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Interactions between baclofen and DC-induced plasticity of afferent fibres within the spinal cord

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Authors contributions

The experiments were performed at the Department of Physiology, University of Gothenburg. FB, EJ and IH contributed to the design of the experiments, the collection, analysis, and interpretation of the data and the drafting of the article. RE was involved in the analysis, interpretation of the data and the drafting of the article. All authors approved the final version of the manuscript.

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Conflict of interest: none

Highlights

- Baclofen interacts differentially with DC effects on epidurally or intraspinally stimulated muscle and skin afferent fibres. (123/125)
- DC/baclofen interactions are consistent with different mechanisms of plasticity in parent afferent fibres and their terminals. (125/125).
- DC applied epidurally may facilitate the effects of epidural stimulation in combination with treatment with baclofen. 118/125

Abstract

The aims of the study were to compare effects of baclofen, a $GABA_B$ receptor agonist commonly used as an antispastic drug, on direct current (DC) evoked long-lasting changes in the excitability of afferent fibres traversing the dorsal columns and their terminal branches in the spinal cord, and to examine whether baclofen interferes with the development and expression of these changes. The experiments were performed on deeply anaesthetized rats by analyzing the effects of DC before, during and following baclofen administration. Muscle and skin afferent fibres within the dorsal columns were stimulated epidurally and changes in their excitability were investigated following epidural polarization by 1.0-1.1 µA subsequent to i.v. administration of baclofen. Epidural polarization increased the excitability of these fibres during post-polarization periods of at least one hour. The facilitation was as potent as in preparations that were not pretreated with baclofen, indicating that the advantages of combining epidural polarization with epidural stimulation would not be endangered by pharmacological antispastic treatment with baclofen. In contrast, baclofen reduced effects of intraspinal stimulation combined with intraspinal polarization (0.3 µA) of terminal axonal branches of the afferents within the dorsal horn or in motor nuclei, whether administered ionophoretically or intravenously. Effects of DC on monosynaptically evoked synaptic actions of these fibres (extracellular field potentials) were likewise reduced by

baclofen. The study thus provides further evidence for differential effects of DC on afferent fibres in the dorsal columns and the preterminal branches of these fibres and their involvement in spinal plasticity. 246/250 words

Abbreviations

DC, direct current; DR, dorsal root; EPSP, excitatory postsynaptic potential; L, lumbar; PAD, primary afferent depolarization; Per, peroneal; S, sacral; Sur, sural; T, threshold; tDCS, transcranial direct current stimulation; tsDCS, trans-spinal direct current stimulation; VR, ventral root.

Introduction

Spinal actions of direct current (DC) are of both theoretical and therapeutic interest. Theoretical, because DC-evoked long-term increases in the excitability of spinal nerve fibres provide new clues on the mechanisms underlying spinal plasticity. While DC has been demonstrated to affect afferent nerve fibres traversing the dorsal columns as well as the terminal branches of these fibres in the grey matter, the increases in the excitability developed more rapidly and were stronger and longer lasting in the dorsal columns (Jankowska et al., 2016, 2017). Observations that myelinated nerve fibres and terminal branches are differentially modified by the K^+ channel blocker 4-amino-pyridine (4-AP) indicate a differential contribution of potassium membrane channels to axonal plasticity (Jankowska et al., 2016, Bolzoni et al., 2017, Kaczmarek and Jankowska, 2018).

Extending these observations, one of the main aims of the present study has been to investigate the effects of the $GABA_B$ receptor agonist baclofen with effects opposite to those of 4-AP. Baclofen has been demonstrated to decrease the duration of action potentials in terminal branches without affecting those in myelinated nerve fibres (Curtis et al., 1997,1998, 1981). The effects of baclofen were therefore considered of particular interest for delineating the mechanisms of differential effects of DC on nerve fibre excitability.

Clinical interest in spinal effects of DC arose following the progress of transcranial direct current stimulation (tDCS) and its wide application range (Priori, 2003, Nitsche et al., 2008, Brunoni et al., 2012, Giordano et al., 2017, Jamil et al., 2017, Lefaucheur et al., 2017). DC-evoked changes in spinal activity were first analyzed following trans-spinal application of DC (tsDCS) and demonstrated to have wide-spread effects (Aguilar et al., 2011, Ahmed, 2011, Cogiamanian et al., 2011, Cogiamanian et al., 2012, Ahmed, 2013, 2014a, b, 2016), Bączyk et al, 2018). Effects of

intra-spinally and epidurally applied DC were more restricted (for review see Jankowska, 2017) with those of epidural polarization found to be particularly potent. Effects of epidural polarization thus appeared to be more promising for therapeutic purposes, especially in combination with epidural stimulation. A particular advantage of DC-evoked increases in the excitability of epidurally stimulated fibres would be that they are very potent and long-lasting. The beneficial effects of DC might nevertheless depend on the medication administered to the patients (for the latest review see e.g. Czesnik and Paulus, 2017, Lefaucheur et al., 2017, McLaren et al., 2018). This factor might be of particular importance for patients treated pharmacologically for central injuries associated with spasticity but it has remained an open question to what extent antispastic drugs would potentiate or counteract combined effects of DC and epidural stimulation.

The second main aim of the present study has, therefore, been to compare spinal effects of DC in the presence or absence of baclofen, commonly used as the antispastic drug (Davidoff and Sears, 1974, Fox et al., 1978, Curtis et al., 1981, Capek and Esplin, 1982, Curtis and Malik, 1985, Curtis et al., 1997). Baclofen acts by weakening synaptic transmission in spinal reflex pathways (Lev-Tov et al., 1988, Edwards et al., 1989, Jimenez et al., 1991, Quevedo et al., 1992, Nance, 1994, Azouvi et al., 1996, Li et al., 2004, Schechtmann et al., 2010). However, the modulatory actions of baclofen may vary at different sites within spinal neuronal networks and the interactions between baclofen and DC might accordingly differ at these sites. In order to investigate these interactions, they were analyzed following intraspinally and epidurally applied DC using minimal effective current intensities (of the order of microamperes, for review, see Mushahwar et al., 2000, Holinski et al., 2016, Prochazka, 2016, Jankowska, 2017). We aimed at investigating them at the level of myelinated afferent fibres traversing the dorsal columns as well as at the level of the terminal branches of these fibres within their projection areas in the motor nuclei (for group Ia muscle afferents) and in the dorsal horn (for low threshold skin and for group II muscle afferents).

The experiments were performed in deeply anaesthetized rats, using electrophysiological techniques to monitor DC evoked changes in the excitability of muscle and skin nerve fibres before and after administration of baclofen. Increases or decreases in nerve volleys evoked in peripheral nerves by near threshold spinal stimulation were used as a measure of the excitability of the stimulated afferent fibres while changes in extracellular field potentials monosynaptically evoked by these fibres were used to evaluate changes in their direct synaptic actions.

Methods

All the main experimental procedures were as described in detail by Bolzoni and Jankowska (2015, Jankowska et al. (2017), Kaczmarek and Jankowska (2018).

Ethical approval

All experiments were approved by the Regional Ethics Committee for Animal Research (Göteborgs Djurförsöksetiska Nämnd) and followed EU guidelines for animal care (86/609/EEC). The animals were housed under veterinary supervision at the Laboratory of Experimental Biomedicine at Sahlgrenska Academy where the experiments were carried out. Particular measures were taken to minimize the number of animals used as well as animal discomfort.

Preparation

The experiments were performed on 36 adult rats of both sexes (Wistar, 250-550 g). Anaesthesia was induced with isoflurane (4% in air) (Baxter Medical AB, Kista, Sweden) followed by i.p. administration of α-chloralose (Acros Organics, Geel, Belgium, or Rhône-Poulenc Santé, France), 30-40 mg/kg, together with pentobarbital sodium (Apoteksbolaget, Göteborg, Sweden), (25-30 mg/kg). During the course of the experiment, the anaesthesia was supplemented with 2-3 additional doses of α-chloralose (10 mg/kg, up to 60 mg/kg). The preliminary dissection included tracheal intubation, cannulation of the tail veins, dissection of the sural (Sur) and peroneal (Per) nerves and the exposure of the 2^{nd} to 5^{th} lumbar (L2-L5) spinal segments by laminectomy. Paraffin oil pools were constructed by skin flaps above the dissected tissues. During experiments, the neuromuscular transmission was blocked by gallamine triethiodide (Sigma, G8134-5G) or pancuronium bromide (Pavulon Jelfa, Poland), applied i.v. (via the tail vein) at an initial dose of about 10 mg/kg and supplemented, when needed. Artificial ventilation was applied using a respiratory pump (CWE; 50-70/min and 0.3-0.4 ml/min volume depending on the animal size) to maintain the expired CO_2 level at 3-4%.

The core body temperature was maintained at approximately 38^{o}C by servo-controlled heating lamps. In order to compensate for fluid loss and to prevent the deterioration of the state of the animals, 10-20 ml of acetate buffer were injected subcutaneously at the beginning of the experiments. The experiments were terminated by a lethal dose of pentobarbital followed by excision of the heart.

Recording

DC-induced changes in fibre excitability were estimated from responses of sensory fibres to stimuli applied either epidurally or intraspinally within the terminal projection areas of these fibres, as indicated in Fig. 1A and B respectively. Antidromically evoked responses of the stimulated fibres were recorded from Sural (Sur) and Peroneal (Per) nerves transected distally, at the level of Achilles tendon and the entry of the anterior tibial nerve to the muscle, respectively, to allow their dissection over a distance of at least 10-15 mm. The nerves were mounted on a pair of silver/silver chloride electrodes in a paraffin oil pool. The effects of DC on synaptic actions of Per and Sur afferents were examined on monosynaptic field potentials evoked in motor nuclei and in the dorsal horn (Fig. 1C). The effects of DC on field potentials evoked by epidurally stimulated fibres were examined only in the dorsal horn. The field potentials were recorded with glass microelectrodes (see Fig. 1) filled with a 2M solution of NaCl (tip diameter approximately 2 μm, impedance 1.5-5 MΩ) and a conventional high-impedance amplifier (low-pass filters 15 or 1 Hz, high-pass filter 5 or 3 kHz). Afferent volleys following nerve stimulation were recorded with a silver-silver chloride ball electrode in contact with the surface of the spinal cord at the L2 spinal level against a reference electrode inserted into the back muscles at the same segmental level and as a triphasic or a diphasic potential preceding the extracellularly recorded field potentials. Both original records and averages of records evoked by 10 or 20 stimuli were stored online.

Fig. 1 near here

Stimulation.

Epidural stimulation (10-20 μA) of fibres running within the dorsal columns was applied via an electrode placed on the dura mater and carefully forwarded under microscopic control until the dura mater was indented to a point where the distance between the dura and the surface of the dorsal columns was reduced to a minimum, or when contact between them occurred, as described by Jankowska et al.(2017). The stimuli were applied at a position half-way between the midline and the dorsal root entry zone. Intraspinal stimuli of 3-10 μA, 0.2 ms were applied within motor nuclei to terminal branches of group I muscle afferents or within the dorsal horn to skin and group II muscle afferents, at sites at which stimulation of these afferent fibres evoked distinct monosynaptic field potentials. In both cases, constant current stimuli were delivered via a tungsten needle electrode (200-500 kΩ) insulated except for a tip of 20-30 μm (Microneurography active needle, UNA35FNM, FHC, Bowdoin, ME, USA) against a reference electrode inserted in back

muscles just rostral to the laminectomy at the midline. The intensity of the stimuli was adjusted to be near-threshold while still evoking reliably measurable nerve volleys; for examples of the selected nerve volleys see Fig. 3A and Fig. 4A. Near-threshold stimuli were selected to ensure the highest probability of facilitation of their effects by the conditioning polarization.

The Sur and Per nerves were stimulated via a pair of silver/silver chloride electrodes in a paraffin oil pool using constant voltage current pulses (0.2 ms). The intensity of the stimuli was expressed in multiples of threshold stimuli (as defined by records of incoming volleys from the surface of the spinal cord). Per was stimulated at 1.5-1.8 times threshold (T) or at 5T to activate group I and II muscle afferents and Sur at 2-3T stimuli. Such stimuli were used to evoke field potentials in the dorsal horn and to compare latencies of afferent volleys monitored by records from the cord dorsum and of nerve volleys evoked by epidural stimulation.

The tip of the tungsten electrode used for intraspinal stimulation was aligned at a distance of approximately 10-50 µm from the tip of the glass microelectrode used to record field potentials (for details see Bolzoni and Jankowska, 2015). The positioning of the microelectrode within the Per motor nucleus (usually at 1.0-1.2 mm depth from the cord dorsum, at the angle of 5-10 degrees) was guided by records of antidromic field potentials evoked by stimulation of the Per nerve and/or of monosynaptic field potentials from the group I muscle afferents. In the dorsal horn, the electrodes were positioned at the depth (usually 0.65-0.8 mm from the cord dorsum) at which large monosynaptic field potentials were evoked by stimulation of low threshold skin afferents in the Sur nerve or of both skin and group II muscle afferents in the Per nerve.

DC polarization

The polarization was applied using a custom designed, battery-driven, constant current stimulator (D. Magnusson, University of Gothenburg) by passing a continuously monitored direct current of 1.1 µA epidurally and of 0.2 or 0.3 µA intraspinally, within ranges we previously demonstrated to be highly efficient but with a minimal risk of evoking anodal block (Bolzoni and Jankowska, 2015, Jankowska et al., 2017). The current was passed via the same tungsten electrode that was used for the activation of the fibres, as DC was demonstrated not to interfere with these stimuli (Baczyk and Jankowska, 2014; see their Fig. 2). The polarizing current was applied for 1-2 min (epidurally) or for a total of 25 min (intraspinally; five polarization periods of 5 min separated by a 5 min interval) as different current parameters are needed for evoking long-lasting post-

polarization increases in the excitability of dorsal column fibres and their terminal branches respectively (Bolzoni and Jankowska, 2015, Jankowska et al., 2017)

Drug application

(-)- baclofen hydrochloride (Sigma-Aldrich) was applied either intravenously or ionophoretically. Baclofen was administered intravenously by slow injection of 2-4 mg/kg (from a solution of 1 mg/ml) during 5-10 min. These doses were higher than doses of 0.5-2 mg/kg most often used in cats, taking into account that higher doses are generally required of all drugs (including anaesthetics and gallamine triethiodide) in the rat than in the cat. According to Curtis et al. (1997) intravenous administration of 0.5 mg/kg of baclofen in the cat might result in intrathecal concentrations of the order of 0.2 μM , and monosynaptic reflexes in the rat spinal cord under in vitro conditions are abolished by concentrations of the order of 0.5 μM, likewise justifying the use of relatively high doses of baclofen in the present series of experiments. The effectiveness of baclofen was verified by a decrease in field potentials or nerve volleys induced by either intraspinal or epidural stimuli.

When applied ionophoretically, baclofen was ejected as cation from a glass micropipette (filled with a 2 mM solution in saline; tip broken to about 2 μM, impedance 5-7μΩ) passing a current of 10 or 20 nA. The drug-containing micropipette used for ionophoresis was mounted in a step-motor operated microdrive, attached to the same arc as the double-headed manipulator holding the glass microelectrode and the tungsten stimulating/polarizing electrodes, and connected to a high-impedance amplifier. It was advanced into the spinal cord only after the other two electrodes were positioned and just before the beginning of the drug application. The tip was positioned between the recording microelectrode and the tungsten electrode used for stimulation, just medial to their sites of entry (using a micro cross-table attached to the third micro-drive) and about 0.3-0.4 mm below the surface of the spinal cord, using a retaining current of 10 nA to prevent drug diffusion prior to commencing the ionophoresis. The baclofen containing micropipette was verified to record the same field potentials as the deeper located microelectrode and calibration pulses delivered through this micropipette were monitored to ascertain that current was successfully passed during the whole period of the ionophoresis. The effectiveness of ionophoresis was ascertained by verifying that dorsal horn field potentials were depressed within minutes of baclofen application, as expected based on previous studies (Quevedo et al., 1992, Curtis et al.,

1997, Hammar and Jankowska, 2003). Baclofen was applied for 5-10 min before being combined with DC. After terminating the ionophoresis, the baclofen-containing micropipette was withdrawn and positioned above the surface of the spinal cord.

Previous reports concerning the effects of baclofen on nerve fibre excitability were inconsistent. In the *in vitro* frog spinal cord preparation, reduced excitability and hyperpolarization of dorsal root fibres were described by Davidoff and Sears (1974). In the cat, Fox et al. (1978) reported that intravenously applied baclofen (0.1-5 mg/kg) induced not only a strong (up to 30%) depression of EPSPs in motoneurones but also weak changes in the excitability of primary afferent fibres ("mostly between 2 and 10%") and that "comparable changes in excitability were recorded when stimulating peripheral axons…or afferent terminals". However, the excitability of fibres stimulated within the intermediate zone was reported to remain unaltered by intravenously applied baclofen (1-2 mg/kg in the cat; Jimenez et al., 1991). Curtis et al. (1997) failed to find a decrease in the excitability of fibres stimulated within the dorsal columns during a few minutes of baclofen ionophoresis, while the excitability of afferent fibres within their intraspinal projection areas was depressed and the duration of action potentials in preterminal branches was reduced under the same experimental conditions.

Expecting that an increased dosage or a longer period of baclofen application would increase the probability of affecting fibres in the dorsal columns, we used doses within the upper range of those of Fox et al. (1978) and applied ionophoresis for longer periods. As illustrated in Fig. 2B, administration of baclofen i.v. in doses of 3-4 mg/kg depressed fibre excitability in the dorsal columns to 70-80% of baseline within 20 min. When the observation period was extended to include an additional 20 min, the fibre excitability remained at the same level or was reduced even further. Baclofen ionophoresis reduced intraspinal fibre excitability to a similar degree, 75-80% within 20 min (Fig. 2D), which was further reduced to about 60% when the ionophoresis was continued. The effects remained for at least 30 min following termination of ionophoresis. When DC application was commenced 20 min after the onset of baclofen administration, any additional depressive effects of baclofen developing during the subsequent period thus had to be considered.

Fig. 2 near here

When baclofen was injected i.v, only one region of the spinal cord could be explored in each experiment in view of its generalized longlasting effects. Hence, only one sequence of records was

obtained to compare the effects of epidural stimulation before and following baclofen injection. When baclofen was applied ionophoretically, i.e. locally, the effects were investigated in 2-3 regions of the spinal cord located a few mm apart and the results from each of these regions were considered independent. However, in both cases, several measures were taken to increase the outcome of the experiments and to reduce the number of the experimental animal to the minimum. Firstly, the nerve volleys on which the effects of the conditioning stimuli were examined were recorded in parallel from two peripheral nerves (doubling the number of these series of records per experiment). Secondly, the test field potentials recorded within the dorsal horn were evoked by stimuli applied alternately to two peripheral nerves and epidurally (likewise increasing the number of series per experiment). Thirdly, whenever possible effects of the same conditioning stimuli were examined in parallel on nerve volleys and onfield potentials (for details see the results). Using these measures we collected data from various combinations of conditioning and testing stimuli in at least 6-8 series of records in at least 2-3 rats. Both the number of series and the number of rats are indicated in the results.

Analysis

The tested responses (field potentials and nerve volleys in peripheral nerves) were compared when evoked (i) under control conditions, (ii) during DC application, (iii) during baclofen application, (iv) during combined DC and baclofen application and (v) during the post-polarization period. Changes in the area and/or the latencies of the potentials were evaluated from averages of 10 successive potentials recorded with the sampling frequency of 33 kHz. The area of the field potentials was measured within a time window of 0.4 - 0.9 ms from their onset, usually within the rising phase of these potentials and within <1 ms from the afferent volley, in order to restrict the comparison to the earliest, predominantly monosynaptically evoked components. The measurements of the area of the nerve volleys evoked by intraspinal stimuli were likewise restricted to the earliest components, evoked at the same latency (± 0.3 ms) as the latency of afferent volleys induced by stimulation of the same nerve. The normalized areas from all experiments were averaged for each testing period before proceeding with the comparisons between the periods.

When normal distribution was ascertained (by means of Shapiro Wilk-Test) the comparisons between the periods were performed with RM ANOVA. The equal variance was then verified by

applying Mauchly's sphericity test. The Greenhouse-Geisser correction was used for repeated-measures ANOVA when the assumption of sphericity was violated. Dunnett's post-hoc tests were run to compare the values obtained after baclofen administration or the values obtained during and following DC application to control mean values. When normal distribution was not found (in one case), a non-parametric test (Friedman ANOVA for the main effect) was run. Wilcoxon signed-rank test was then used in order to asses significant differences against the control value and the Bonferroni correction was applied taking into account the number of the comparisons.

Results

Effects of combined application of baclofen and of DC on the fibre excitability

Interactions between the effects of baclofen and DC were found to depend on whether DC was applied epidurally (to fibres in the dorsal columns) or intraspinally (to the terminal branches of these fibres). Fig. 2A shows that the administration of baclofen i.v. did not prevent the increase in the excitability of dorsal column fibres during and following epidurally applied DC (Jankowska et al., 2017). During 1 min of DC polarization (Fig 2A), nerve volleys evoked by stimulation of afferent fibres within the dorsal columns increased to 976±149% *(*mean ±SE) compared to control nerve volleys and the range (299-2668%) overlapped with that in preparations not treated with baclofen (Jankowska et al., 2017, Kaczmarek and Jankowska, 2018). Following baclofen application, the increases in the fibre excitability evoked by DC were maintained at about the same level (437±50% of control) throughout the post-polarization period of at least 40 min even though it declined to about one half of that found during DC application. In contrast, baclofen interfered with post-polarization effects under conditions when terminal axonal branches of afferent fibres were stimulated and polarized intraspinally (as in Fig. 1B). Fig. 2C shows that during intraspinal polarization, effects of baclofen were initially only moderate, as the fibre excitability increased during the first 2-3 periods of DC application that coincided with the baclofen ionophoresis, while the increases were only marginal during the next periods. Furthermore, intraspinally applied DC failed to evoke post-polarization increases in the excitability as the excitability remained below that of baseline values in all tested fibres (cf Fig. 2C and Fig. 2D). The nerve volleys remained reduced to 60-80% of control volleys for 10-15 min after the termination of both baclofen ionophoresis and DC application although a slowly developing increase was observed within the next 20-30 min.

In order to verify that the reported effects of epidural polarization were indeed restricted to the dorsal columns (as schematically indicated by the dotted area in Fig. 1A), and hardly engaged fibres in the spinal grey matter, the following control experiments were performed.

The effects of epidural polarization on nerve volleys evoked by epidural stimulation were compared with its effects on nerve volleys evoked by stimuli applied within the dorsal horn. As shown in Fig. 3D-F and G, 1 µA epidural DC decreased rather than increased those evoked by intraspinal stimulation. The mean area of these nerve volleys amounted to 92±5% during 1 min of polarization and to 78±5%, 64±6% and 67±10% after 1, 10 and 15-20 min of the post-polarization period respectively.

Fig 3 near here.

In Fig. 3 the contrast between effects of epidural polarization on epidurally (A-C) and intraspinally (D-E) stimulated fibres is illustrated with data obtained while effects of DC were tested on nerve volleys evoked by alternating application of epidural and intraspinal stimuli. The intraspinal stimuli were applied in the dorsal horn at 0.7 mm depth from the surface of the spinal cord and within 1 mm radius from the site of the epidural stimulation. The comparison for the two samples is shown in Fig. 3G which in addition provides a comparison with the effects of DC applied within the dorsal horn on the excitability of fibres stimulated at this location. In contrast to the potent effects elicited in fibres in the dorsal columns, the intraspinally evoked nerve volleys increased only marginally (to 132%) during 1 min of 0.3 µA DC. During the subsequent post-polarization period the volleys appeared only to reflect effects of the previously administered baclofen (with a decrease to approximately 70% of their original size) and were difficult to differentiate from the effects of baclofen alone.

Fig, 4 near here

Effects of combined application of DC and of baclofen on synaptic actions of the stimulated afferents

Action potentials induced in epidurally stimulated afferent fibres are conducted not only antidromically, but also centrally via their intraspinal axon collaterals, as indicated in Fig. 1A, where they induce field potentials within the terminal projection areas of the stimulated fibres illustrated in Fig. 4D. When epidurally applied DC increases fibre excitability, the increased number of fibres excited by the same epidural stimuli would, therefore, also be reflected in

changes in the field potentials. Effects of epidural polarization on peripherally recorded nerve volleys evoked by epidural stimulation and on monosynaptically evoked field potentials induced in parallel by the same stimuli are illustrated in Fig. 4 A-C and D-F respectively. The illustrated increases in field potentials were recorded at a location where the excitability of terminal branches of fibres that gave rise to them, was decreased rather than increased by epidural polarization (see previous section and Fig. 3 D-F). The increases in field potentials evoked by epidural stimulation under these conditions are thus fully compatible with an increase in the number of fibres stimulated within the dorsal column.

Baclofen had by itself similar effects on epidurally and peripherally evoked field potentials and reduced these potentials to 70-80% within about 20 min, whether applied intravenously (Fig. 5B) or ionophoretically (Fig. 5 D). However, the effects of the joint application of baclofen and DC differed, depending on whether DC was applied epidurally or intraspinally.

Fig. 5 near here

Epidurally applied DC facilitated field potentials evoked by epidurally stimulated fibres in the presence of baclofen, i.v., during at least 1 hour of the post-polarization period (Fig. 5A). In contrast, when DC was applied intraspinally, in conjunction with ionophoretically or i.v. administered baclofen, it failed to increase field potentials evoked by peripheral nerve stimulation. Neither did an increase of these potentials occur during the post-polarization period. Instead, they were depressed within 5-15 min following the DC application (Fig. 4 J-L, Fig. 5C). Thereafter, the field potentials gradually returned to control levels even though the depressive effects of baclofen on its own persisted for at least 30 min after the termination of its iontophoresis (Fig. 5D).

These results might indicate that the depression of field potentials evoked by joint actions of baclofen and intraspinal polarization was weaker than the depression evoked by baclofen by itself and that this is compatible with facilitatory effects of DC being evoked in parallel with depressive effects of baclofen. If so, the main difference between interactions of baclofen with effects of epidurally and intraspinally applied DC might lie in the degree to which the effects of DC and baclofen summate. However, if DC modulates the excitability of myelinated axons and of their terminal branches via different membrane mechanisms, the possibility that baclofen interferes with these mechanisms in a differential manner might be considered.

Further observations on mechanisms of intraspinal effects of DC

The results presented in the previous section did not allow an estimate of whether effects of baclofen (decreasing fibre excitability) merely summated with the DC induced facilitation or if baclofen interfered with the induction of the facilitation by intraspinally applied DC. In order to address this question, we performed a further series of experiments in which we reversed the order of baclofen and DC application. Assuming that the two effects summate, baclofen would reduce fibre excitability whether administered during, after or before DC application.

In a series of control experiments, illustrated in Fig.6, baclofen ionophoresis was accordingly initiated only at a point when the fibre excitability was already increased by intraspinally applied DC (0.3 μA). Under these conditions, the facilitatory effects of DC continued to increase during the post polarization period in a manner similar to that previously demonstrated in rats not treated with baclofen (see Fig. 2 in Bolzoni and Jankowska, 2015). The facilitation reached 154±44% 10 min after the final period of DC application (15 min after the beginning of baclofen ionophoresis) and remained at a similar level for the next 30-40 min (corresponding to the time of the maximal depressive effects of continuously ionophoresed baclofen illustrated in Fig. 2 D). The post-polarization facilitation during baclofen ionophoresis initiated during the intraspinal DC application was thus in contrast to the post-polarization depression occurring when baclofen ionophopresis preceded the DC application (plotted in Fig. 2C and replicated in crosses in Fig. 6 for comparison). In addition, when the means of all the post polarisation values in Fig. 2C and in Fig. 6 (74±6% and 164±16%) were compared, the difference between them was found to be statistically significant ($p<0.05$, t-test for paired samples). It is, therefore, possible that baclofen primarily interferes with the induction but not with the expression of DC-evoked long-lasting post polarization changes, thus acting in a manner similar to that of 4-AP (Kaczmarek and Jankowska, 2018).

Fig. 6 near here

Discussion

The results of this study demonstrate that nerve fibres are differently affected by baclofen when traversing the dorsal columns and at the level of their intraspinal branches. Thereby, we provide further indications that distinct mechanisms may be involved in the facilitatory actions of epidurally and intraspinally applied DC. The results also show that long-term facilitation of epidural stimulation by epidurally applied DC is potent in preparations pretreated with baclofen

and do not provide any counter-indications against the combined use of epidural stimulation and polarization together with baclofen treatment for clinical purposes.

Indications for different mechanisms underlying effects of DC on afferent fibres in the dorsal columns and within their terminal projection areas

Differences in interactions between baclofen and either epidurally or intraspinally applied DC were found under experimental conditions where baclofen by itself potently reduced the excitability of electrically stimulated sensory nerve fibres.

We do not have any ready explanations for why baclofen reduced the excitability of myelinated fibres under our experimental conditions but failed to do so in the studies of Curtis et al.(1997) and Quevedo et al. (1992), except that we used higher doses of baclofen (0.3-0.4 mg/kg in the rat as compared to 0.1-0.2 mg/kg in the cat), that the fibres were stimulated epidurally rather than within dorsal columns and that we might have waited for longer periods of time to allow baclofen effects to develop. We cannot estimate which of these factors were decisive and can only note that the reported observations were all made in preparations in which the baclofen-induced reduction in fibre excitability or in monosynaptic field potentials evoked by these fibres amounted to at least 20%. The investigation of interactions between effects of baclofen and of DC was restricted to time periods during which the excitability of the tested fibres was reduced by baclofen.

We verified that under our experimental conditions DC applied epidurally has as local effects as those estimated to be evoked under clinical conditions (Holsheimer, 2002, Ramasubbu et al., 2013, Holsheimer and Buitenweg, 2015), as epidurally applied DC increased the excitability of nerve fibres within the dorsal columns but not the excitability of their intraspinally stimulated preterminal branches within a radius of about 1 mm . Thereby, the observed differences in interactions between baclofen on the one hand and epidurally or intraspinally applied DC on the other may be linked to differential effects of baclofen on afferent fibres in the dorsal columns and on preterminal axonal branches within the grey matter. These differences provide further indications for the differential effects of DC upon them based on differences in the degree and timing of the increases in the excitability evoked by epidural and intraspinal polarization (Jankowska et al., 2017) and different effects of the K^+ channel blocker 4-AP on preterminal axonal branches and fibres stimulated within the dorsal columns (Kaczmarek and Jankowska, 2018).

It would be of great interest to establish whether the DC-evoked long-term changes in properties of myelinated nerve fibre are related to other forms of nonsynaptic axonal plasticity (Debanne, 2004, Debanne et al., 2011) including those in the respiratory system (Fuller and Mitchell, 2017) and in peripheral nerves (Ardolino et al., 2005, Ahmed, 2014b, Bolzoni et al., 2017) and which mechanisms might underlie this phenomenon. The delineation of mechanisms via which DC induces long-lasting post-polarization plastic changes in terminal branches of afferent nerve fibres but not in their parent axons might be aided by the similarities in interactions between effects of baclofen and 4-AP and effects of intraspinally applied DC. It might be particularly relevant that baclofen counteracted effects of intraspinally applied DC only under conditions when it was administered prior to, but not after DC, so that once the altered excitability had been established it was unaffected by baclofen (Fig. 6). The same was true for interactions between DC and 4-AP (Kaczmarek and Jankowska, 2018). Thus, both baclofen and 4-AP appear to prevent the induction of the sustained facilitatory effects of DC but not their expression. For the future identification of these mechanisms it might be also relevant that baclofen shortens the duration of action potentials in terminal branches of these afferents but not in the stem axons (Curtis et al., 1981, Curtis et al., 1997, Curtis and Lacey, 1998) and that actions of 4-AP are not restricted to voltage-dependent potassium channels (for references see e.g. Dunn and Blight, 2011).

Could DC be used to facilitate long-lasting effects of epidural stimulation together with antispastic medication with baclofen?

The results of the present study give a positive answer to this question, provided that DC is applied under conditions when its direct effects are restricted to afferent fibres traversing the dorsal columns, i.e. when DC is applied epidurally. As discussed previously, epidural stimulation at intensities tolerated by human subjects is effective primarily for fibres in the most external layers of the dorsal columns (see Holsheimer, 2002, Ramasubbu et al., 2013) because it is shunted by lower resistant tissue overlying the spinal cord and the cerebrospinal fluid. DC applied epidurally under the conditions of the present study would be expected to be shunted to a similar extent but the effects were potent enough to increase the excitability of epidurally stimulated dorsal column fibres, both during DC application and during a considerable post-polarization period (Jankowska et al., 2017, Kaczmarek and Jankowska, 2018). Nevertheless, even if effects of polarization of fibres in the dorsal columns are not counteracted by baclofen, interactions between effects of baclofen and the much stronger (2-3 mA) trans-spinal polarization, used both clinically

and experimentally, with a spread of current to deeper parts of the grey matter might be possible. Hence, facilitation of intraspinal excitability equivalent to that evoked by intraspinally applied local DC might be counteracted by baclofen (as in Fig. 2D, Fig. 5D). As baclofen practically eliminated post-polarization effects of intraspinally applied DC, it might restrict the time period of facilitatory effects of DC to the period coinciding with the tsDCS application. tsDCS would thus be unlikely to induce any facilitatory effects during the post-polarization period in patients under antispastic treatment with baclofen.

A long-lasting enhancement of synaptic actions would provide an additional opportunity of restoring the deficient spinal reflex functions by spacial and temporal facilitation of peripherally and epidurally evoked synaptic actions during extended sessions of rehabilitation. As discussed by Kaczmarek and Jankowska, 2018), enhancing synaptic actions of epidurally stimulated fibres in subjects with pathologically deficient reflex actions might greatly increase the probability of activation of motoneurones as well as of neuronal networks providing input to motoneurones. The long-term facilitation of synaptic actions of epidurally polarized fibres might thus be particularly beneficial for the enhancement of effects of epidural stimulation. As such facilitation occurs in the presence as well as in the absence of baclofen, the study leads to the conclusion that the advantages of combining epidural polarization with epidural stimulation would not be counteracted by pharmacological antispastic treatment with baclofen.

Figures and legends

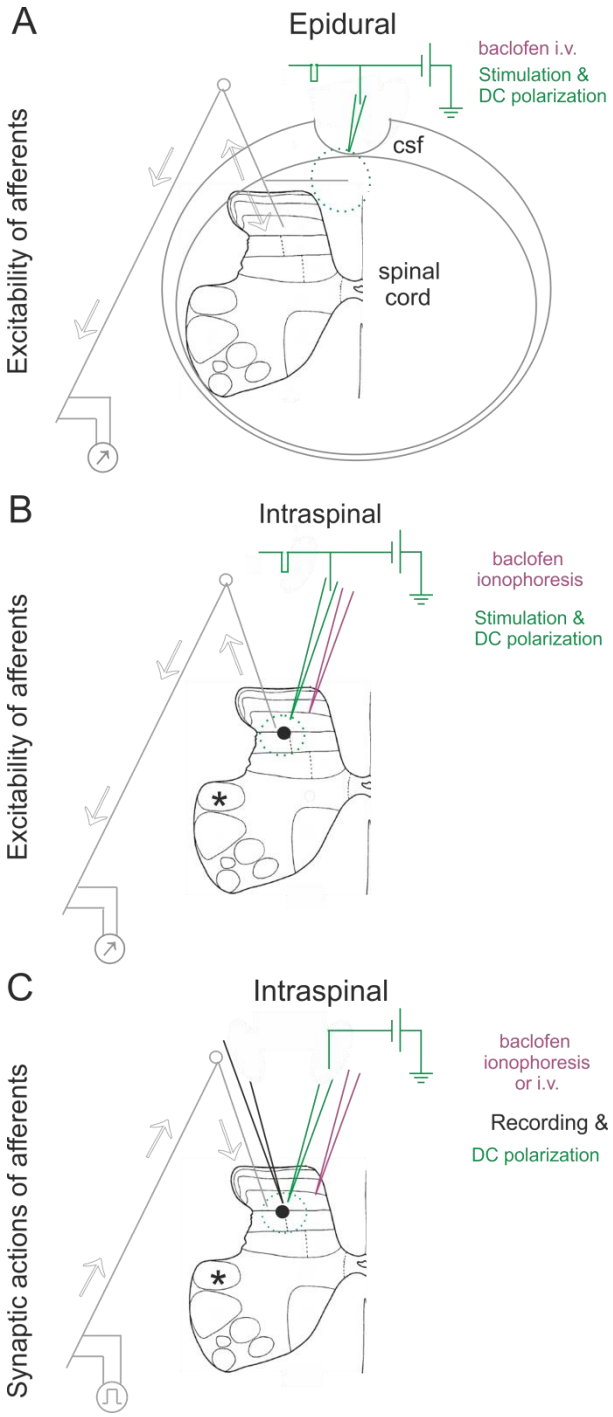

Figure 1. Experimental design.

A and **B,** Diagrams of the setup used to examine changes in the excitability of afferent fibres stimulated epidurally(**A**) and intraspinally (**B**) respectively. The same tungsten electrode was used to stimulate and to polarize the fibres. **C,** Diagram of the setup used to examine the effects of DC on postsynaptic potentials evoked by stimulation of a peripheral nerve. The potentials (extracellular field potentials) were recorded with a glass microelectrode while a tungsten electrode was used to deliver the polarizing current. Their tips were separated by 50-100 µm; for details see e.g. Bolzoni and Jankowska (2015). The dotted circles around the tips of the stimulating electrodes in **A** and **B** indicate the regions of the most potent effects of the stimulation. The intraspinal sites of stimulation in **B** and of recording in **C** are within the dorsal horn; those within the peroneal motor nucleus are indicated by asterisks. Nerve volleys were usually recorded simultaneously from the sural and the peroneal nerves. Drugs were applied intravenously or ionophoretically, from a micropipette inserted separately in the setups **B** and **C**.

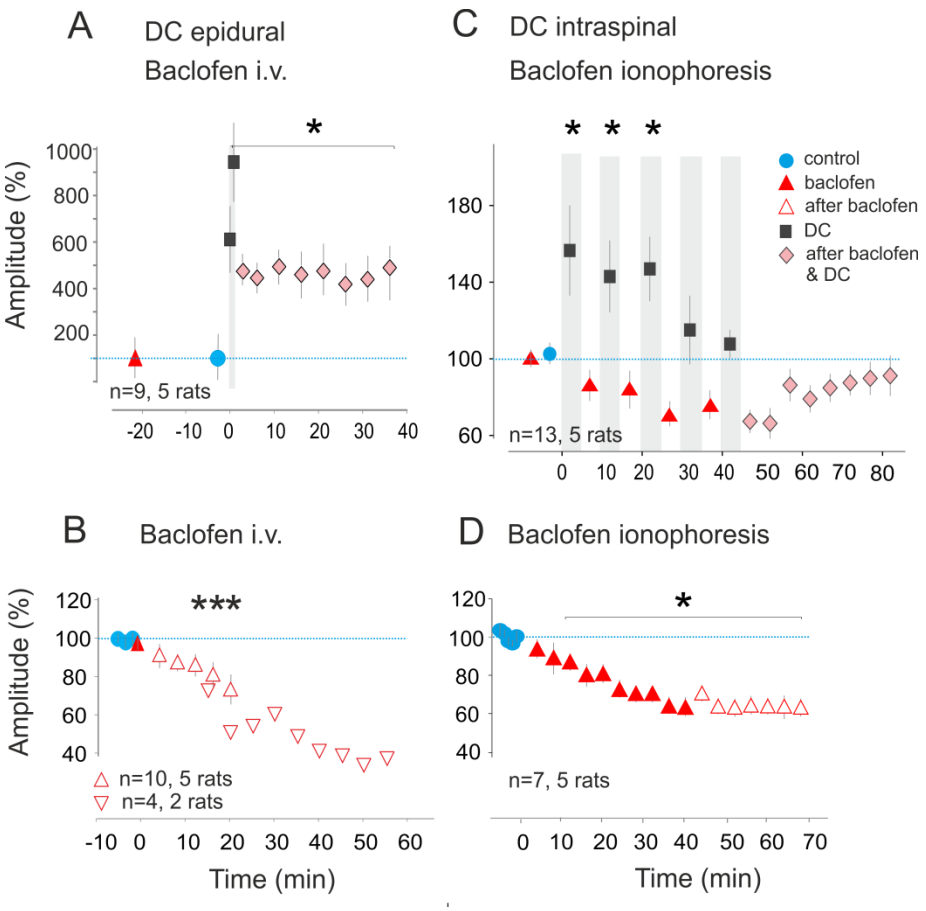

Figure 2. Interactions between effects of baclofen and cathodal DC on the excitability of afferent fibres. Plots of the areas of the early components of the nerve volleys (ordinate) evoked using the setups outlined in Fig. 1A and B. **A**, Changes in the areas of nerve volleys (in % control) plotted against time intervals from the onset of DC application (single period of 1 µA application; grey column). **B**, Effects of baclofen without DC application. The two symbols are for data points from two samples within different time periods. **C**, as in **A,** but after ionophoretic application of baclofen and effects of five periods of 0.3 µA DC applications; note different ordinate scales. **D**, as in B but for effects of ionophoreically applied baclofen. Data points represent mean areas with standard errors after having pooled together all data points during the indicated periods. Statistically significant differences (with respect to mean control values) are indicated by asterisks. **A** and **C,** Data normalized with respect to nerve volleys preceding DC application; main effect: $F_{(2.70,21.64)}$=9.1.13 P≤0.0.1 Dunnet (asterisk) always P≤0.05 and $F_{(3.48,41.80)}$=7.72 P≤0.0.1 Dunnet (asterisk) always P≤0.05, respectively) **B**, main effect: $F_{(5,45)}$=5.5430 P≤0.01, Dunnet (asterisk) always P≤0.05. Triangles in the reversed direction, data from a series of records continued for a

longer period, **D**, Data for changes evoked when baclofen alone was applied ionophoretically; main effect: $F_{(17,102)}$=14.997 P≤0.01, Dunnet (asterisk) always P≤0.05. Horizontal dotted lines indicate the control levels.

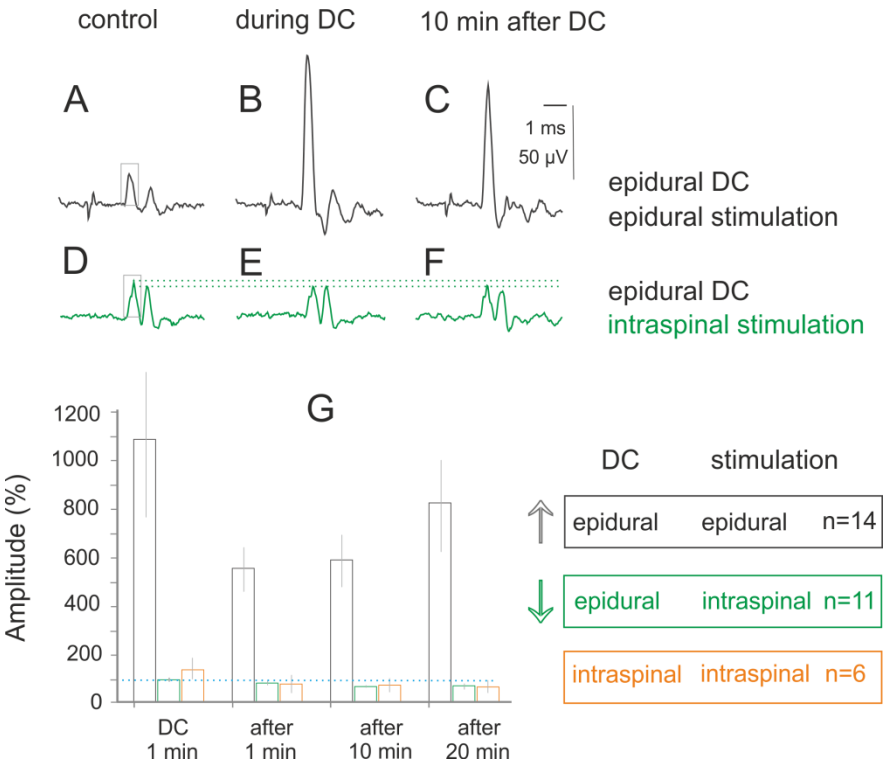

Figure 3. Comparison of effects of epidural DC on the excitability of epidurally and intraspinally stimulated nerve fibres.

A-C and **D-F**, examples of nerve volleys evoked by stimuli applied alternately epidurally (16 µA; as in Fig. 1A) and in the dorsal horn (3 µA; as in Fig. 1B) in the same experiment. Averages of 10 single records obtained before, during and after epidurally applied DC (1.1 µA for 1 min) in a preparation that was treated with baclofen. Boxed areas in **A** and **D**, time windows used for measurements. Dotted horizontal line, the control level for **D-F**. **G**, comparison of mean areas of the earliest components of nerve volleys in three samples during and following DC application. Pooled data for preparations that were (n=3) or were not (n=2) pretreated with baclofen systemically.

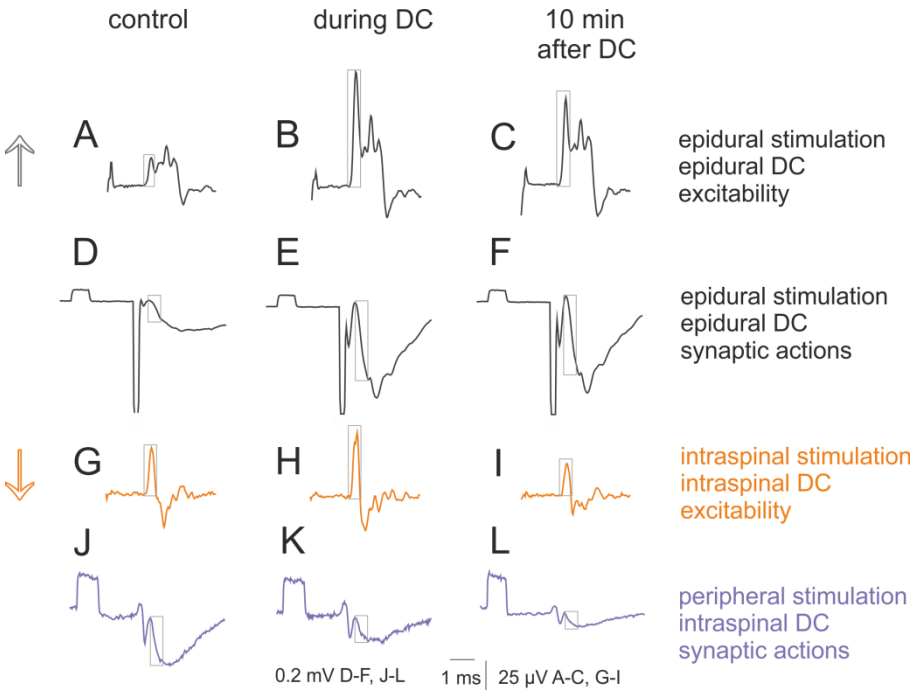

Figure 4. Changes evoked by combined application of baclofen i.v. and DC

A-C, nerve volleys in the peroneal nerve evoked by epidural stimulation (22 µA). **D-F**, field potentials evoked by epidural stimulation (12µA) the in the dorsal horn. **G-H**, nerve volleys in the peroneal nerve evoked by stimuli applied in the dorsal horn (3.5 µA). **J-L,** field potentials evoked by group I afferents (1.5T) in the peroneal motor nucleus. Rectangular calibration pulses (0.2 mV) are for records of field potentials. Left column, control records, 20 min after baclofen i.v. application (A, D) or before baclofen ionophoresis (G, J). Middle and right columns, records of responses evoked by the same stimuli but during the first minute of DC and 10 min after the termination of DC application, respectively. Note that during the post-polarization period, epidurally applied DC increased both nerve volleys and field potentials evoked by fibres stimulated in the dorsal column while intraspinally applied DC decreased them, as indicated by the arrows to the left of the records. Note also that field potentials in E and F were preceded by considerably increased presynaptic volleys while presynaptic volleys in K and L were reduced. Boxes indicate time windows within which the areas of the early components of the nerve volleys and of the field potentials were measured for the plots of the time course of the effects in Fig 2 and in Figs. 5-6.

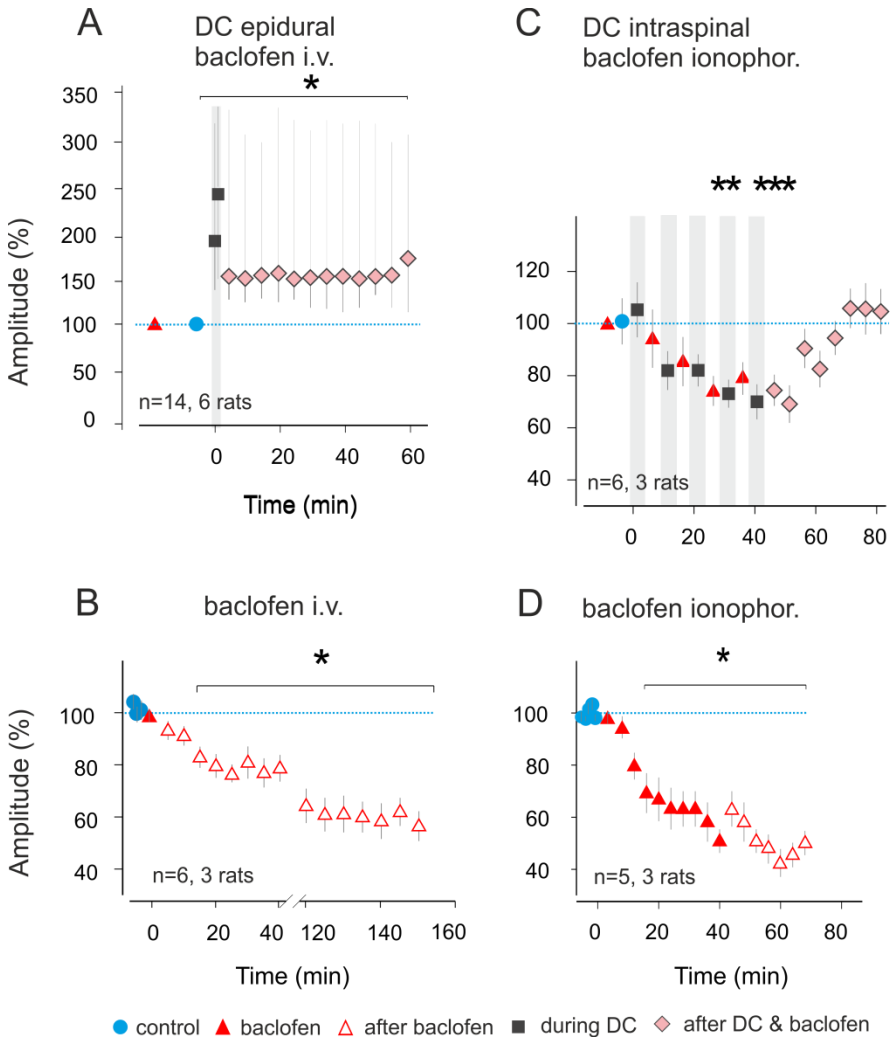

Figure 5. Interactions between effects of baclofen and cathodal DC on field potentials.

Left column, changes in field potentials, evoked by epidural stimulation using the setup outlined in Fig. 1A, following i.v. administration of baclofen. Right column, changes in field potentials, evoked by stimulation of peripheral nerves, using the setup outlined in Fig. 1C, following ionophoretic application of baclofen. **A**, Changes in the area of the early components of field potentials evoked by epidural stimulation illustrated in Fig. 4 D-F (boxed) in preparations in which DC was applied epidurally 20 min after administration of baclofen. Since the distribution of the data points was not compatible with a normal distribution, the points represent their median values and the error bars the 75th and 25th percentiles. **B**, Effects of baclofen i.v. on field

potentials evoked by epidural stimulation, as in A but without DC application. **C,** Mean areas and standard errors of the early components of field potentials illustrated in Fig. 4 J-L plotted against the time intervals from the onset of DC (grey columns) as in Fig. 2. **D**, as in **B** but for effects of ionophoretically applied baclofen. Note different ordinate scales. Note also that the data are for 2 ranges of intervals, 0-40 and 120-150 ms. Statistically significant differences (with respect to the control values) are indicated by asterisks. A and C, main effect:$\chi 214=56.29$ $P\leq 0.05$, Wilcoxon signed-rank test, Bonferroni corrected (asterisk) always $P\leq 0.05$ and $F_{(17.85)}=3.672$ $P\leq 0.01$, Dunnet (asterisk) always $P\leq 0.05$) **B** and **D,** main effects: $F_{(35.185)}=9.942$ $P\leq 0.01$, Dunnet (asterisk) always $P\leq 0.05$ and $F_{(17,68)}=12.538$ $P\leq 0.01$, Dunnet (asterisk) always $P\leq 0.05$.

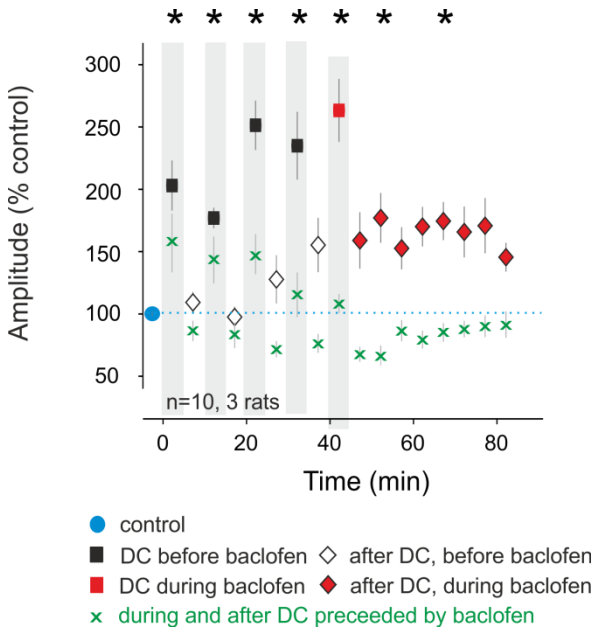

Fig 6. Interactions between effects of baclofen and DC on the excitability of intraspinally stimulated and polarized afferent fibres when the order of application of baclofen and DC was reversed.

Changes in the areas of nerve volleys evoked by intraspinal stimuli plotted against time intervals from the onset of the first period of the cathodal DC application through the same electrode (0.3 µA, grey columns). Baclofen was ionophoresed during the last polarization period and over the whole period of testing (for 45 min). Note increases in the volleys both during and following the later periods of DC. Changes indicated by * were statistically significant. Main effect: $F_{(3.82,34.34)}=7.69$ $P\leq0.01$ Dunnet (asterisk) always $P\leq0.05$. Crosses, changes in the areas of nerve volleys in the series of records plotted in Fig 2C illustrating the post-polarization decrease in the excitability of intraspinally stimulated fibres when the administration of baclofen preceded the intraspinal application of DC.

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