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DOI:
10.1038/sj.bjp.0706407

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):
Webster, RG, Brain, K, Wilson, RH, Grem, JL & Vincent, A 2005, 'Oxaliplatin induces hyperexcitability at motor and autonomic neuromuscular junctions through effects on voltage-gated sodium channels', *British Journal of Pharmacology*, vol. 146, no. 7, pp. 1027-1039. https://doi.org/10.1038/sj.bjp.0706407

British Journal of Pharmacology (2005) 146, 1027–1039
www.nature.com/bjp

Oxaliplatin induces hyperexcitability at motor and autonomic neuromuscular junctions through effects on voltage-gated sodium channels

*,[1]Richard G. Webster, [2]Keith L. Brain, [3]Richard H. Wilson, [3]Jean L. Grem & [1]Angela Vincent

[1]Neurosciences Group, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DS; [2]University Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT and [3]Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A.

1 Oxaliplatin, an effective cytotoxic treatment in combination with 5-fluorouracil for colorectal cancer, is associated with sensory, motor and autonomic neurotoxicity. Motor symptoms include hyperexcitability while autonomic effects include urinary retention, but the cause of these side-effects is unknown. We examined the effects on motor nerve function in the mouse hemidiaphragm and on the autonomic system in the vas deferens.

2 In the mouse diaphragm, oxaliplatin (0.5 mM) induced multiple endplate potentials (EPPs) following a single stimulus, and was associated with an increase in spontaneous miniature EPP frequency. In the vas deferens, spontaneous excitatory junction potential frequency was increased after 30 min exposure to oxaliplatin; no changes in resting Ca^{2+} concentration in nerve terminal varicosities were observed, and recovery after stimuli trains was unaffected.

3 In both tissues, an oxaliplatin-induced increase in spontaneous activity was prevented by the voltage-gated Na^+ channel blocker tetrodotoxin (TTX). Carbamazepine (0.3 mM) also prevented multiple EPPs and the increase in spontaneous activity in both tissues. In diaphragm, β-pompilidotoxin (100 μM), which slows Na^+ channel inactivation, induced multiple EPPs similar to oxaliplatin's effect. By contrast, blockers of K^+ channels (4-aminopyridine and apamin) did not replicate oxaliplatin-induced hyperexcitability in the diaphragm.

4 The prevention of hyperexcitability by TTX blockade implies that oxaliplatin acts on nerve conduction rather than by effecting repolarisation. The similarity between β-pompilidotoxin and oxaliplatin suggests that alteration of voltage-gated Na^+ channel kinetics is likely to underlie the acute neurotoxic actions of oxaliplatin.
British Journal of Pharmacology (2005) **146,** 1027–1039. doi:10.1038/sj.bjp.0706407;
published online 17 October 2005

Keywords: Oxaliplatin; neuromyotonia; neurotoxicity; hyperexcitability; carbamazepine; β-pompilidotoxin; voltage-activated Na^+ channels; neuromuscular junction

Abbreviations: 4-AP, 4-aminopyridine; EJP, excitatory junction potential; EPP, endplate potential; MEPP, miniature endplate potential; sEJP, spontaneous excitatory junction potential; TTX, tetrodotoxin

Introduction

Oxaliplatin (*trans*-1-diaminocyclohexane oxaliplatinum) is a widely used and effective treatment in combination with 5-fluorouracil for colorectal cancer. Its efficacy was first demonstrated in the metastatic setting (for review, see Cvitkovic & Bekradda, 1999; Wiseman *et al*., 1999; Misset *et al*., 2000). It has recently been shown to improve disease-free survival (although not overall survival) in the adjuvant treatment of colorectal cancer (Andre *et al*., 2004). Although it has a good safety profile, its use is associated with sensory, motor and autonomic neurotoxicity. A common neurotoxic manifestation is motor neuron hyperexcitability (Wilson *et al*., 2002), with spasms, myotonia and fasciculations of the limb and jaw muscles occurring during and for a period of hours after drug infusion (Raymond *et al*., 1998). Autonomic manifestations of oxaliplatin toxicity are not so common (Quasthoff & Hartung, 2002), but atonic bladder has been reported (Taieb *et al*., 2002). Acute sensory neurotoxicity includes parasthesiae, cold-induced dysasthesia, reversible visual field changes and pharyngolaryngodysesthesias (Wilson *et al*., 2002). The acute neurotoxicity associated with oxaliplatin treatment is distinct from the chronic sensory neurotoxicic action on the dorsal root ganglia, which is associated with long-term treatment with a variety of platinum analogues (Meijer *et al*., 1999), which produces a chronic sensory neuropathy with parasthesia and sensory loss (Extra *et al*., 1998). The central nervous system is relatively protected from the toxic effects of the platinates, probably because of their poor permeability through the blood–brain barrier, although occasionally central neurotoxicity (such as ototoxicity) does occur (Extra *et al*., 1998).

The acute symptoms of oxaliplatin treatment are often exacerbated by exposure to cold temperatures, and may respond to some extent to antiepileptic drugs such as carbamazepine (Lersch *et al*., 2002), but the relief of symptoms is not always evident (Wilson *et al*., 2002). It is important that

*Author for correspondence: E-mail: richard.webster@imm.ox.ac.uk

we understand more about the molecular mechanisms by which oxaliplatin affects the function of the peripheral nervous system to provide a rational approach for prevention or treatment of neurotoxicity. Oxaliplatin has been shown to alter Na^+ channel properties in cultured dorsal unpaired median neurons from cockroach (Grolleau *et al*., 2001), rat peripheral sensory nerve preparations and cultured dorsal root ganglia cells (Adelsberger *et al*., 2000). Oxaliplatin has also been shown to modify intracellular Ca^{2+} handling within the cell bodies of cultured neurons (Grolleau *et al*., 2001). Either of these mechanisms could occur in the peripheral nervous system, but there have been no direct studies of peripheral neuromuscular or autonomic transmission.

The mouse phrenic nerve/diaphragm preparation allows investigation of both spontaneous and nerve-evoked release of acetylcholine at the neuromuscular junction and is the site of action of many drugs and toxins that increase motor nerve excitability. The mouse vas deferens is densely innervated by sympathetic nerves (Taxi, 1965) that release noradrenaline and ATP (Burnstock, 1976), making it a useful preparation for the selective investigation of sympathetic transmission. Although autonomic pathology is rare, this preparation allows one to study regulation of intracellular Ca^{2+} (Brain & Bennett, 1997; O'Connor *et al*., 1999; Brain *et al*., 2001; Jackson *et al*., 2001), which has not been achieved so far at the mature mammalian skeletal neuromuscular junction. In this study, therefore, we investigated the acute *in vitro* effects of oxaliplatin on transmission in the mouse diaphragm by intracellular recordings of spontaneous miniature endplate potentials (MEPPs) and nerve-evoked EPPs, and on changes in the vas deferens by intracellular recordings and measurement of intraterminal Ca^{2+}. We compared the findings with those in the presence of a variety of drugs and toxins that alter nerve excitability.

Methods

Mouse preparations

For the phrenic nerve hemidiaphragm preparation, adult C57/BL6 mice were killed by exposure to a raised CO_2 atmosphere followed by cervical dislocation. Phrenic nerve/hemidiaphragm preparations were dissected from mouse thorax and bathed in Krebs solution, bubbled with 95%O_2/5%CO_2. Preparations were pinned out in a Sylgard-coated Petri dish containing bubbled Krebs solution. Preparations were allowed at least 30 min of equilibration before commencement of experimental procedures. For the vas deferens preparations, 8–12-week-old Balb/c mice (Harlan, U.K.) were killed by cervical fracture and both vasa deferentia removed. The connective tissue around each vas deferens was carefully dissected in order to obtain clear images and to remove any ganglia or isolated nerve cell bodies located close to the vas deferens.

Solution and drugs

The bath solution (Krebs) for dissection and recording was of the following composition (in mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.9, Glucose 10, $CaCl_2$ 2.5. The following drugs (source) were made as stock solutions at the stated (concentration) in distilled H_2O and stored frozen in aliquots until required, then added to Krebs to reach the required concentration: μ-conotoxin GIIIB (Peptide Institute, Japan) (0.1 mM); excipient-free oxaliplatin (kind gift of Sanofi Synthelabo) (15 mM); β-pompilidotoxin (Tocris Cookson Ltd, U.K.) (10 mM); tetrodotoxin (TTX) (Sigma-Aldrich Company Ltd, Dorset, U.K.) (3 mM); 4-aminopyridine (4-AP) (Sigma) (100 mM); apamin (Sigma) (1 mM). carbamazepine (Sigma) was dissolved in bubbled Krebs at the required concentration and stirred vigorously until just prior to use.

Phrenic nerve hemidiaphragm preparation

The phrenic nerve was stimulated *via* a suction electrode coupled to a pulse generator (GRASS instruments S48, solid-state square wave stimulator, Quincy, U.S.A.) with an associated stimulus isolation unit. To enable measurement of evoked potentials, the muscle action potential and contraction were blocked with 2.5 μM μ-conotoxin GIIIB. Neuromuscular transmission viability was checked before contractile blockade. Recordings were made at room temperature (20–22°C) *via* an Axoclamp-2A amplifier (Axon Instruments, Union City, CA, U.S.A.).

Nerve-evoked EPPs and spontaneous EPPs (MEPPs) were recorded intracellularly with conventional borosilicate glass electrodes filled with 3 M KCl (10–15 MΩ resistance; Harvard Apparatus, Edenbridge, Kent, U.K.) and filtered at 1 kHz. Electrodes were positioned above endplate regions, as visualised with a stereomicroscope, under micromanipulator control. Impalement adjacent to an endplate was indicated by a fast EPP rise time (less than 2 ms).

To evoke an EPP the nerve was stimulated supramaximally with platinum wire electrodes. Upon impalement, a 30 s period of equilibration was allowed before MEPP recordings began. If the membrane potential drifted by more than 5 mV or depolarised below -55 mV the recording from that endplate was abandoned. Data signals were passed through a 'Humbug 50 Hz noise eliminator' (Quest Scientific *via* Digitimer, Welwyn Garden City, U.K.) to reduce electrical noise on recordings. MEPPs were autodetected with Winwcp software (Whole cell program, Strathclyde University, U.K.); autodetection was set such that all MEPPs with amplitude greater than the magnitude of the residual electrical noise were detected. Up to 30 MEPPs followed by up to 30 EPPs (stimulated at 1 Hz) were recorded per endplate for later offline analysis. MEPP frequency was determined by two methods, either by recording the time between detected events (detected) or counting events over a longer time course recording (direct).

Data analysis

Recorded MEPPs and EPPs were digitised at 12.5 kHz (CED 1401 interface, Cambridge Electronic Design, Cambridge, U.K.) and stored on computer hardware. Each MEPP and EPP was visually inspected and rejected if of poor quality or if noise was autodetected. Records were analysed with Winwcp software or pClamp 9 programs (Axon Instruments, Union City, CA, U.S.A.). All MEPP and EPP amplitudes were standardised to a resting membrane potential of -80 mV to correct for changes in driving force due to altered postjunctional membrane potential (Katz & Thesleff, 1957). Mean

quantal content was calculated by the direct method as described previously (Giovannini *et al*., 2002).

Mouse vas deferens

Conventional intracellular recording techniques were used to record excitatory junction potentials (EJPs) in smooth muscle cells (see Brock & Cunnane, 1992). Each vas deferens was superfused with physiological salt solution (PSS) containing: NaCl 118.4, $NaHCO_3$ 25, NaH_2PO_4 1.13, KCl 4.7, $CaCl_2$ 1.8, $MgCl_2$ 1.3 and glucose 11.1. The pH was maintained at 7.4, and the solution oxygenated, by continuously bubbling with 95% O_2/5% CO_2. Oxaliplatin was applied by swapping the perfusion solution to one containing 0.5 mM oxaliplatin. Stimuli (rectangular pulses, 0.01 ms duration, amplitude 10 V) were delivered through Ag/AgCl electrodes positioned around the proximal end of the vas deferens. Microelectrodes were filled with 5 M potassium acetate and had tip resistances of 40–80 MΩ.

Intracellular recordings were acquired with a PowerLab 4SP (ADInstruments Ltd, Chalgrove Oxon, U.K.) attached to a Macintosh computer running Chart v.4.2 (ADInstruments). Data analysis was performed offline and automated using custom-written macros. Spontaneous excitatory junction potentials (sEJPs) were detected using the 'template' extension; this algorithm correlates a rolling, normalised segment of the trace with a user-selected template (a typical sEJP). A correlation of >0.9 was used to define the occurrence of an sEJP; only sEJPs with an amplitude of >1 mV were counted. The sensitivity and specificity of this technique (compared to manual counting) were greater than 0.9. A burst of sEJPs is arbitrarily defined as three sEJPs within 1 s.

Confocal microscopy

The cut prostatic end of the vas deferens was secured in a glass micropipette containing the Ca^{2+} indicator Oregon Green 488 BAPTA-1, 10 kDa dextran (Oregon-BAPTA-1; Molecular Probes, OR, U.S.A.), using a protocol previously described (Brain & Bennett, 1997). The preparation was left for 5 h at room temperature, then removed from the pipette and washed in Krebs for a further 5 h. The vas deferens was placed in a chamber that was continuously perfused with PSS (bath temperature 31–32°C). The base of the chamber was a coverslip; images were acquired with a Leica NT inverted confocal microscope. Field stimuli (pulse width 0.6 ms; amplitude 10 V) were applied with parallel platinum electrodes located near the prostatic end of the vas deferens; stimuli were synchronised with the start of image acquisition, so that the interval between each stimulus and recording was fixed. A total of 128, 256×256 pixel images were acquired at the fastest possible speed (4.67 Hz) every 3 min. Field stimuli were triggered on the 56th–60th frames, inclusive (i.e. five impulses at about 5 Hz).

Image analysis

Image analysis was performed with NIH Image version 1.63 (from http://rsb.info.nih.gov/nih-image/). The fluorescent intensity in individual nerve terminal varicosities was measured, accounting for any movement of the preparation, using custom-written macros as previously described (Brain & Bennett, 1997).

Statistics and data handling

Values are expressed as mean ± s.e.m., and statistical analysis was performed with InStat software (GraphPad Software, Inc., San Diego, CA, U.S.A.), a P-value of >0.05 was considered to demonstrate the lack of a significant difference between groups. It is assumed that each nerve terminal varicosity or endplate behaves independently. The kinetics of Ca^{2+} recovery in nerve terminal varicosities following nerve stimulation was modelled as the sum of two exponentials, using GraphPad Prism (GraphPad Software Inc.).

Results

Effects of oxaliplatin on neuromuscular transmission in the mouse diaphragm

Recordings from independent endplates were made as frequently as possible (usually every 2–5 min). Spontaneous MEPP amplitude, MEPP frequency, EPP amplitude and quantal content remained relatively constant for over 70 min when only vehicle (66 μl H_2O in 2 ml Krebs) or oxalic acid, a metabolite of oxaliplatin (final concentration, 0.5 mM) were added to the incubation (Table 1 and Figure 1a).

Oxaliplatin (0.5 mM) had no immediate effect on neurotransmission. However, after an average of 37.1 ± 1.7 min ($n=10$ preparations), changes began to be observed (Figure 1b). Double responses to single stimuli appeared but, in this early phase, not every stimulus evoked a multiple response (Figure 1bi, ii). At this stage, EPP amplitudes and MEPP amplitudes (Table 1 and Figure 1biii) were no different from control. However, in the continued presence of oxaliplatin, the evoked potentials became increasingly abnormal with trains of potentials evoked by a single stimulus (Figure 1ci, ii). Eventually, 20–30 min after the initial changes, EPPs were unattainable following stimulation at some endplates; while at others spontaneous activity was very frequent (Figure 1ciii) and individual EPPs could not be evoked by stimulation. If oxaliplatin was washed out after the late phase had begun, disruption of neurotransmission was not reversed.

MEPPs were not altered in amplitude or frequency during the preliminary incubation in oxaliplatin, and were normal in amplitude and frequency during the early phase when multiple EPPs began to appear (Table 1). However, during the late phase, when trains of EPPs were evoked by single stimuli, MEPP frequency was significantly increased (Figure 1d and Table 1). During runs of overlapping MEPPs, EPPs could sometimes not be evoked upon stimulation.

Interestingly, MEPP frequency elevation by oxaliplatin was not observed when the tissue was left unstimulated during 80 min of oxaliplatin incubation (Figure 2a; Table 2), but a single stimulus at 80 min resulted in a massive production of overlapping MEPPs (Figure 2b), suggesting that oxaliplatin is able to change the underlying motor nerve function in the absence of nerve stimulation, although its effects are not seen until nerve stimulation occurs. When stimulated after 80 min in oxaliplatin without stimulation, neither single EPPs nor trains of EPPs could be evoked. This is presumably because the early

Table 1 Effects of oxaliplatin on spontaneous and evoked potential characteristics in mouse hemidiaphragm

Treatment	*Vehicle control*	*Oxaliplatin (0.5 mM)*			*Oxalic acid (0.5 mM)*
Time after intervention when recording made	Between 5 and 71 min	Between 27 and 40 min Multiple EPPs only	Between 40 and 50 min	Between 51 and 80 min	Between 35 and 82 min
EPP amplitude (mV)	29.08±0.80	29.83±4.10	—	—	27.34±1.15
% Of preincubation	91	97	—	—	97
MEPP amplitude (mV)	1.18±0.04	1.06±0.12	—	—	1.16±0.04
% Of preincubation	88	81	—	—	101
MEPP frequency (detected, Hz)	0.84±0.05	1.07±0.20	—	—	1.18±0.10
% Of preincubation	87	86	—	—	93
MEPP frequency (direct, Hz)	1.07±0.08 $n_e=19$	1.33±0.31 $n_e=7$	7.74±3.12[a]	32.10±7.49[a]	1.47±0.13 $n_e=19$
% Of preincubation	73	100	784	3251	106
Number of endplates (n_e)	55	9–13	17	19	41
Number of preparations	4	5	4	4	4

% Of preincubation: comparison with mean of three to five endplates per preparation measured before interventions began.
MEPP frequency (detected): MEPP frequency determined by measurement of interval between detected events.
MEPP frequency (direct): MEPP frequency determined by counting events during time course recording.
Multiple EPPs only: Only measurements from endplates with observed multiple EPP were included.
[a]Denotes a significant difference from vehicle with a P-value <0.05.

phase of the oxaliplatin effect had already occurred and the tissue had entered the unresponsive late phase described above.

To see, therefore, whether the changes were dependent on nerve conduction, we tested the effects of the Na^+ channel blocker, TTX (1 μM). When TTX was applied before oxaliplatin, no multiple EPPs or trains were elicited by phrenic nerve stimulation (as expected since TTX blocks nerve conduction), and the oxaliplatin-induced increase in MEPP frequency was also prevented (Figure 2c; Table 2). Similarly, if TTX was applied after oxaliplatin-induced multiple EPPs had occurred, the subsequent increase in MEPP frequency was prevented (Table 2). These findings, combined with those described above (Figure 2a), indicate that nerve conduction is required for oxaliplatin to achieve an effect on spontaneous activity, but any of the processes between the initiation of the action potential by nerve stimulation and the release of transmitter from the neuromuscular junction could be involved.

Pharmacological investigation of potential oxaliplatin targets in mouse diaphragm

K^+ channels We tried various drugs to determine whether they either blocked or reproduced the effect of oxaliplatin. We started by looking at K^+ channels because their blockade can lead to motor nerve hyperexcitability. Apamin (10 μM) is a known inhibitor of small conductance Ca^{2+}-activated K^+ channels (SK channels) (Carignani *et al*., 2002) which are present at the neuromuscular junction and are thought to play a role in after-hyperpolarisation of the motor nerve terminal (Roncarati *et al*., 2001). Apamin did not replicate the effects of oxaliplatin (Table 3 and Figure 3a, $n=5$) and had no significant effects on MEPP amplitude or frequency, or EPP amplitude.

4-AP (0.3 mM) an inhibitor of delayed rectifier voltage-activated K^+ channels at the neuromuscular junction (Thomsen & Wilson, 1983) also failed to replicate the multiple EPPs elicited by oxaliplatin. However, this treatment significantly increased EPP amplitude and delayed repolarisation, such that EPP area was significantly increased compared to control (406.4±42.1 vs 135.8±7.5 mV ms, $n_e=16$ and 54, $n_p=5$, respectively) (Figure 3b). MEPP amplitude was not significantly increased, when measured between periods of stimulation (Figure 3b; Table 3), but increased MEPP frequency occurred during the period immediately following stimulation. Since neither of these drugs caused oxaliplatin-like effects, it is likely that neither SK channels nor delayed rectifier K^+ channels are the targets for oxaliplatin at the neuromuscular junction.

Na^+ channels The effects of TTX described earlier, showed that oxaliplatin has no effect in the absence of a stimulation-induced action potential. Carbamazepine, a drug used clinically to treat some forms of temporal lobe epilepsy and trigeminal neuralgia, acts by delaying the recovery of Na^+ channels from the inactivated state, thus decreasing the availability of Na^+ channels (Wang *et al*., 2002; Yang & Kuo, 2002). In the mouse diaphragm, carbamazepine (0.3 mM) reduced MEPP and EPP amplitudes by 16.1 and 21.6% (after about 20 min incubation), respectively, from preincubation values (Table 3). The decay of both MEPPs and EPPs were prolonged and were fitted by two exponential decay functions (Figure 3c). Addition of oxaliplatin in the presence of carbamazepine did not lead to multiple EPPs or to an increase in MEPP frequency (Table 3), while EPP and MEPP amplitudes continued to decrease. During continued exposure to carbamazepine the stimulus required to evoke an EPP increased and there was an evident delay between the stimulus artefact and the evoked response (Figure 3c and d); at some endplates no EPP could be evoked, even at the maximum stimulus voltage. These results indicate that carbamazepine does protect the motor nerve terminal against the effects of oxaliplatin at this concentration, but has the potential to reduce the efficacy of neuromuscular transmission by its effects on MEPP and EPP amplitudes. However, lower therapeutic doses of ~25 μM may preclude effects on MEPP and EPP amplitude. These findings confirm that the effect of oxaliplatin is crucially dependent on normal Na^+ channel function, and is unlikely to be due to an independent effect on motor nerve excitability.

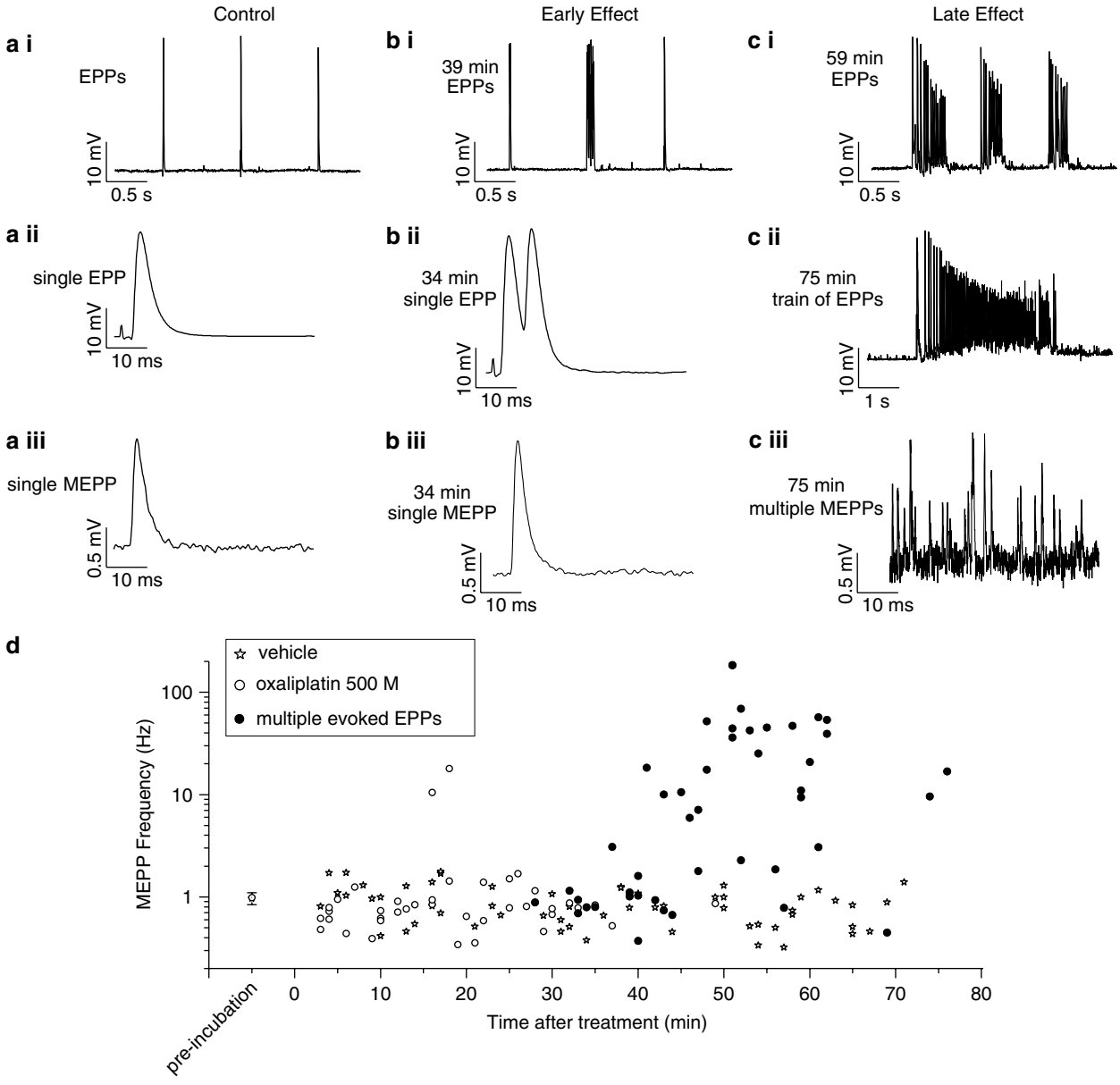

Figure 1 Oxaliplatin induces multiple EPPs in response to a single stimulation and increases MEPP frequency. Example traces of (a) control, (b) early effect (c) late effect of 500 μM oxaliplatin on mouse phrenic nerve/hemidiaphragm preparation. (ai) Evoked potentials at a low temporal resolution, (aii) single EPPs signal averaged from 30 consecutive potentials, (aiii) single spontaneous MEPP record, signal averaged from 30 detected events. (bi) Low temporal resolution EPPs 39 min after application of oxaliplatin, on some occasions a single stimulus evoked multiple EPPs. (bii) Example of a multiple EPP from a single stimulus, typical of an early oxaliplatin effect. (biii) Signal averaged MEPP at 34 min oxaliplatin exposure. (ci) Repeated trains of EPPs following single stimuli at 59 min oxaliplatin exposure, typical of a late effect. (cii) An example of a sustained train of excitation following a single stimulus, recorded 75 min after oxaliplatin exposure. (ciii) Multiple overlapping MEPP activity observed between stimuli after 75 min oxaliplatin exposure. (d) Changes in MEPP frequency during exposure to H_2O vehicle (star, $n=4$ preparations), or 500 μM oxaliplatin (circle, $n=4$). For each treatment group and each preparation, three to four preincubation recordings were made and the mean $\pm$ s.e.m. are displayed, labelled preincubation on x-axis. Time on x-axis indicates time after addition of vehicle or oxaliplatin. Oxaliplatin MEPP frequency data were directly counted from continuous records. Vehicle MEPP frequency data was either calculated from interval between detected events ($n_p=3$) or counted from continuous records ($n_p=1$).

β-pompilidotoxin, isolated from the venom of the solitary wasp, acts on Na^+ channels to delay entry into the inactivated state (Sahara *et al*., 2000). This class of toxin has also been shown to induce repetitive action potential firing in lobster walking leg preparation (Sahara *et al*., 2000) and can differentiate between rat cardiac and neuronal Na^+ channels (Kinoshita *et al*., 2001). In the mouse diaphragm, β-pompilidotoxin (0.1 mM) induced multiple EPPs resulting from a single stimulus within 5 min of application (Figure 4a). There was no significant reduction in either MEPP or EPP amplitude compared with preincubation recordings from the same preparation. Multiple EPPs induced by β-pompilidotoxin had two different characteristics: the majority were similar to the late stage oxaliplatin-induced multiple EPPs (Figure 4ai),

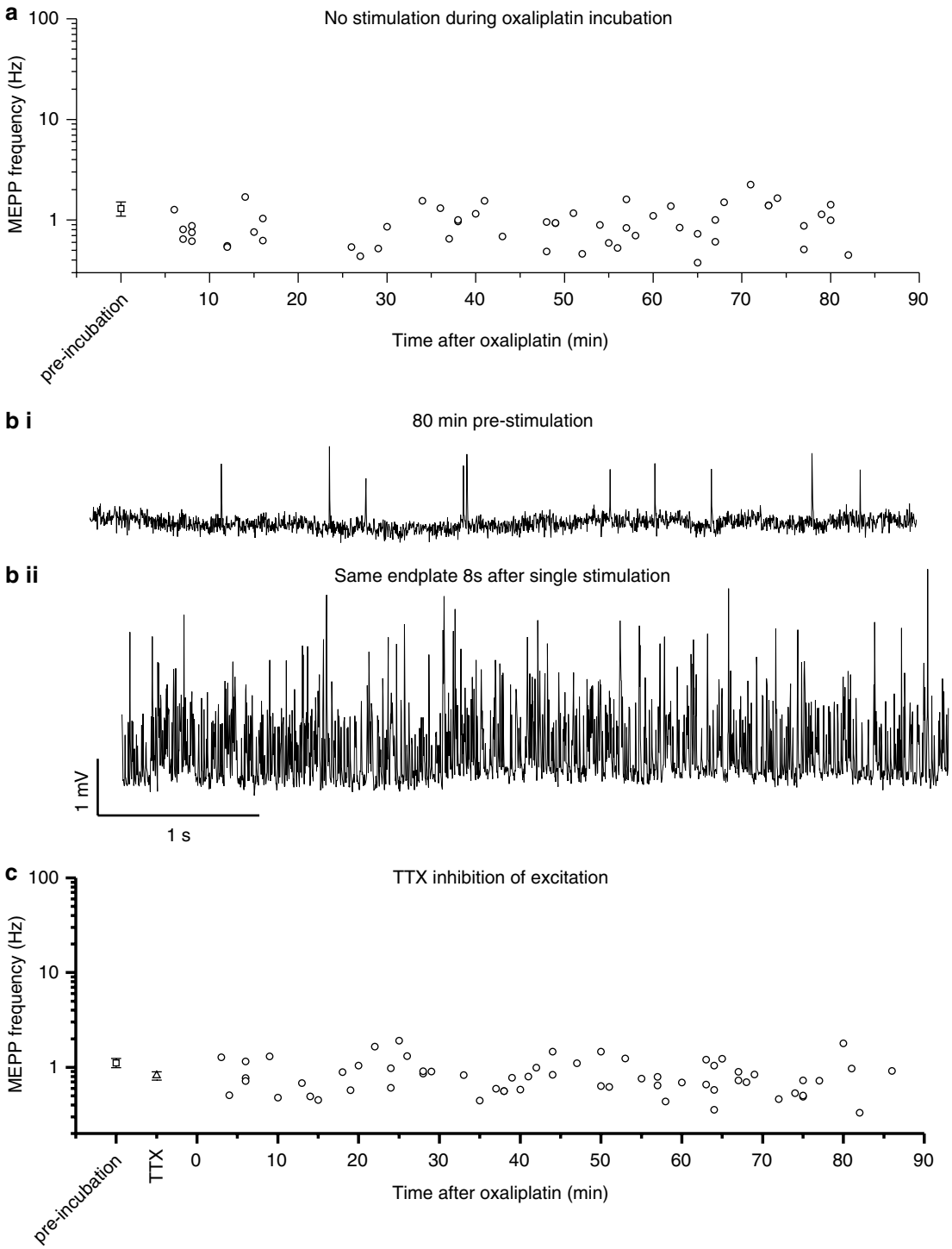

Figure 2 Oxaliplatin has no effect when muscle is not stimulated. (a) During oxaliplatin treatment the muscle was not stimulated and MEPP frequency was directly assessed from continuous records ($n=4$). Three to four endplates were studied pretreatment and the mean$\pm$s.e.m. plotted as preincubation. An example of MEPP activity after 80 min is shown in (bi), at this time the muscle was subjected to a single stimulus, while in the maintained presence of oxaliplatin, and the resultant massive increase in MEPP activity is shown in (bii) 8 s after the single stimulus. (c) Neuronal Na-channels were blocked by TTX (1 μM), MEPP frequency was directly assessed from continuous records ($n=4$). Three to four endplates were studied pretreatment and the mean$\pm$s.e.m. plotted as preincubation, the effect of TTX alone was assessed and the mean$\pm$s.e.m. plotted as TTX. Between each assessment of MEPP frequency the phrenic nerve was stimulated for 1 min at 1 Hz.

but some were associated with substantial MEPP release after the initial EPP, without further EPPs being induced (Figure 4aii). These two phenomena could be seen in the same endplate, separated by only a few stimuli (Figure 4b). There was no increase in MEPP frequency between stimuli, in those endplates that displayed multiple EPPs over the period studied (up to 23 min), see Figure 4c. It is interesting to note that from the time of the initial effect only 55% of endplates studied had

Table 2 Nerve conduction required for increased MEPP frequency

Treatment	*Oxaliplatin (0.5 mM) no stimulation*	*Oxaliplatin (0.5 mM) TTX (1 μM) pretreatment*	*Oxaliplatin (0.5 mM) TTX (1 μM) post-treatment*[a]
Period of measurement	Between 36 and 82 min	Between 38 and 93 min	Between 5 and 33 min after addition of TTX
MEPP frequency (direct, Hz)	1.00±0.07	0.80±0.05	1.03±0.17
Number of endplates	37	39	22
Number of preparations	4	4	4

MEPP frequency (detected): MEPP frequency determined by measurement of interval between detected events.
[a]TTX (1 μM) added after 50.5±3.7 min incubation with oxaliplatin (0.5 mM), in all preparations multiple EPPs had been observed in several endplates.

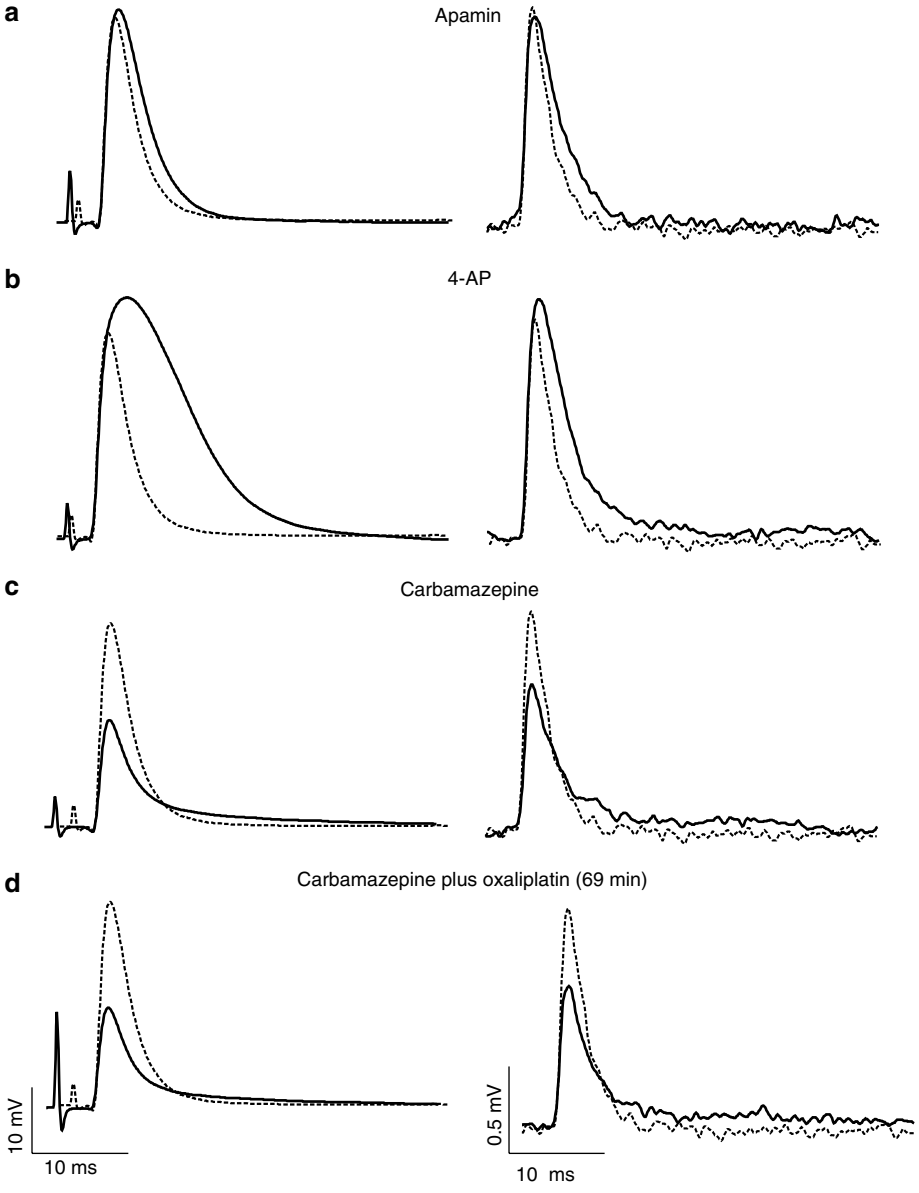

Figure 3 Pharmacological investigation of oxaliplatin effect. Example traces of signal averaged EPPs and MEPPs (up to 30 records) are shown for each treatment (solid lines), for reference a control trace is aligned for each example (broken line). (a) Apamin (10 μM) after 61 min incubation. (b) 4-AP (0.3 mM) after 6 min incubation. (c) Carbamazepine (0.3 mM) after 14 min incubation. (d) Carbamazepine (0.3 mM) and oxaliplatin (0.5 mM) after 69 min oxaliplatin incubation.

Table 3 Effects of channel modulators on neurotransmission in mouse hemidiaphragm

Treatment	*Apamin (10 μM)*	*4-AP (0.3 mM)*	*Carbamazepine (0.3 mM)*	*Carbamazepine (0.3 mM) then oxaliplatin (0.5 mM)*	*β-pompilidotoxin (0.1 mM)*
Period of measurement	Between 10 and 66 min	Between 5 and 34 min	Between 5 and 19 min	Between 35 and 82 min after addition of oxaliplatin	Between 2 and 40 min multiple EPPs only
EPP amplitude (mV)	30.83±1.05	38.70±2.43**	22.47±2.03*	16.97±0.92**	24.85±3.11
% Of preincubation	103	138	78	59	86
MEPP amplitude (mV)	1.18±0.04	1.22±0.07	1.09±0.07	0.87±0.03*	0.97±0.11
% Of preincubation	101	108	84	67	84
MEPP frequency (detected, Hz)	0.93±0.05	2.30±0.19**	0.69±0.04	0.93±0.14	2.01±0.47**
% Of preincubation	87	205	62	84	220
MEPP frequency (direct, Hz)	1.16±0.08 $n_e=29$	2.19±0.18** $n_e=34$	—	—	1.03±0.14 $n_e=10$
Number of endplates (n_e)	66	31	14–18	31–41	8–10
Number of preparations.	5	5	4	4	4

% Of preincubation: comparison with mean of three to five endplates per preparation measured before interventions began.
MEPP frequency (detected): MEPP frequency determined by measurement of interval between detected events.
MEPP frequency (direct): MEPP frequency determined by counting events during time course recording.
Multiple EPPs only: Only measurements from endplates with observed multiple EPP were included.
* and ** denote a significant difference from vehicle with a P-value <0.05 or 0.01, respectively.

multiple EPPs with β-pompilidotoxin. This compares with 87% of endplates showing multiple EPPs after the start of the oxaliplatin-induced disruption. Overall, the effects of β-pompilidotoxin were more variable, but the toxin did reproduce some of the changes seen with oxaliplatin.

Effects of oxaliplatin on autonomic transmission in the mouse vas deferens

The vas deferens preparation is particularly suitable for testing the effects of drugs on autonomic transmission, and allows one to look for possible effects on intra-terminal Ca^{2+}. Spontaneous sEJPs were measured with standard intracellular electrode recording techniques. After 20–30 min in oxaliplatin (0.5 mM) the frequency of sEJPs began to increase, with an average 3.4-fold increase (from a resting frequency of $0.11\pm0.03\,s^{-1}$; $n_p=7$; Figure 5a) during the period between 30 and 60 min. Many of the sEJPs in the presence of oxaliplatin occurred in bursts. In each of the seven preparations there was a marked increase in the frequency of bursts of sEJPs ($0.042\pm0.015\,s^{-1}$; a 60-fold increase over controls; Figure 5b and c). Indeed, in four out of seven preparations, no bursts were detected in the controls.

To determine whether bursts of spontaneous transmitter release were generated by bursts of nerve terminal action potentials, TTX was applied. TTX (100 nM) abolished all EJPs (Figure 5e) in normal tissue, confirming that action potential generation was abolished. Upon the addition of oxaliplatin (0.5 mM), still in the presence of TTX, there was no increase in the frequency of sEJPs or bursts of sEJPs (Figure 5e; $n=3$), suggesting that, as in the diaphragm preparation, the oxaliplatin effect was dependent on Na^+ channel activity.

Carbamazepine (0.3 mM) reduced the amplitude of EJPs by 55±12% ($n=5$; $P<0.05$). However, the subsequent application of oxaliplatin (0.5 mM) in the continued presence of carbamazepine (0.3 mM) resulted in no further change in the amplitude of EJPs (+4±23%) (Figure 5f). Both the rate of spontaneous EJPs ($0.18\pm0.05\,s^{-1}$) and bursts of sEJPs ($0.009\pm0.005\,s^{-1}$) were significantly lower than when oxaliplatin alone was present ($P<0.05$ using an unpaired t-test). There was no change in the smooth muscle resting membrane potential (1±1 mV).

Confocal Ca^{2+} imaging

It has been suggested that oxaliplatin might act by altering nerve terminal Ca^{2+} regulation (Grolleau *et al*., 2001). We studied the effect of oxaliplatin on Ca^{2+} concentration in nerve terminal varicosities ($[Ca^{2+}]_v$), both at rest and following nerve stimulation. Following a nerve terminal action potential the $[Ca^{2+}]_v$ rose rapidly (Figure 6a), before recovering to its resting concentration over a period of seconds as previously described (Brain & Bennett, 1997). Oxaliplatin (0.5 mM) had no effect on the amplitude of the residual $[Ca^{2+}]_v$ transient following either single nerve terminal action potentials (+1.3±0.8%; number of nerve terminal varicosities, $n_v=18$; $n_p=5$) or trains of five action potentials at 5 Hz (−7±10%; $n_v=14$; $n_p=4$; Figure 6b).

In order to investigate cytoplasmic Ca^{2+} buffering and sequestration mechanisms, the kinetics of $[Ca^{2+}]_v$ recovery following trains of five action potentials at 5 Hz was investigated. There was no change in the time course of the first (and dominant) component of $[Ca^{2+}]_v$ recovery (Figure 6c; 0.41±0.07 s in the control compared to 0.43±0.06 s in oxaliplatin). Significant spontaneous contraction and subsequent movement in the presence of oxaliplatin meant that the slower component of recovery (previously described with a time course of about 4 s) could not be adequately assessed.

In the presence of oxaliplatin, spontaneous bursts of rapid whole nerve terminal Ca^{2+} transients (consistent with spontaneous action potentials) were observed in two out of five preparations. In one of these preparations, the action potential bursts were associated with local tissue contraction, suggesting that they had initiated local transmitter release.

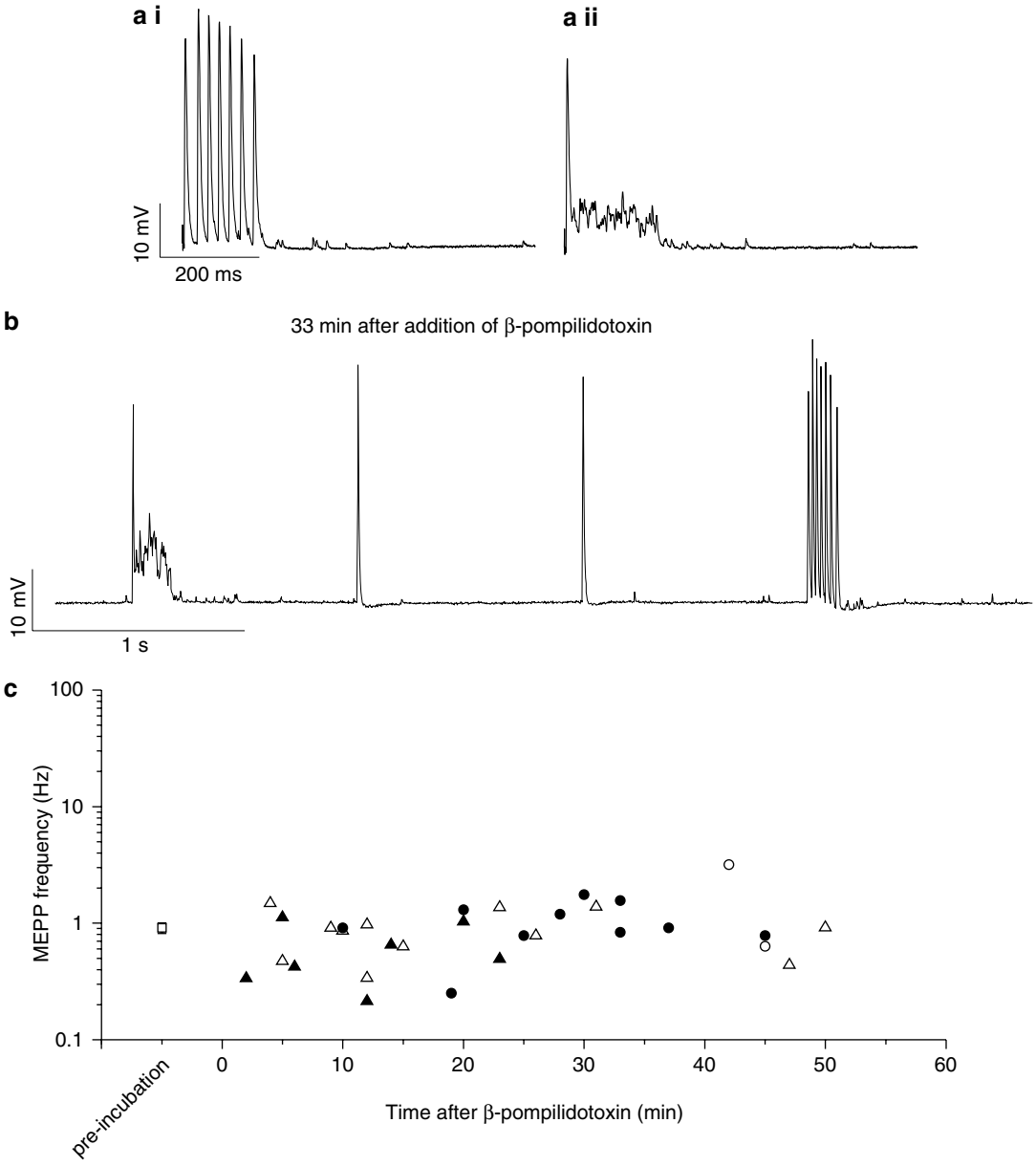

Figure 4 β-pompilidotoxin mimics some of the actions of oxaliplatin. (ai) Example trace of β-pompilidotoxin induced multiple EPPs following stimulation. (aii) Example trace of an EPP associated with elevated MEPP activity in presence of β-pompilidotoxin. (b) Example of two variations of β-pompilidotoxin-induced EPP abnormalities within one continuous trace recorded from a single endplate, 33 min after toxin application. (c) MEPP frequency during β-pompilidotoxin incubation ($n=4$), three to four endplates were studied pretreatment in each preparation and the mean $\pm$ s.e.m. plotted as preincubation (error bars are contained within the symbol). Triangles are MEPP frequencies calculated from the interval between detected events, circles are directly measured in continuous records, each symbol represents an individual endplate.

Discussion

Although the neurotoxic effects of oxaliplatin have been recognised for some time, there have been no previous studies on the tissue preparation that represents one of the main target tissues *in vivo*. Here we show that oxaliplatin increased both evoked and spontaneous neurotransmitter release in the motor nerve terminal of the phrenic nerve hemidiaphragm preparation, and these effects did not occur if the preparation was first blocked by TTX. Indeed, slowing Na^+ channel recovery from inactivation with the antiepileptic carbamazepine substantially reduced the effects of oxaliplatin. Interestingly, the effects of oxaliplatin were replicated to a large extent by the wasp toxin, β-pompilidotoxin (Sahara *et al*., 2000). β-pompilidotoxin, examined for the first time at the mammalian neuromuscular junction, produced repetitive activity and some hyperexcitability, probably *via* a mechanism which delays entry of Na^+ channels into an inactivated state.

These observations suggest that oxaliplatin has an action on the kinetics of voltage-gated Na^+ channels. There are many different sites of action of sodium channel effectors and neurotoxin sites 3, 4 and 5 could be involved in the effects of

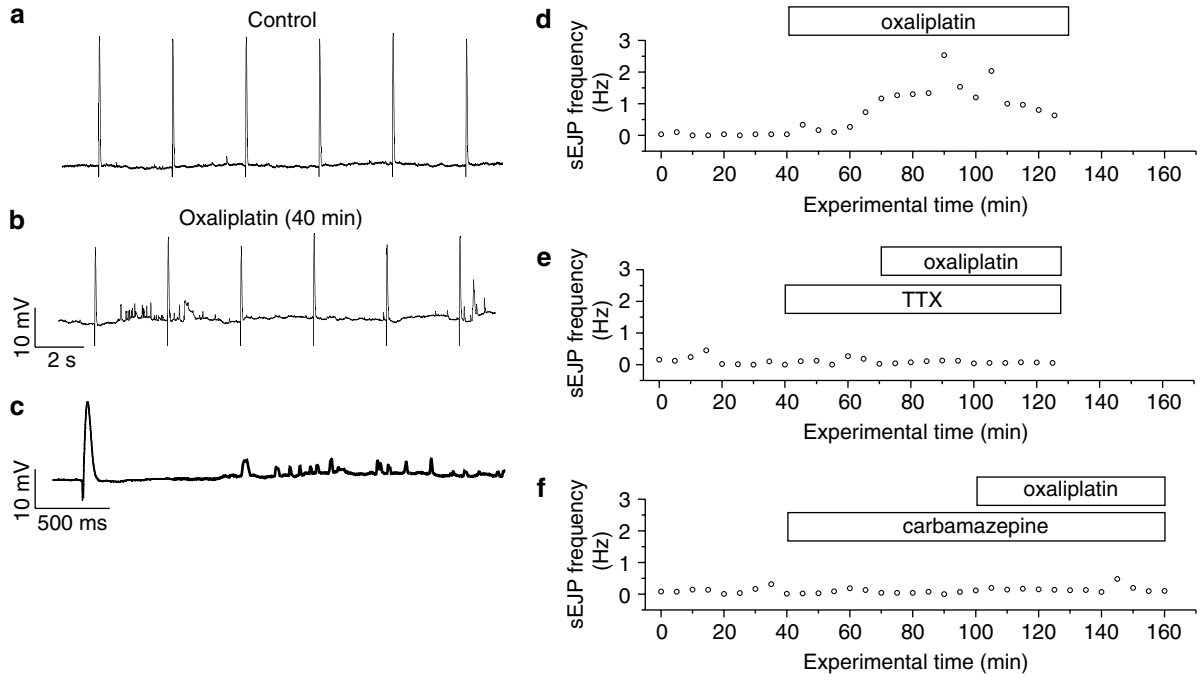

Figure 5 Oxaliplatin reduces the amplitude of EJPs and increases sEJP frequency in the vas deferens. (a) Shows a representative trace of the membrane potential recorded in the mouse vas deferens. Field stimuli were applied every 3 s (arrows) and result in a stimulus artefact (downward deflection) and excitatory junction potential (depolarisation). sEJPs, which report the spontaneous release of ATP, are occasionally detected. After 40 min in oxaliplatin (0.5 mM); (b) the amplitude of EJPs was lower and bursts of sEJPs were recorded. The first few seconds of this recording have been magnified in (c), and the frequency of sEJPs over time is shown in (d), with the data collected into 5 min bins. Pretreatment with either TTX (e) or carbamazepine (f) prevents oxaliplatin's effect on sEJP frequency. In parts (d), (e) and (f) data (collected into 5 min bins) from representative experiments are plotted.

oxaliplatin (Cestele & Catterall, 2000). Neurotoxins active at these sites can produce increases in spontaneous activity in motor nerves similar to β-pompilidotoxin, *via* shifts in voltage dependence of activation and/or prolonging Na^+ channel openings (Oliveira *et al*., 1989; Molgo *et al*., 1990; Gilles *et al*., 2000). For example, oxaliplatin was found to have type-3-like actions in cultured dorsal root ganglia neurons, where slowed Na^+ channel inactivation kinetics was accompanied by a shift in the voltage dependence of activation (Adelsberger *et al*., 2000). While in cultured dorsal unpaired median neurons of the cockroach, oxaliplatin reduced the peak Na^+ current, a type-4 action (Grolleau *et al*., 2001), and in a third tissue, cultured hippocampal neurons, oxaliplatin had no effect (Adelsberger *et al*., 2000). These differences probably reflect differential expression of Na^+ channel subtypes in these different tissues. Given the variation in effects in different tissues, and since β-pompilidotoxin does not fully replicate the actions of oxaliplatin, the effects of oxaliplatin may be due to alteration in either the voltage dependence of activation or the kinetics of inactivation.

In fact, the results were partially different in the vas deferens. In this preparation, oxaliplatin initiated bursts of sEJPs, reflecting bursts of transmitter release from sympathetic nerves. These bursts of transmitter release are probably generated by bursts of nerve terminal action potentials as they did not occur in the presence of TTX, and oxaliplatin-induced bursts of nerve terminal action potentials were observed during confocal Ca^{2+} imaging. These results are consistent with a direct effect of oxaliplatin on voltage-gated Na^+ channels on the nerve varicosities. At the mouse motor nerve terminal, there are no voltage-gated Na^+ channels and the oxaliplatin effect on EPPs and spontaneous release are likely, instead, to be dependent on the passive depolarisation caused by prolonged or repetitive motor nerve action potentials. Previous work had suggested that alteration in intracellular Ca^{2+} regulation by oxaliplatin was responsible for the modification of voltage-gated Na^+ channel activity (Grolleau *et al*., 2001). Our results, showing that autonomic hyperexcitability occurred in the vas deferens preparation in the absence of changes in Ca^{2+} handling, do not support a role for calcium regulation in the effects of oxaliplatin in this tissue. Moreover, carbamazepine both protected autonomic nerves from a decrement in evoked transmitter release and reduced the rate of spontaneous transmitter release, consistent with an effect of oxaliplatin on Na^+ channel kinetics. The decline in the amplitude of the EPP and EJP induced by carbamazepine alone may be due to a partial Ca^{2+} channel block (Elliott, 1990).

This study addressed the acute hyperexcitability associated with oxaliplatin therapy (Lehky *et al*., 2004) rather than the axonal neuropathy and cell death that follows chronic treatment and appears similar to that of other platinum-based chemotherapeutic agents. We used a concentration of oxaliplatin that is used in therapeutic infusions, and within the range used in other studies of oxaliplatin neurotoxicity in tissues and cell-based assays (25–250 μM; Adelsberger *et al*., 2000; and 100–500 μM; Grolleau *et al*., 2001). As in other studies (Adelsberger *et al*., 2000) we found similar effects with lower concentrations, but they took longer to develop. The concentration we used was higher than that achieved in the plasma of treated patients but may be similar to that in the tissues. Pharmacokinetic studies of oxaliplatin (130 mg/m^2

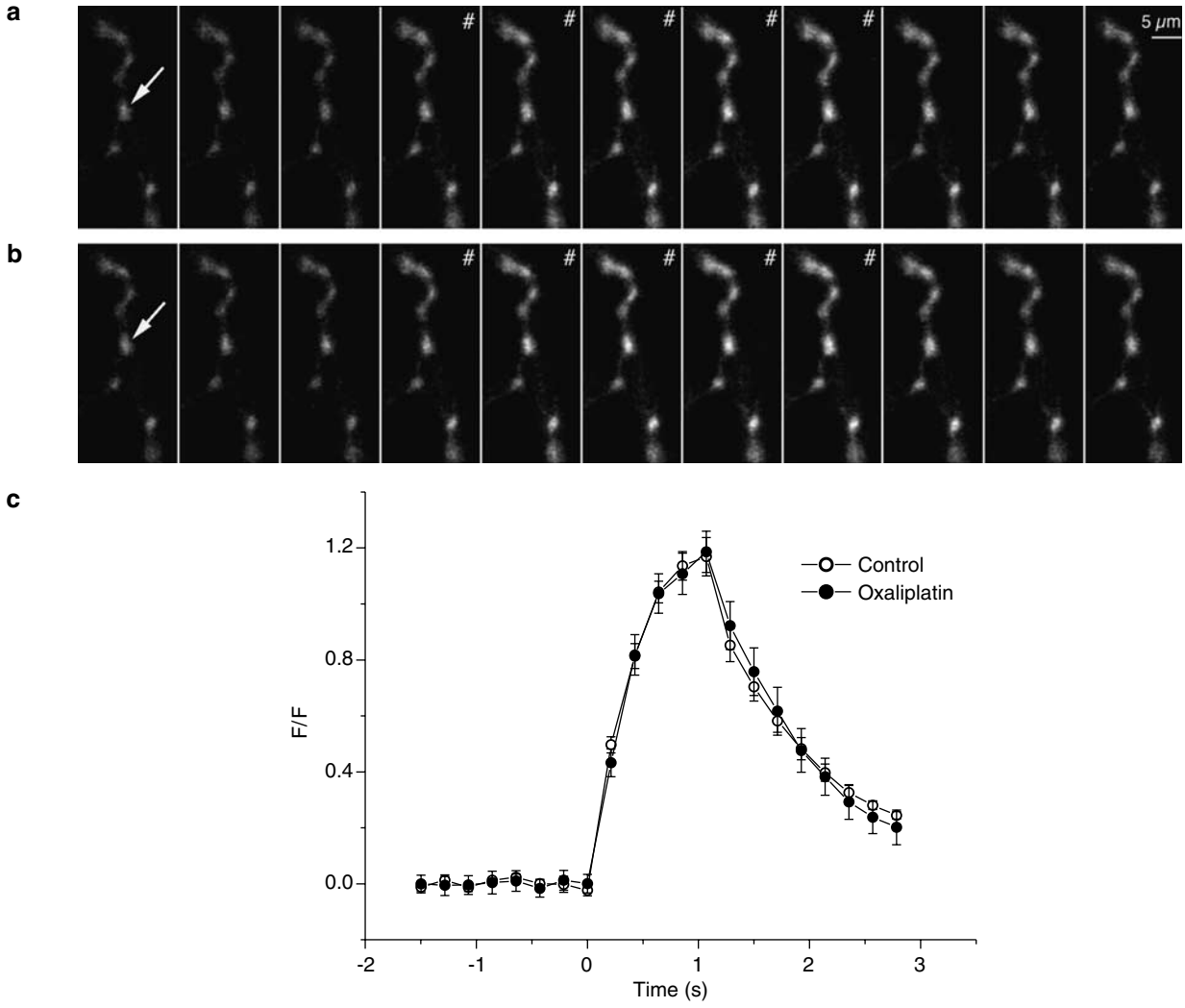

Figure 6 Confocal nerve terminal Ca^{2+} imaging in the presence of oxaliplatin. (a) Shows consecutive confocal images of part of a nerve terminal in the mouse vas deferens filled with the Ca^{2+} indicator Oregon-BAPA-1. A nerve terminal varicosity is marked with an arrow on the first frame. Images were acquired every 0.214 s and field stimuli (sufficient to induce a single nerve terminal action potential) applied at the #. This results in an increase in the fluorescent intensity, indicating an increase in Ca^{2+} concentration within every nerve terminal varicosity ($[Ca^{2+}]_v$). Upon cessation of the stimulus train, the $[Ca^{2+}]_v$ returns towards its resting concentration. (b) Shows the same site 40 min after the application of oxaliplatin. The change in the fluorescent signal from the varicosity marked with the arrow is quantified in (c). This figure shows the relative change in fluorescence ($\Delta F/F$), averaged over eight recordings taken 3 min apart, in absence and then presence of oxaliplatin. The error bars show the s.e.m. Oxaliplatin had no significant effect on the stimulation-evoked change in $[Ca^{2+}]_v$.

oxaliplatin as a 4 h infusion) revealed a peak concentration of free platin of about 1600 ng/ml (or 5.6 μM; Kern *et al*., 1999), with a terminal half-life of 27 h, implying that the body is exposed to oxaliplatin for a significant length of time. Moreover, oxaliplatin's large volume of distribution (free platin, 350 l; protein-bound platin 180 l) implies that there are areas outside the circulation in which oxaliplatin accumulates at much higher concentrations. Intracellular accumulation has been confirmed, at least in erythrocytes (Pendyala & Creaven, 1993) and human ovarian carcinoma cell lines (Pendyala *et al*., 1995).

The reason for the delay in the effects of oxaliplatin on evoked transmitter release is unclear. Oxaliplatin is taken up by cells and metabolised to various platinum biotransformation products, including 1,2-diaminocyclohexane-platinum. This compound enters the nucleus where it acts as an alkylating agent to form inter- and intracross links within DNA, leading eventually to apoptosis (Wiseman *et al*., 1999; Misset *et al*., 2000). The delayed results could be because of delayed entry of oxaliplatin into the nerve terminals, where it might act to alter calcium release from intracellular stores, but the lack of effect on calcium release in the presence of TTX argues against this. Another oxaliplatin breakdown product is oxalate. Oxalate increases the uptake of Ca^{2+} into intracellular stores (Grover & Kwan, 1984); oxalate enters the ER and then binds Ca^{2+} in the ER lumen (Waldron *et al*., 1995). A French group used infusions of calcium gluconate and magnesium sulphate before and after oxaliplatin as oxalate chelators and suggested that Ca/Mg infusions reduced incidence and intensity of acute oxaliplatin-induced symptoms and might delay cumulative

neuropathy (Gamelin *et al*., 2004). However, the lack of evidence for a change in Ca^{2+} regulation in the nerve terminal following action potential-induced Ca^{2+} influx in the vas deferens, makes it unlikely that oxalate release is an important mechanism of action of the drug, at least in autonomic terminals. In addition, we saw no effect of 0.5 mM oxalic acid on neurotransmission in the mouse hemidiaphragm preparation.

The role of defects on Na^+ and K^+ channels in neurological diseases is well recognised. Slowing of Na^+ channel inactivation kinetics has been implicated in many of the myotonias (Cannon, 1997), diseases which cause periodic muscle stiffness and/or weakness. In these cases, the altered kinetics of the muscle Na^+ channels allow greater Na^+ current to pass and result in repetitive firing. It is also interesting to note that the altered kinetics of this mutant channel is exacerbated by cold (Bouhours *et al*., 2004), similar to the exacerbation of sensory signs with oxaliplatin-induced neurotoxicity. Reduced K^+ channel activity, due to genetic mutations, autoantibodies, drugs or toxins, can also cause similar symptoms in nerve or muscle diseases (Hart *et al*., 1997; Kinali *et al*., 2004). Indeed, a previous clinical study of oxaliplatin-induced hyperexcitability and neuropathy described symptoms reminiscent of neuromyotonia (Lehky *et al*., 2004), a condition that results from impairment of K^+ channel-dependent membrane repolarisation and is often caused by autoantibodies to K^+ channels (Newsom-Davis, 2004). However, the lack of similar oxaliplatin-like effects with K^+ channel blockade suggests that reduction in K^+ channel function is unlikely to be the cause of hyperexcitability in the hemidiaphragm preparation.

In the tissues we examined, therefore, the most likely mechanism of oxaliplatin neurotoxicity is a direct effect on voltage-gated Na^+ channels rather than an effect on K^+ channels or nerve terminal Ca^{2+} regulation. Although no effect of carbamazepine was seen in one study (Wilson *et al*., 2002), both carbamazepine and gabapentin, which prolong Na^+ channel inactivation, have been used clinically to reduce the neurotoxic effects of oxaliplatin (Lersch *et al*., 2002). Our results suggest that targeting Na^+ channels will continue to provide the best approach to counteract the acute neurotoxic effects of oxaliplatin.

We thank Sanofi-aventis for the kind gift of excipient-free oxaliplatin for use in these studies. The authors also thank Dr Tom Cunnane (Pharmacology, Oxford) for the generous provision of equipment for autonomic tests. R.G.W. was supported by grants from Muscular Dystrophy Campaign.

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(Received May 17, 2005
Revised July 26, 2005
Accepted August 26, 2005
Published online 17 October 2005)