Electrophysiological evidence showing muscarinic agonist-antagonist activities of *N*-desmethylclozapine using hippocampal excitatory and inhibitory neurons

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Abbreviations

oxo-M, oxotremorine M

NDMC, *N*-desmethylclozapine

PLC, phospholipase C

NMDA, N-methyl-D-aspartate

EPSC, excitatory postsynaptic current

IPSC, inhibitory postsynaptic current

TTX, tetrodotoxin

Abstract

The atypical antipsychotic clozapine is widely used for treatment-resistant schizophrenic patients. Clozapine and its major active metabolite, N-desmethylclozapine (NDMC), have complex pharmacological properties, and interact with various neurotransmitter receptors. There are several biochemical studies reporting that NDMC exhibits a partial agonist profile at the human recombinant M_1 muscarinic receptors. However, direct electrophysiological evidence showing the ability of NDMC to activate native M_1 receptors in intact neurons is poor. Using rat hippocampal neurons, we previously demonstrated that activation of muscarinic receptors by a muscarinic agonist, oxotremorine M (oxo-M), induces a decrease in outward K^+ current at -40 mV. In the present study, using this muscarinic current response we assessed agonist and antagonist activities of clozapine and NDMC at native muscarinic receptors in intact hippocampal excitatory and inhibitory neurons. Suppression of the oxo-M-induced current response by the M_1 antagonist pirenzepine was evident only in excitatory neurons, while the M_3 antagonist darifenacin was effective in both types of neurons. Muscarinic agonist activity of NDMC was higher than that of clozapine, higher in excitatory neurons than in inhibitory neurons, sensitive to pirenzepine, and partially masked when co-applied with clozapine. Muscarinic antagonist activity of clozapine as well as NDMC was not different between excitatory and inhibitory neurons, but clozapine was more effective than NDMC. These results demonstrate that NDMC has the ability to activate native M_1 receptors expressed in hippocampal excitatory neurons, but its agonist activity might be limited in clozapine-treated patients because of the presence of excessive clozapine with muscarinic

antagonist activity.

Key Words:

N-desmethylclozapine, clozapine, muscarinic agonist, muscarinic antagonist, hippocampus, K^+ channel

1. Introduction

Clozapine is a unique antipsychotic drug, and currently classified as first-line treatment in treatment-resistant schizophrenia (Buchanan et al., 2010; Dold and Leucht, 2014; Hasan et al., 2012; Leucht et al., 2003). Clozapine improves positive and negative symptoms, while inducing minimal extrapyramidal side effects (Divac et al., 2014). Clozapine and its predominant active metabolite, *N*-desmethylclozapine (NDMC), have complex pharmacological profiles, and interact with a wide range of neurotransmitter receptors including dopamine, serotonin, norepinephrine, histamine and acetylcholine receptors (Correll, 2010; Lameh et al., 2007; Mendoza and Lindenmayer, 2009; Weiner et al., 2004). Although the antipsychotic efficacy of clozapine is thought to be based primarily on its interaction with dopamine and serotonin receptors, the interactions of clozapine and NDMC with muscarinic receptors could also contribute to the therapeutic efficacy (Lameh et al., 2007; Maehara et al., 2011a; Maehara et al., 2011b). Among the muscarinic receptor subtypes (M_1-M_5), much attention has been given to M_1 receptors, because it is implicated in learning and memory processes (Anagnostaras et al., 2003; Shirey et al., 2009). Biochemical studies demonstrated that NDMC exhibits a partial agonist profile at

human recombinant M_1 receptors (Davies et al., 2005), which seems to be unique and not shared by any other antipsychotics. However, direct electrophysiological evidence showing the ability of NDMC to activate native M_1 receptors in intact neurons is poor.

We previously reported that in rat cultured hippocampal neurons the activation of muscarinic receptors by applying the muscarinic agonist oxotremorine M (oxo-M) decreased the steady-state outward current at -40 mV, which was attributed to inhibition of K^+ channels sensitive to the M-current blocker XE991 (Sugawara et al., 2013). This muscarinic current response was inhibited by the phospholipase C (PLC) inhibitor U73122, but not its inactive analogue U73343, indicating the involvement of PLC-coupled types of muscarinic receptors including M_1 and M_3 subtypes (Sugawara et al., 2013). In the present study, using this muscarinic current response we assessed muscarinic agonist and antagonist activities of clozapine and NDMC in intact hippocampal excitatory and inhibitory neurons.

2. Results

2.1 Effects of the muscarinic agonist oxo-M

In cultured hippocampal neurons, we previously demonstrated that the activation of muscarinic receptors by the muscarinic agonist oxo-M decreases the steady-state outward K^+ current at -40 mV. To examine whether there are cell-type differences in this coupling of muscarinic receptors to K^+ channels, effects of oxo-M application on the outward current were compared between excitatory and inhibitory neurons. The steady-state outward current before the oxo-M application varied from neuron to neuron. On average,

it was larger in inhibitory neurons (209.8 ± 10.5 pA, n = 123) than in excitatory neurons (132.9 ± 5.8 pA, n = 84). Bath application of 3 μM oxo-M markedly decreased the outward current in both types of neurons (Fig. 1A). The percentage reduction of the outward current by oxo-M was slightly larger in excitatory neurons than in inhibitory neurons, but the difference was not significant (Fig. 1B, $P = 0.068$, unpaired t test). These results indicate that both types of neurons express muscarinic receptors that negatively regulate the K^+ channels active at -40 mV.

2.2 Effects of subtype-selective muscarinic antagonists

Using relatively selective antagonists for M_1 (pirenzepine) and M_3 (darifenacin) subtypes of muscarinic receptors, contributions of M_1 and M_3 subtypes to the oxo-M-induced current responses were examined. The M_1 antagonist pirenzepine (0.1 μM) partially suppressed the current response in excitatory neurons (Fig. 2A, 2C), but not in inhibitory neurons (Fig. 2B, 2C). The difference between excitatory and inhibitory neurons was significant ($P < 0.05$, unpaired t test). On the other hand, the M_3 antagonist darifenacin (1 μM) suppressed the current response strongly in both excitatory and inhibitory neurons (Fig. 3). The antagonistic effect of darifenacin was larger in inhibitory neurons than in excitatory neurons (Fig. 3C), but the difference was not significant ($P = 0.071$, unpaired t test). These results indicate that relative contributions of M_1 and M_3 receptors to the channel modulation are different between excitatory and inhibitory neurons, and that M_1 receptors contribute to the channel modulation selectively in excitatory neurons.

2.3 Agonist activities of clozapine and NDMC at muscarinic receptors

To assess the agonist activity at muscarinic receptors, effects of clozapine (Fig. 4A) and NDMC (Fig. 4B) on the holding current at -40 mV were examined in excitatory (left) and inhibitory neurons (right) separately. To mimic intracerebral conditions of clozapine-treated patients, we also examined effects of co-application of NDMC and clozapine (Fig. 4C). If they function as muscarinic agonists, they are expected to decrease the outward current. A two-way ANOVA (cell type × drug) revealed a significant interaction effect of cell type and drug ($F = 6.68$, $P < 0.01$). Bath-applied clozapine (3 μM) induced no or only a small effect in both types of neurons (Fig. 4A, 4D). Application of 3 μM NDMC greatly decreased the outward current in excitatory neurons, but not in inhibitory neurons (Fig. 4B). The NMDA-induced current response was significantly larger in excitatory neurons than in inhibitory ones (Fig. 4D, $P < 0.01$). In excitatory neurons, NDMC alone was more effective than either clozapine alone ($P < 0.05$) or the combination of clozapine and NDMC ($P < 0.05$) (Fig. 4D). The effect of NDMC in excitatory neurons was significantly suppressed by 0.1 μM pirenzepine ($P < 0.05$) and 1 μM atropine ($P < 0.001$) (Fig. 5). These results indicate that NDMC has an agonist activity at native M_1 receptors of hippocampal excitatory neurons.

2.4 Antagonist activities of clozapine and NDMC on muscarinic receptors

To assess the antagonist activity at muscarinic receptors, effects of clozapine and NDMC, alone or in combination, on the holding current at -40 mV were examined after application of oxo-M. If the drug has an antagonist activity, the suppression of the

outward current by oxo-M is expected to be reversed upon its application. When clozapine or NDMC, or both, was applied in the presence of oxo-M, the outward current was markedly reversed in both excitatory and inhibitory neurons (Fig. 6). A two-way ANOVA (cell type × drug) showed no significant interaction effect of cell type and drug ($F = 0.721$, $P = 0.491$). There was a significant main effect of drug ($F = 5.02$, $P < 0.05$), but not cell type ($F = 1.04$, $P = 0.314$). The antagonist activity of clozapine was significantly larger than that of NDMC (Fig. 6D, $P < 0.01$).

3. Discussions

The present study is the first attempt to examine agonist and antagonist activities of clozapine and NDMC at PLC-coupled types of muscarinic receptors including M_1 and M_3 subtypes by monitoring muscarinic current responses in intact hippocampal excitatory and inhibitory neurons separately. Our data confirmed that clozapine acts primarily as an antagonist, whereas NDMC acts as a mixed agonist-antagonist. The muscarinic agonist activity of NDMC was found to be higher in excitatory neurons than in inhibitory neurons, and sensitive to pirenzepine. We also confirmed cell-type differences in the effects of pirenzepine and darifenacin on oxo-M-induced current responses, indicating that the contribution of M_1 receptors to the muscarinic responses is evident only in excitatory neurons. These results strongly suggest that the agonist activity of NDMC depends on the M_1, rather than M_3, subtype, in agreement with some biochemical studies reporting that NDMC exhibits a partial agonist profile at M_1 receptors (Davies et al., 2005; Gregory et al., 2007; Weiner et al., 2004).

Our electrophysiological data suggested that M_3 receptors mediate the muscarinic suppression of potassium channels in both excitatory and inhibitory hippocampal neurons, whereas M_1 receptors contribute to the muscarinic channel modulation only in the excitatory neurons. These data are consistent with previous anatomical and electrophysiological studies on hippocampal M_1 and M_3 receptors. Yamasaki et al. examined cellular and subcellular distribution of M_1 receptors in the cerebral cortex and hippocampus, and demonstrated that M_1 receptors are expressed preferentially in glutamatergic pyramidal neurons, and scarce or undetectable in various GABAergic interneuron subtypes (Yamasaki et al., 2010). Instead of M_1 receptors, M_3 receptors were detectable in interneurons (Yamasaki et al., 2010). Using mice genetically lacking specific muscarinic receptor subtypes, receptor subtypes responsible for cholinergic modulation of ion channels have been identified in hippocampal pyramidal neurons (Dasari and Gulledge, 2011; Rouse et al., 2000). Rouse et al. reported that the muscarinic inhibition of K^+ channels remained intact in the pyramidal neurons prepared from M_1-knockout mice (Rouse et al., 2000). Dasari and Gulledge reported that carbachol-induced depolarization was reduced in neurons from either M_1-knockout or M_3-knockout mice, and was eliminated in M_1/M_3 double-knockout mice (Dasari and Gulledge, 2011). These results are compatible with the idea that both M_1 and M_3 receptors are functional in pyramidal neurons.

There has been some dispute as to whether NDMC has M_1 agonist activity at native rat M_1 receptors. Using rat cerebral cortical and hippocampal membranes, M_1 muscarinic receptor-mediated G_q activation was pharmacologically characterized. The

study failed to detect the M_1 agonist activity of NDMC (Odagaki et al., 2013). By contrast, an electrophysiological study clearly demonstrated that NDMC has an M_1 agonist activity in intact hippocampal neurons (Sur et al., 2003). Using CA1 pyramidal neurons, Sur et al. demonstrated that bath application of NDMC potentiated the current response evoked by fast application (0.5-1.5 s) of N-methyl-D-aspartate (NMDA). Considering these results together with previous reports of M_1-mediated potentiation of NMDA receptors together, the authors concluded that NDMC has an M_1 agonist activity, in agreement with the present study. In the study by Sur et al, however, electrophysiological experiments were carried out to examine only an M_1 agonist action of NDMC, using only pyramidal cells. Therefore, comparisons between pyramidal cells and interneurons, between NDMC and clozapine, and between agonist and antagonist actions were not performed. In the present study, we used both excitatory and inhibitory neurons, and examined both agonist and antagonist activities of NDMC and clozapine at muscarinic receptors. Our experimental data highlight that NDMC, but not clozapine, has the potential to depolarize excitatory neurons selectively by inhibiting potassium channels.

We observed that the M_1 agonist action of NDMC was largely masked when the same concentration of clozapine was co-applied. In clozapine-treated patients, the median plasma clozapine/NDMC concentration ratio has been reported to be 1.25 and 2.08 at plasma clozapine concentrations < 0.35 mg/L and > 1.0 mg/L, respectively (Couchman et al., 2010). Experimental studies in laboratory animals demonstrated that the clozapine/NDMC ratio in the brain is higher than that in the plasma (Gershkovich et al., 2010; Weigmann et al., 1999). Accordingly, the activation of M_1 receptors by NDMC in

the brain seems to be limited in most clozapine-treated patients, because of the presence of excessive clozapine. In some patients with a low clozapine/NDMC ratio, however, it is still possible that M_1 receptors are partially activated by NDMC. In line with this possibility, a recent study in clozapine-treated patients demonstrated that the clozapine/NDMC ratio, but not individual clozapine and NDMC concentrations, was significantly and negatively associated with working memory performance after controlling for age, gender, education, and symptom severity (Rajji et al., 2015).

4. Conclusions

In the present study, we assessed agonist and antagonist activities of clozapine and its major active metabolite, NDMC, at native muscarinic receptors in rat intact hippocampal excitatory and inhibitory neurons, by monitoring muscarinic current responses. Our data clearly demonstrated that clozapine acts primarily as an antagonist, whereas NDMC acts as a mixed agonist-antagonist. The agonist activity of NDMC, which was explained by its action on the M_1, rather than M_3, subtype, was largely masked when the same concentration of clozapine was co-applied. These results suggest that the clozapine/NDMC concentration ratio in clozapine-treated patients might determine the degree of M_1 receptor activation by NDMC.

5. Experimental Procedure

5.1 Preparation of neurons

All experiments were performed in accordance with the guidelines set by the animal

welfare committee of Kanazawa University. Hippocampal neurons were prepared from newborn Sprague-Dawley rats, as described previously (Ohno-Shosaku et al., 2001). Briefly, following isoflurane anesthesia, rats were decapitated and cells were mechanically dissociated from the hippocampi and plated onto culture dishes (35 mm) pretreated with poly L-ornithine (0.01 %). The cultures were kept at 36 °C in 5 % CO_2 for 13-19 days before use.

5.2 Electrophysiology

All experiments were performed at room temperature. Neurons were whole-cell voltage clamped using patch pipettes (3 –5 MΩ) filled with an internal solution containing (in mM) 100 K-gluconate, 15 KCl, 10 HEPES, 10 EGTA, 6 $MgCl_2$, 4.65 $CaCl_2$, 40 KOH, 5 Na_2ATP and 0.2 Na_2GTP (pCa 7, pH 7.3 adjusted with KOH). The whole-cell current was measured with a patch-clamp amplifier (EPC9/3, HEKA Electronics, Germany). A standard external solution contained (in mM) 140 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose (pH 7.3 adjusted with NaOH).

The hippocampus contains excitatory (glutamatergic) and inhibitory (GABAergic) neurons. The cell type was identified electrophysiologically in double whole-cell recordings, where two neurons were whole-cell voltage clamped at -80 mV. One neuron was stimulated by applying positive voltage pulses (to 0 mV, 2 ms), and the evoked excitatory or inhibitory postsynaptic currents (EPSCs or IPSCs) were recorded from the other neuron. IPSCs and EPSCs are easily distinguished by their decay time constants, reversal potentials and sensitivities to $GABA_A$ and AMPA receptor antagonists

(Ohno-Shosaku et al., 2011). When the postsynaptic currents were EPSCs or IPSCs, the presynaptic neuron was identified as inhibitory or excitatory, respectively.

The steady-state outward current was measured at the holding potential of -40 mV. To suppress the generation of action potentials and spontaneous synaptic currents, 0.1 μM tetrodotoxin (TTX) was added to the standard external solution. The holding current was recorded for 100 ms (sampling rate, 1 kHz) once every second, except for the experiments shown in Fig. 2 and 3, where the current was recorded every 200 ms. In figures, the averaged current values of each sweep are plotted as a function of time. The recording chamber was perfused with an external solution with or without drugs at a flow rate of 1-3 ml/min.

To assess agonist and antagonist activities at muscarinic receptors, the steady-state outward current at -40 mV was monitored. For agonist activity, the amplitude of the outward current before agonist application was taken as 100 % and the percentage reduction of the current was calculated. For antagonist activity, the amplitude of the current reduction induced by application of the muscarinic agonist oxo-M was taken as 100 % and the percentage recovery of the current around 60 s after the application of the drug with antagonist activity was calculated.

In some experiments (Fig. 2 and 3), the external solution containing oxo-M was locally applied for 5 s through a capillary tube (250 μm inner diameter) using a perfusion valve controller (VC-6M, Warner Instrument, CT). The applied agonist was rapidly washed out by continuous bath perfusion. When neurons were exposed to the antagonist (pirenzepine or darifenacin), it was added to both the bath solution and the local

application solution containing oxo-M. To calculate the antagonistic effect of these drugs, oxo-M was applied three times at intervals longer than 1 min in each neuron. In experiments with pirenzepine, the amplitude of oxo-M-induced current response was measured before (R1) and during the treatment with the drug (R2), and after its washout (R3). The antagonistic effect (E) was calculated by the following equation: $E=1-2\times R2/(R1+R3)$. Data were discarded when R3 is less than 50% of R1. In experiments with darifenacin, the amplitude of oxo-M-induced current response was measured twice before the treatment (R1 and R2) and once during the treatment with the drug (R3). The antagonistic effect (E) was calculated by the following equation: $E=1-R3/R2$. Data were discarded when R2 is less than 70% of R1.

In all experiments, recordings were discarded when series resistance was > 20 MΩ at the beginning or > 25 MΩ at the end of experiments, when the holding current at -40 mV was < 40 pA at the beginning of experiments or < 0 pA during oxo-M application, and when the amplitude of current response to oxo-M application was < 30 pA.

5.3 Statistics

All data are expressed as mean ± S.E.M., with numbers of neurons given in parentheses. Statistical significance was evaluated by Student's unpaired t test and two-way ANOVA for 2 factors followed by post hoc unpaired t test with the use of the Bonferroni method. The differences with $P < 0.05$ were taken as significant. Single, double, and triple asterisks in figures indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

5.4 Drugs

Oxo-M, pirenzepine, clozapine and NDMC were purchased from Tocris Cookson (UK), TTX and atropine were from Nacalai (Japan), and darifenacin was from Santa Cruz (USA). Darifenacin, clozapine and NDMC were dissolved in DMSO as stock solutions.

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Figure legends

Figure 1

A comparison of current responses to the muscarinic agonist oxo-M between excitatory and inhibitory neurons. A: Representative current responses induced by bath application of 3 μM oxo-M in excitatory (left) and inhibitory (right) neurons. B: Mean data for the reduction of the outward current by oxo-M in excitatory (left) and inhibitory neurons (right).

Figure 2

The antagonistic effect of pirenzepine on oxo-M-induced current responses in excitatory and inhibitory neurons. A, B: Representative examples of the experimental records from excitatory (A) and inhibitory (B) neurons. In each neuron, local application of oxo-M (3 μM) was repeated in the absence and presence of pirenzepine (0.1 μM). C: Mean data for the suppression of the oxo-M-induced current response by pirenzepine in excitatory (left) and inhibitory neurons (right).

Figure 3

The antagonistic effect of darifenacin on oxo-M-induced current responses in excitatory and inhibitory neurons. A, B: Representative examples of the experimental records from excitatory (A) and inhibitory (B) neurons. Local application of oxo-M (3 μM) was repeated in the absence and presence of darifenacin (1 μM). C: Mean data for the suppression of the oxo-M-induced current response by darifenacin in excitatory (left) and

inhibitory neurons (right).

Figure 4

Abilities of clozapine and NDMC to decrease the outward current in excitatory and inhibitory neurons. A-C: Representative current responses induced by bath application of 3 μM clozapine (A), 3 μM NDMC (B), and the mixture of 3 μM clozapine and 3 μM NDMC (C) in excitatory (left) and inhibitory (right) neurons. D: Mean data for the reduction of the outward current by clozapine (left), NDMC (middle) and the mixture (right) in excitatory (white bars) and inhibitory neurons (gray bars).

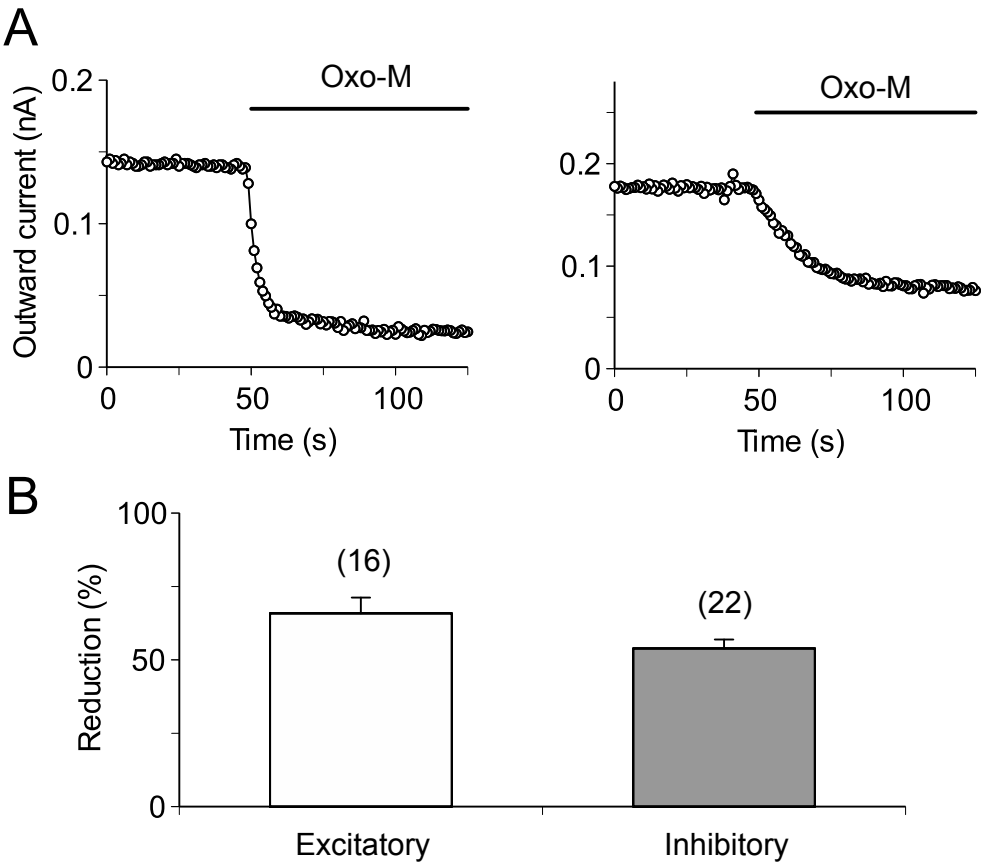
Figure 5

Antagonistic effects of pirenzepine and atropine on NDMC-induced current responses in excitatory neurons. A: Representative current responses induced by bath application of 3 μM NDMC in the absence (left) or presence of 0.1 μM pirenzepine (middle) or 1 μM atropine (right). B: Mean data for the reduction of the outward current by NDMC with or without pirenzepine or atropine.

Figure 6

Abilities of clozapine and NDMC to recover the outward current from the oxo-M-induced suppression in excitatory and inhibitory neurons. A-C: Representative current responses induced by bath application of 3 μM clozapine (A), 3 μM NDMC (B), and the mixture of 3 μM clozapine and 3 μM NDMC (C) in the presence of 3 μM oxo-M in excitatory (left)

and inhibitory (right) neurons. D: Mean data for the percentage recovery of the current from the oxo-M-induced suppression by clozapine (left), NDMC (middle) and the mixture (right) in excitatory (white bars) and inhibitory neurons (gray bars).



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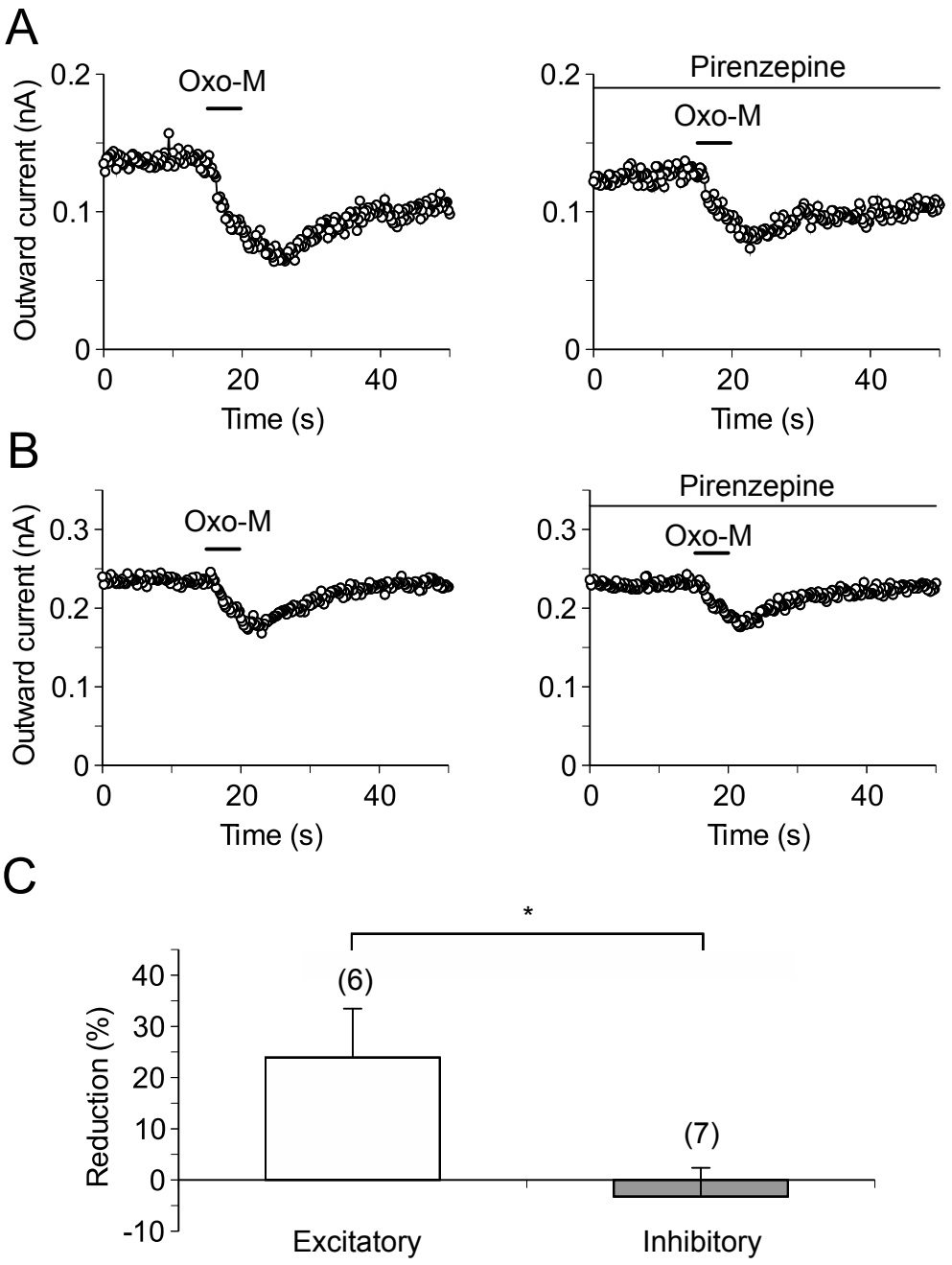

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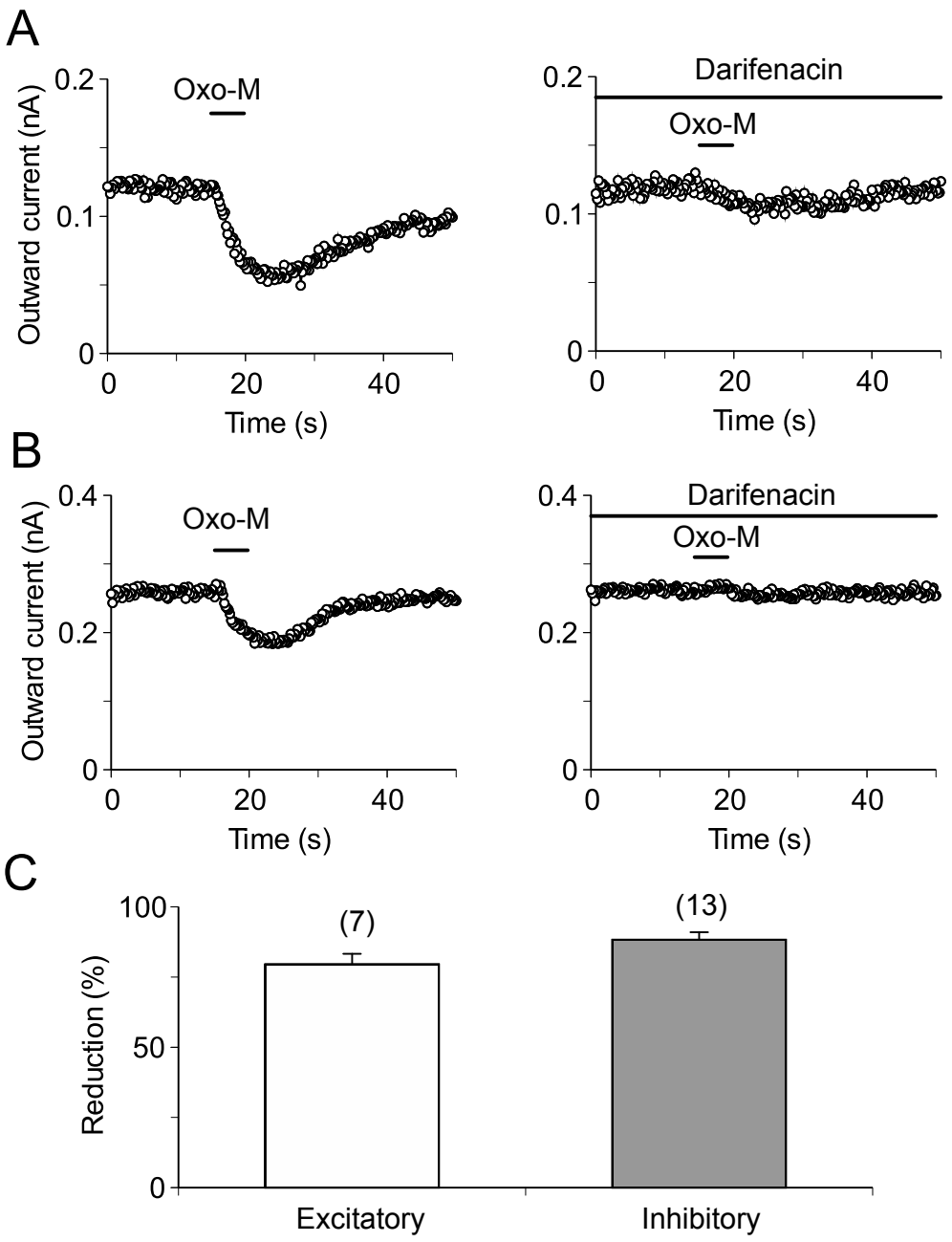

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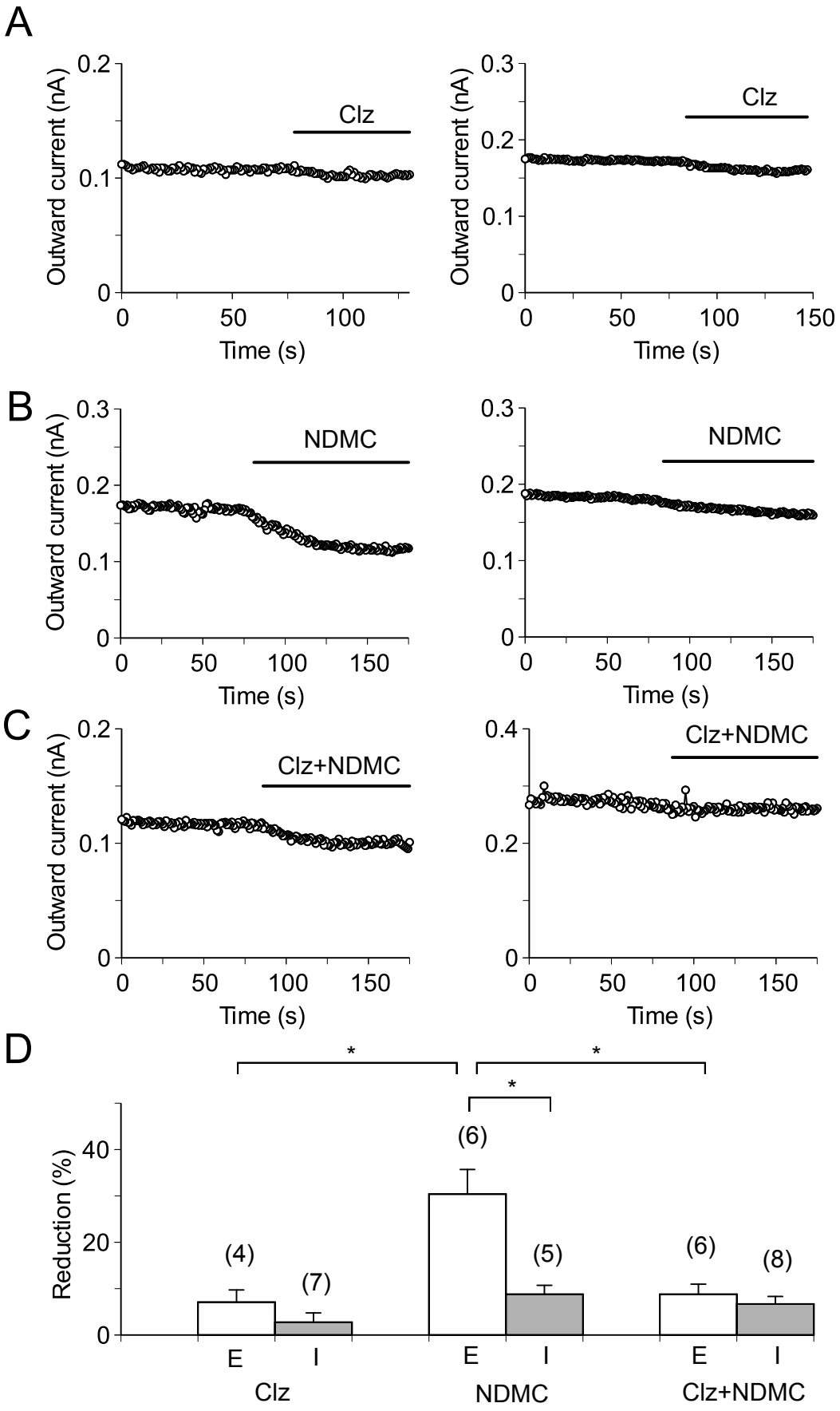

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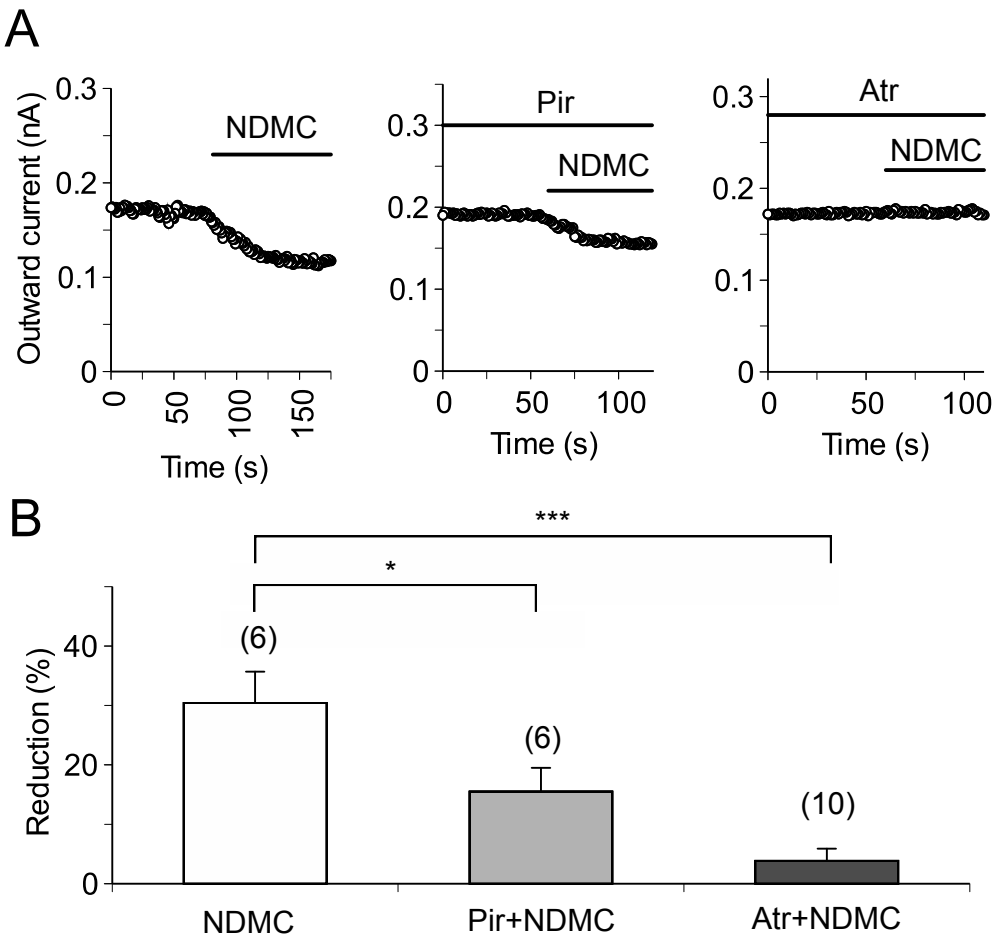

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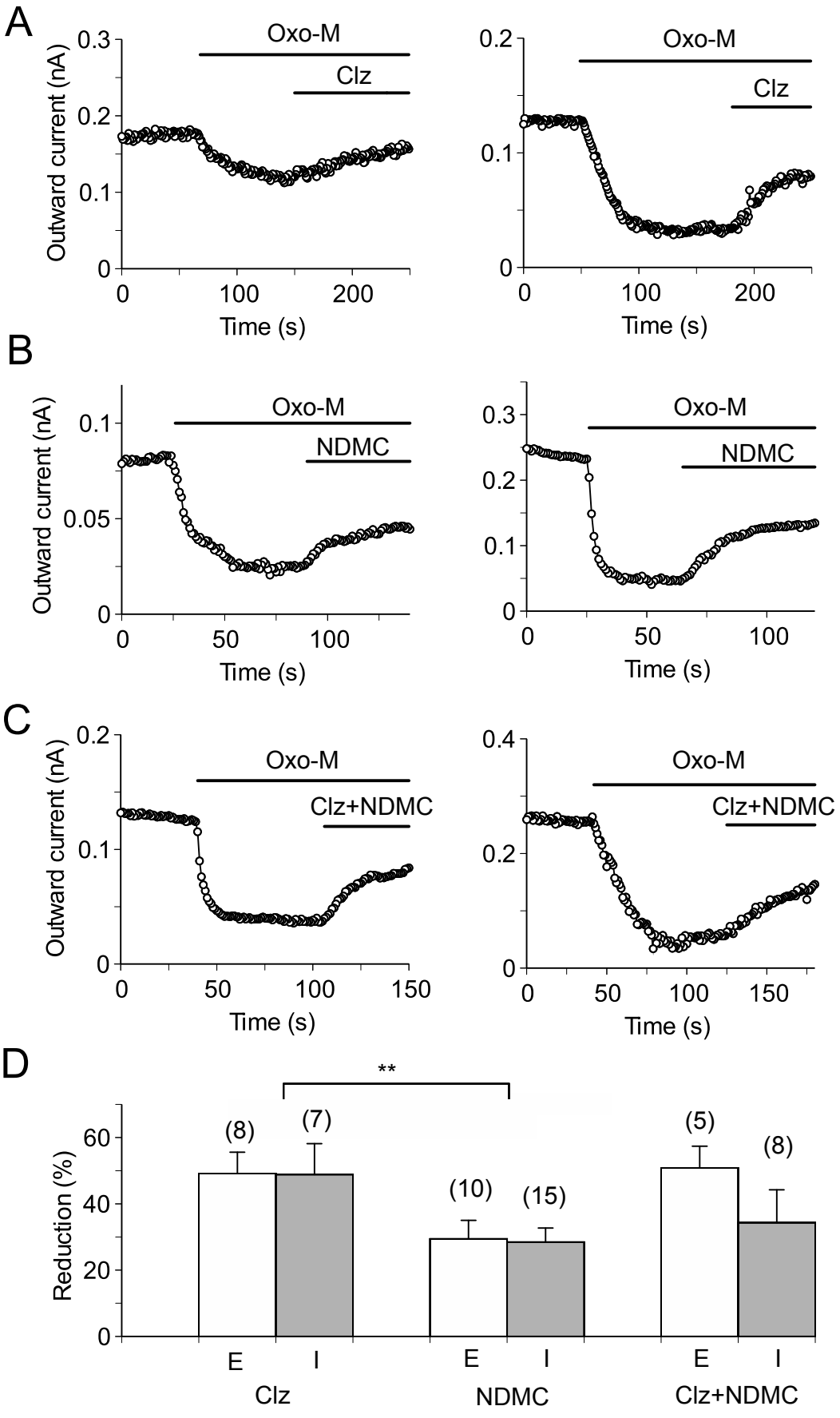

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