in nerve terminals. The altered currents can be directly analyzed by the voltage-clamp method in larger cells, such as muscle fibers.

It is worth noting that the DTX effect is specific but relatively weak compared with the actions of other channel-specific toxins or drugs. As in *Drosophila* larval muscle, DTX reduces but does not eliminate the K^+ currents in different vertebrate preparations (Weller *et al.* 1985; Halliwell *et al.* 1986; Penner *et al.* 1986; Stansfeld *et al.* 1986). At mouse and frog neuromuscular junctions, DTX enhances transmission by a mild increase in the quantal content of EPPs and the occasional appearance of repetitive EPPs (Anderson & Harvey, 1985; Harvey & Anderson, 1985; Harvey, 1987). As in vertebrate preparations, the effect of DTX at *Drosophila* neuromuscular junctions is relatively mild (Fig. 1). Similar but more extreme effects are observed when I_A is eliminated by *Sh* mutations or treatment with 4-AP (Figs 2 and 3). Previous extracellular nerve recordings and focal EJP recordings in *Sh* and *eag* larvae and in normal larvae treated with 4-AP or tetraethylammonium show that the abnormal EJPs reflect predominantly presynaptic defects rather than a failure of postsynaptic membrane repolarization.

The nerve terminals fail to repolarize properly, resulting in repetitive firing of the axon and release of an abnormally large amount of transmitter (Jan *et al.* 1977; Ganetzky & Wu, 1982). The results of the present study indicate that DTX facilitates larval neuromuscular transmission, probably by reducing I_A but not other K^+ currents in the nerve terminal.

We found that the relatively mild action of DTX on synaptic transmission can, nevertheless, be enhanced and clearly demonstrated through its synergistic interactions with mutations or drugs that reduce different K^+ currents (Figs 4 and 5). The synergistic effect of DTX on *Sh*, *eag* and *slo* mutants could be readily studied to obtain information regarding the type of K^+ currents (i.e. I_A, I_K and I_{CF}) in the nerve terminal affected by DTX. The results were also corroborated by examining the synergism of DTX with drugs blocking I_A or I_K. At the neuromuscular junction, DTX effectively augmented the effect of quinidine or the *eag* mutation but did not further increase the effect of 4-AP or the *Sh* mutation.

Two conclusions may be drawn from these results. First, the I_A channels in the nerve terminal and muscle membrane may be very similar or identical in their properties, as suggested by their similar sensitivity to DTX as well as to 4-AP and the *Sh* mutation. Second, the regulation of synaptic transmission by interplay of different K^+ currents is intricate and can produce results in a wide dynamic range. Blocking I_K produced a relatively smaller effect on EJPs than blocking I_A (compare Figs 2B and 3A with 4B). Partial blockade of I_A by DTX resulted in only mild effects on EJPs at physiological concentrations of Mg^{2+} (Fig. 1A,B) but could lead to explosive discharges of repetitive or prolonged giant EJPs (Figs 4D and 5C) when I_K was also blocked or reduced. Since multiple K^+ conductances are also present in vertebrate nerve terminals (David & Yaari, 1985; Mallart, 1985), our understanding of the ionic mechanisms underlying the generation of repetitive nerve spikes and increased synaptic activity in certain human pathological conditions (Dichter & Ayala, 1987) may be improved by further studies of the functional consequences of K^+ channel-specific toxins and mutations.

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