Descending inhibitory reflexes involve P2X receptor-mediated transmission from interneurons to motor neurons in guinea-pig ileum

Author:
Bian, X.-C.; Bertrand, Paul P.; Bornstein, J. C.

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DESCENDING INHIBITORY REFLEXES INVOLVE P2X MEDIATED TRANSMISSION FROM INTERNEURONS TO MOTOR NEURONS IN GUINEA-PIG ILEUM

Running title: P2X mediated transmission in guinea-pig ileum

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Xiaochun Bian, Paul P. Bertrand and Joel C. Bornstein
Department of Physiology University of Melbourne, Parkville VIC 3010, Australia

Address for correspondence:
Dr X.-C. Bian
Department of Physiology
University of Melbourne
Parkville, VIC 3010
Australia
Ph 61-3-8344-4466
Fax 61-3-8344-5818
Email: x.bian@physiology.unimelb.edu.au

SUMMARY

1. The role of P2X receptors in descending inhibitory reflexes evoked by distension or mucosal distortion in the guinea-pig ileum was studied using intracellular recording from the circular muscle in a two chambered organ bath. This allowed separate superfusion of the sites of reflex stimulation and recording, thereby allowing drugs to be selectively applied to different parts of the reflex pathway.

2. Inhibitory junction potentials (IJPs) evoked by electrical field stimulation (EFS) in the recording chamber were compared with those evoked during reflexes to control for effects of P2 receptor antagonists on neuromuscular transmission.

3. The P2 receptor antagonists, suramin (100 μM) and pyridoxal-phosphate-6-azophenyl-2'4'-disulphonic acid (10 and 60 μM; PPADS), when added to the recording chamber, depressed reflexly evoked IJPs significantly more than those evoked by EFS. In particular, 10 μM PPADS depressed IJPs evoked by distension or mucosal distortion by about 50%, but had little effect on IJPs evoked by EFS.

4. Blockade of synaptic transmission in the stimulation chamber with a low Ca^{2+}, high Mg^{2+} solution depressed, but did not abolish, IJPs evoked by distension. The residual reflex IJPs were unaffected by PPADS (10 μM), hyoscine (1 μM), hyoscine plus hexamethonium (200 μM), or hysocine plus hexamethonium plus PPADS in the recording chamber.

5. We conclude that P2X receptors are important for synaptic transmission from descending interneurons to inhibitory motor neurons in descending inhibitory reflex pathways of guinea-pig ileum. Transmission from anally directed axons of distension-sensitive intrinsic sensory neurons to inhibitory motor neurons is unlikely to involve P2X, muscarinic or nicotinic receptors.

INTRODUCTION

Studies of the mechanisms underlying intestinal motility date back over a century, but the roles of the different reflexes and motor patterns that have been identified either *in vivo* or *in vitro* remain poorly understood. The “Law of the Intestine”, that a physiological stimulus in the isolated intestine triggers excitation of the circular muscle on the oral side (ascending excitation) and inhibition on the anal side (descending inhibition), was formulated by Bayliss and Starling at the turn of the last century (Bayliss & Starling, 1899; Bayliss & Starling, 1900). In the guinea-pig ileum, both ascending excitation and descending inhibition have been identified repeatedly in electrophysiological studies of reflexes evoked by distension and mechanical or chemical stimulation of the mucosa (Hirst *et al.*, 1975; Smith & Furness, 1988; Smith *et al.*, 1990; Smith *et al.*, 1991; Yuan *et al.*, 1991; Yuan *et al.*, 1992; Furness *et al.*, 1995; Johnson *et al.*, 1996; Johnson *et al.*, 1998). Similar stimuli also initiate a descending contraction in the guinea-pig ileum (Hirst *et al.*, 1975; Brookes *et al.*, 1999; Spencer *et al.*, 1999; Spencer *et al.*, 2000), although this has not been seen in most electrophysiological studies.

The nature of synaptic transmission within the ascending reflex pathway has now been well characterised as a result of studies using multichambered organ baths in which different parts of the pathway are separately superfused (Tonini & Costa, 1990; Johnson *et al.*, 1996; Johnson *et al.*, 1998). These studies indicate that synaptic transmission along the pathway, i.e. between interneurons and between interneurons and excitatory motor neurons, is predominantly via acetylcholine (ACh) acting at nicotinic receptors. Transmission from the intrinsic sensory neurons to ascending interneurons depends on ACh acting at nicotinic and muscarinic receptors and on tachykinins acting at NK_3 receptors. By contrast, the nature of transmission

in the descending inhibitory reflex pathway is much less well understood. Although minor roles can be identified for ACh acting at nicotinic receptors and for tachykinins at NK_1 and NK_3 receptors (Johnson *et al.*, 1996; Johnson *et al.*, 1998), descending inhibitory reflex responses evoked by either distension or mucosal stimulation are largely unaffected by blockade of these receptors (Smith *et al.*, 1990; Smith *et al.*, 1991; Johnson *et al.*, 1996; Johnson *et al.*, 1998). These results strongly suggest that a transmitter other than acetylcholine or a tachykinin is important for transmisison between neurons of the descending inhibitory reflex pathway.

There is strong evidence that ATP, acting at P2X receptors, mediates fast excitatory postsynaptic potentials (EPSPs) in many myenteric neurons of guinea-pig ileum. This is based on the finding that P2X receptor antagonists such as pyridoxal-phosphate-6-azophenyl-2'4'-disulphonic acid (PPADS) and suramin block hexamethonium resistant fast EPSPs in many myenteric neurons (Galligan & Bertrand, 1994; Zhou & Galligan, 1996; LePard *et al.*, 1997; LePard & Galligan, 1999; Johnson *et al.*, 1999). Studies aimed at identifying which functional types of myenteric neurons exhibit these P2X-mediated fast EPSPs suggest that they are in descending neural pathways (LePard & Galligan, 1999; Johnson *et al.*, 1999) and may be inhibitory motor neurons. However, these studies employed electrical stimulation to excite fast EPSPs in the impaled neurons and such stimuli would excite all neural pathways, so that no conclusions can be drawn about the source of these fast EPSPs or their physiological role. There have been two studies using physiological stimuli that might be expected to be more specific than electrical stimulation. One examined the role of P2X receptors in descending excitation evoked by mechanical stimulation of the mucosa (Spencer *et al.*, 2000) and concluded that such receptors may have a role in transmission between interneurons

in this pathway. The other (Johnson *et al.*, 1999) studied descending inhibitory reflexes evoked by distension, but only examined transmission between intrinsic sensory neurons and descending interneurons and between descending interneurons. This study concluded that P2X receptors were not involved in transmission at either class of synapse in the physiologically stimulated descending inhibitory pathway. However, whether such receptors are involved in transmission to the inhibitory motor neurons excited by physiologically relevant stimuli applied to the intestinal wall remains undetermined. A major reason for this is that P2 receptors appear to be involved in inhibitory neuromuscular transmission in the guinea-pig ileum (Crist *et al.*, 1992; He & Goyal, 1993). Thus, inhibitory junction potentials (IJPs) may be depressed by P2 antagonists independently of any effects of such antagonists on transmission between neurons.

In the present study, we have examined the role of neuronal P2X receptors in descending inhibitory reflexes using a method that distinguishes the effects of P2 antagonists on neurons from those on inhibitory neuromuscular transmission. The effects of submaximal concentrations of P2 antagonists on reflexly evoked IJPs in the circular muscle were compared with those on IJPs evoked by electrical stimulation of inhibitory motor neurons. Thus, any reduction in synaptic transmission between neurons was seen as a greater reduction in reflexly evoked IJPs than in electrically evoked IJPs.

Previous studies of descending inhibitory reflexes have shown, by blocking synaptic transmission in the stimulation chamber of a multichamber organ bath, that some distension-sensitive intrinsic sensory neurons have long anally projecting axons that act in parallel to descending interneurons (Fig. 1*A*) (Johnson *et al.*, 1996; Johnson *et al.*, 1998). A similar method was used in the present study to determine

whether P2X receptor mediated synaptic transmission arose from intrinsic sensory neurons or from descending interneurons. Anatomical studies indicate that long descending intrinsic sensory neurons probably make up only about 10% of the total population of myenteric sensory neurons (Brookes *et al.*, 1995). This still, however, represents a large proportion of anally directed axons, because 20-30% of all myenteric neurons are sensory and descending interneurons make up around 10% of myenteric neurons (Costa *et al.*, 1996).

METHODS

Guinea-pigs of either sex (200 to 350 g) were killed by a blow to the occipital region of the head and severing the carotid arteries. A 5-10 cm segment of ileum approximately 10 – 25 cm from the ileocaecal junction was removed and placed into physiological salt solution (composition in mM: NaCl 118; KCl 4.8; NaH_2PO_4 1.0; $NaCO_3$ 25; $MgSO_4$ 1.2; D-Glucose 11.12 and $CaCl_2$ 2.5) containing nicardipine (3×10^{-6} M) and bubbled with 95% O_2 plus 5% CO_2 at room temperature. After the contents of the intestine were flushed away with physiological saline solution, the segment was opened along its mesenteric border and mounted flat, mucosa uppermost in an organ bath. This bath was divided into two chambers with a method modified from a previous study (Yuan *et al.*, 1994). A rubber balloon was embedded in the base of one chamber (stimulation or oral chamber) and the preparation was pinned so that the serosa was in contact with the surface of the balloon. Inflating the balloon stretched the intestinal wall, thus mimicking physiological distension, without disturbing the mucosa (Smith *et al.*, 1990; Smith *et al.*, 1992). A compressor was also used to distort the mucosal villi in the oral chamber (Yuan *et al.*, 1991; Yuan *et al.*, 1994). The anal end of the preparation in the other chamber was rolled over to expose the longitudinal muscle, thereby allowing direct penetration through the

longitudinal muscle of circular muscle cells, whose the electrical activity was monitored with standard intracellular recording methods (Yuan *et al.*, 1994; Johnson *et al.*, 1996). Throughout the experiments, the preparation in both the recording and stimulation chambers was superfused with the physiological saline containing nicardipine (see above) to suppress smooth muscle contractions. A pair of silver wires (0.5 mm diameter) was placed in the recording chamber above and below the preparation and parallel to the circular muscle, so that electric field stimulation (EFS) could be applied to enteric neural pathways (Fig. 1).

The distance from the centre of the balloon, or the compressor, in the stimulation chamber to the recording sites was approximately 15 mm. The distance from the stimulating electrode in the recording chamber to the recording sites was approximately 4 mm. Thus, responses to distension or compression were due to descending reflex activity conducted from remote neurons, but responses to EFS were due to direct stimulation of local neurons many of which directly innervated the circular muscle from which recordings were made (Bornstein *et al.*, 1986) (Figure 1*B*).

IJPs in circular muscle were evoked by EFS and by distension or, in one series of experiments, by mucosal compression. In most experiments, the magnitudes of both the EFS and the distension were adjusted to produce IJPs of about 12 mV in amplitude, in most cases this involved a supramaximal distension. This allowed direct comparison of effects on the two responses without the need to correct for nonlinear summation (Bornstein *et al.*, 1986). Once control data were obtained, antagonists were added to the superfusing solution in the recording chamber and IJPs evoked by EFS and by reflex stimulation were recorded after 5 to 40 min in the presence of the antagonists.

Statistical analysis was performed using Student's paired *t* tests to compare mean IJP amplitudes before and during application of the drug. Analysis of variance (ANOVA) was used to determine the significance of changes in IJP amplitudes when more than one drug was applied in sequence. When ANOVA revealed a significant difference, the significance of individual differences was determined with a Tukey-Kramer Multiple Comparisons Test. Statistical comparisons of relative changes in responses with different stimulation regimes were made using the Wilcoxon signed rank test. In all statistical comparisons performed, differences were considered significant if $P < 0.05$. All data presented in the Results are expressed as mean $\pm$ standard error of the mean (S.E.M.).

The drugs used in this study were hexamethonium, hyoscine (scopolamine), nicardipine, suramin, pyridoxal-phosphate-6-azophenyl-2'4'-disulphonic acid (PPADS) (Sigma Chemical Co., MO, USA). Although the P2 antagonists used in this study are not particularly selective for P2X over P2Y receptors, it is likely that any effects on neurons in the recording chamber would be on P2X receptors. While many myenteric neurons respond to ATP with slow depolarizations that are probably mediated by metabotropic P2Y receptors (Katayama & Morita, 1989), these neurons are probably intrinsic sensory neurons and would not be activated in the recording chamber under the conditions of the present experiments. By contrast, P2X mediated fast EPSPs are commonly observed in non-sensory neurons of the descending reflex pathways (Galligan & Bertrand, 1994; LePard *et al.*, 1997; LePard & Galligan, 1999; Johnson *et al.*, 1999) and these would be expected to be excited in the recording chamber. Accordingly, neuronal effects of the P2 antagonists added to the recording chamber have been assumed to be on P2X receptors.

The composition of the low Ca^{++}/high Mg^{++} saline (in mM) was: NaCl 118; KCl 4.8; NaH_2PO_4 1.0; $NaCO_3$ 25; $MgSO_4$ 12; D-glucose 11.12 and $CaCl_2$ 0.25.

RESULTS

IJPs were recorded from circular muscle cells after direct excitation of inhibitory motor neurons by EFS in the recording chamber and after initiation of a descending reflex by distension of the intestinal wall (Fig. 2) or compression of the mucosa in the oral chamber. In most cases, recorded IJPs were biphasic with a fast initial component followed by a slower component (Johnson *et al.*, 1996; Johnson *et al.*, 1998). Where biphasic IJPs were seen the amplitudes measured were those of the largest peak.

Effect of the P2X antagonists, suramin and PPADS, on IJPs

Suramin (100 μM), when added to the recording chamber, reduced the peak amplitudes of IJPs evoked by EFS from 12.4 ± 0.1 mV to 7.8 ± 0.6 mV and those of IJPs evoked by distension from 12.4 ± 0.1 mV to 5.9 ± 0.5 mV without altering the resting membrane potential. The reduction in the IJPs evoked by distension (54%) was significantly larger than that of IJPs evoked by EFS (37%) ($P < 0.01$, Wilcoxon signed rank test, n = 8) (Figure 2).

Addition of PPADS (10 μM) to the recording chamber had little effect on the peak amplitudes of IJPs evoked by EFS which fell from 11.2 ± 0.6 mV to 10.2 ± 0.4 mV, but reduced the peak amplitudes of IJPs evoked by distension from 11.1 ± 0.5 mV to 5.8 ± 0.3 mV. Neither 10 μM (PPADS) nor 60 μM PPADS (see below) had any significant effect on the resting membrane potential. The reduction of IJPs evoked by distension (48%) was significantly greater than the reduction of those evoked by EFS (9%) ($P < 0.05$, Wilcoxon signed rank test, n = 12) (Fig. 3). In a subsequent series of experiments, although PPADS (10 μM) had no significant effect on IJPs

evoked by EFS (control 10.0 ± 0.9 mV; PPADS 9.1 ± 0.3 mV), addition of hexamethonium (200 μM) to the recording chamber with PPADS still present significantly depressed IJPs evoked by EFS (mean 7.0 ± 0.7 mV). Nevertheless, hexamethonium (200 μM) together with PPADS (10 μM) had no greater effect on IJPs evoked by distension than PPADS (10 μM) alone (control 10 ± 0.8 mV, PPADS 5.4 ± 0.4 mV, PPADS plus hexamethonium 5.7 ± 0.6 mV) (Fig. 3).

Descending inhibitory reflexes can also be evoked by mucosal distortion with the initial part of the pathway being separate from that excited by distension, although both pathways employ common final motor neurons (Smith *et al.*, 1991; Smith *et al.*, 1992). The effects of P2X receptor blockade in the recording chamber on responses to mucosal compression were similar to those on responses to distension. Application of PPADS (10 μM) to the recording chamber significantly ($P < 0.05$, Student's paired *t* test, n = 7) reduced the peak amplitudes of IJPs evoked by compression from 7.1 ± 0.4 mV to 3.6 ± 0.5 mV, while IJPs evoked by EFS fell from 10.5 ± 0.5 mV to 8.9 ± 0.5 mV (Fig. 4). Reflexly evoked IJPs were depressed more than those evoked by EFS (50% and 16%, respectively; $P < 0.025$, Wilcoxon signed rank test; n = 7).

The apparent selectivity of PPADS for a neuronal P2X receptor was concentration-dependent, because a higher concentration of the antagonist (60 μM) was much more effective in reducing IJPs evoked by EFS. These fell from 12.5 ± 0.1 mV to 4.7 ± 0.4 mV, a decline of 64%. The reduction in IJPs evoked by distension was slightly, but significantly ($P < 0.05$, single tailed Wilcoxon signed rank test, n = 6) greater, these IJPs fell from 12.3 ± 0.04 mV to 4.0 ± 0.4 mV (71%). Thus, even when PPADS had a substantial effect on inhibitory neuromuscular transmission, it

was possible to detect a component of transmission in the reflex pathway mediated by P2X receptors.

Effects of synaptic blockade in the stimulation chamber on IJPs evoked by distension

To determine whether synaptic transmission mediated by P2X receptors arose from interneurons, sensory neurons or both, the superfusing solution in the stimulation chamber was replaced with a low Ca^{++}/high Mg^{++} solution (see Methods). This solution blocks synaptic transmission between enteric neurons (Dingledine & Goldstein, 1976) and thus would block transmission from sensory neurons to descending interneurons or to long descending inhibitory motor neurons in the stimulation chamber. Thus, the descending inhibitory reflex would only be conducted into the recording chamber by distension-sensitive sensory neurons with long descending axons (Johnson *et al.*, 1996). That is, any contribution to the reflex from activity in descending interneurons would be eliminated. Under these conditions, the amplitudes of IJPs evoked by distension were only 7 mV. The strength of the EFS was adjusted to produce an IJP to match this. Adding PPADS (10 µM) to the recording chamber had no significant effect on responses to either stimulus (Fig. 5). Mean responses to EFS were 7.6 $\pm$ 0.1 mV in control and 7.3 $\pm$ 0.1 mV in PPADS, while those for distension were 7.6 $\pm$ 0.1 mV and 7.3 $\pm$ 0.1 mV, respectively (in each case, $P > 0.05$, Student's paired *t* test, n = 6).

Other antagonists were also tested in the recording chamber when synaptic transmission in the stimulation chamber was blocked (Fig. 6). When hyoscine (1 µM) was superfused into the recording chamber, the sizes of the IJPs evoked by both EFS and distension were visibly increased in 5 of 6 preparations (e.g. Fig. 6*A*), although this increase was not statistically significant (in control 9.1 $\pm$ 0.8 mV, in hyoscine 9.9 $\pm$ 0.3 mV, $P > 0.05$). Adding hyoscine (1 µM) and hexamethonium (200

μM) simultaneously to the recording chamber significantly reduced the peak amplitude of IJPs evoked by EFS by 31% compared to control (the mean amplitude in hyoscine plus hexamethonium was 6.0 ± 0.6 mV, $P < 0.01$, one-way ANOVA; n = 6). The combination of PPADS (10 μM), hyoscine (1 μM) and hexamethonium (200 μM) did not further reduce the electrically evoked IJPs (5.7 ± 0.8 mV, $P > 0.05$; n = 6).

By contrast, hyoscine (1 μM), hexamethonium (200 μM) and PPADS (10 μM), separately and together, had no significant effect on the amplitudes of IJPs evoked by distension during synaptic blockade in the stimulation chamber (control 6.9 ± 0.4 mV, hyoscine 8.2 ± 0.7 mV, hyoscine plus hexamethonium 7.8 ± 0.5 mV, and hyoscine plus hexamethonium plus PPADS 7.9 ± 0.6 mV; $P > 0.05$, one-way ANOVA, n = 6) (see Fig. 6).

DISCUSSION

The results of this study provide strong experimental support for the suggestion that a substantial component of transmission between neurons in the descending inhibitory reflex pathway of the guinea-pig ileum is mediated by P2X purinoceptors (LePard & Galligan, 1999; Johnson *et al.*, 1999). Furthermore, they indicate that this transmission is between descending interneurons and inhibitory motor neurons. Transmission from the long descending axons of sensory neurons to inhibitory motor neurons is not mediated via P2X receptors, muscarinic receptors or nicotinic receptors. The results also provide support for the conclusion that IJPs in the circular muscle of the guinea-pig ileum are mediated by ATP acting at P2 receptors on the muscle (Crist *et al.*, 1992; He & Goyal, 1993).

P2X receptors mediate transmission to inhibitory motor neurons in a descending reflex pathway

When added to the recording chamber of a two chambered organ bath, both suramin and PPADS depressed IJPs evoked by distension to a significantly greater extent than IJPs evoked by electrical stimulation of neurons within the recording chamber (Figs 2 and 3). Thus, the descending inhibitory reflex is more sensitive to blockade of P2 receptors in the recording chamber than is inhibitory neuromuscular transmission. This indicates that transmission between neurons in the recording chamber depends in part on P2X receptors. In a previous study, it was found that application of suramin (100 μM) to the central chamber of a three chambered organ bath had no effect on IJPs evoked in the anal (recording) chamber by distension or mucosal distortion in either the central or oral chamber (Johnson *et al.*, 1999). This indicates that P2X receptors are not involved in transmission from intrinsic sensory neurons to descending interneurons or in transmission between descending interneurons of the inhibitory reflex pathway. Thus, it is unlikely that blockade of P2X receptors in the recording chamber in the present series of experiments affected transmission between interneurons in this chamber. Rather, the substantial depression of the reflex IJPs seen in PPADS, in particular, is probably due to a reduction of synaptic transmission from descending reflex pathways to inhibitory motor neurons. This remains to be confirmed by direct recording from identified inhibitory motor neurons during physiologically evoked descending reflexes.

Electrical stimulation in the recording chamber would excite local inhibitory motor neurons both directly and via synaptic inputs from other neurons within the recording chamber. The latter possibility is consistent with the observation in this, and previous studies (Bornstein *et al.*, 1986), that IJPs evoked by transmural stimulation are depressed, but not abolished, by the nicotinic antagonist hexamethonium. Intracellular studies indicate that the fast EPSPs in all myenteric

neurons are partially mediated through nicotinic receptors (Galligan & Bertrand, 1994; LePard *et al.*, 1997; LePard & Galligan, 1999; Johnson *et al.*, 1999), so that inhibitory motor neurons would be expected to be excited by both nicotinic agonists and purinergic agonists. The failure of hexamethonium to modify reflexly evoked IJPs, however, suggests that many neural pathways excited by EFS are distinct from those excited by distension.

Neurons responsible for P2X mediated transmission to inhibitory motor neurons

Anatomical studies of anally directed neural pathways in the guinea-pig ileum indicate that there are at least two parallel systems that can mediate reflex IJPs (Fig. 1*B*). These are a population of descending interneurons that receive synaptic input from circumferentially placed intrinsic sensory neurons (Mann *et al.*, 1997) and a population of intrinsic sensory neurons that project anally along the intestine for up to 10 cm (Brookes *et al.*, 1995). Studies using three chambered organ baths have indicated that the intrinsic sensory neurons contribute to transmission along the descending inhibitory pathway over the first 1-2 cm, but may have little role over longer distances (Johnson *et al.*, 1996; Johnson *et al.*, 1998). The present results indicate that transmission from the anally directed axons of intrinsic sensory neurons to inhibitory motor neurons is not mediated by P2X receptors. Thus, P2X receptors appear to be involved in transmission from descending interneurons to inhibitory motor neurons.

Two classes of descending interneurons make synaptic connections with inhibitory motor neurons: interneurons immunoreactive for nitric oxide synthase (NOS), vasoactive intestinal peptide and gastrin-releasing peptide; and interneurons immunoreactive for somatostatin (SOM) and choline acetyltransferase (Mann *et al.*, 1997; Young *et al.*, 1995b). At least some of the NOS-immunoreactive interneurons

are also immunoreactive for choline acetyltransferase (Li & Furness, 1998). The present experiments do not allow definitive conclusions as to which of these two populations of interneurons are responsible for the P2X receptor mediated transmission to inhibitory motor neurons. However, it seems likely that the NOS-immunoreactive interneurons are involved, because there is physiological, neuroanatomical and pharmacological evidence that these neurons are the first interneurons of the descending inhibitory reflex pathway (Yuan *et al.*, 1995; Stebbing & Bornstein, 1996; Pompolo & Furness, 1998). By contrast, the SOM-immunoreactive interneurons do not receive direct synaptic input from the intrinsic sensory neurons (Stebbing & Bornstein, 1996; Pompolo & Furness, 1998) and probably make very little contribution to the conduction of the descending inhibitory reflex over the distances involved in the present study.

A recent study of excitatory reflexes in the guinea-pig ileum concluded that P2 receptors were involved in transmission between interneurons of both the ascending excitatory pathway and a descending excitatory pathway (Spencer *et al.*, 2000). These reflexes were measured by recording contractions of the intestinal smooth muscle and a descending relaxation was not recorded, although a descending inhibition was detected pharmacologically. By contrast, descending inhibition has been observed in every electrophysiological study of reflexes evoked by either distension or mucosal distortion, while descending excitation has rarely been described in such studies (e.g. (Hirst *et al.*, 1975; Smith & Furness, 1988; Smith *et al.*, 1990; Smith *et al.*, 1991; Yuan *et al.*, 1991; Yuan *et al.*, 1995; Johnson *et al.*, 1996; Johnson *et al.*, 1998)). A significant methodological difference is that in all, but one, of the electrophysiological studies smooth muscle contraction was prevented by blockade of L-type calcium channels. One possible explanation for

these differences is that nicardipine preferentially depresses excitatory neuromuscular transmission and the membrane potential changes observed in the present experiments represented the sum of a descending inhibition and a smaller descending excitation. Indeed, in the electrophysiological study of descending reflexes that did not employ an L-type calcium channel blocker, a descending excitation was observed (Hirst *et al.*, 1975). However, this descending excitatory junction potential (EJP) had a substantially longer latency that the descending IJP and was completely abolished by the muscarinic antagonist, atropine. In this present study, no late EJP was observed and blockade of muscarinic receptors with hyoscine had no significant effect on the IJPs indicating that there was no underlying EJP. Why the presence or absence of nicardipine may shift the relative weight of descending reflexes in favour of either excitation or inhibition is not clear, but it suggests that studies of the role of P2X receptors may not be equivalent. For example, some intrinsic sensory neurons respond to generation of active tension in the muscle (Kunze *et al.*, 1999), so that some neural pathways may not be activated in the electrophysiological experiments.

Intrinsic sensory neurons use an unidentified neurotransmitter at some synapses

The intrinsic sensory neurons are immunoreactive for both choline acetyltransferase and tachykinins (Costa *et al.*, 1996; Li & Furness, 1998). However, transmission from the anally directed axons of the intrinsic sensory neurons excited by distension was not only insensitive to P2X receptor blockade, it was also unaffected by nicotinic and muscarinic antagonists (Fig. 6*B*). Thus, neither ATP nor ACh is likely to be important for transmission at synapses between these axons and inhibitory motor neurons. Further, earlier studies using multichambered organ baths and electrophysiological recording to study the role(s) of tachykinins in the

descending inhibitory pathway indicate that transmission from the sensory neurons to inhibitory motor neurons is not blocked by specific NK_1 or NK_3 receptor antagonists (Johnson *et al.*, 1998). NK_1 and NK_3 receptors are widespread on myenteric neurons in guinea-pig ileum (Portbury *et al.*, 1996b; Lomax *et al.*, 1998; Jenkinson *et al.*, 1999), but NK_2 receptors are absent from nerve cell bodies in this preparation (Portbury *et al.*, 1996a). Thus, transmission between anally projecting intrinsic sensory neurons and inhibitory motor neurons is not mediated via ATP acting on P2X receptors, by ACh acting on nicotinic or muscarinic receptor or by tachykinins. This suggests that a hitherto unidentified neurotransmitter is involved in transmission from intrinsic sensory neurons at some of their synaptic connections.

An alternative possibility is that some of the inhibitory motor neurons that have very long anal projections (Bornstein *et al.*, 1986) are mechanosensitive, so that distension would directly excite them. However, in a study of tension-sensitive myenteric neurons, Kunze et al. (Kunze *et al.*, 1998) identified intrinsic sensory neurons and a small population of longitudinal muscle motor neurons, but not inhibitory motor neurons, as being mechanosensitive.

Intracellular recordings from myenteric neurons anal to a distending stimulus have found that such neurons respond to activation with a burst of fast EPSPs (Hirst *et al.*, 1975; Smith *et al.*, 1992); slow EPSPs are almost never observed. Thus, it can be predicted that transmission from intrinsic sensory neurons to inhibitory motor neurons would be via a neurotransmitter and receptor subtype that produces fast EPSPs in myenteric neurons. Two further transmitter/receptor combinations that produce such effects have been identified within the guinea-pig myenteric plexus, 5-HT acting at $5\text{-}HT_3$ receptors (Zhou & Galligan, 1999) and glutamate acting at AMPA receptors (Liu *et al.*, 1997). However, 5-HT is not contained within the intrinsic

sensory neurons (Furness & Costa, 1982) and 5-HT-immunoreactive nerve terminals rarely contact inhibitory motor neurons (Young & Furness, 1995a). Thus, it is highly unlikely that 5-HT mediates transmission from the descending axons of intrinsic sensory neurons to inhibitory motor neurons. This transmission is also unlikely to be mediated by glutamate acting at AMPA receptors. It has recently been found that the responses to glutamate in myenteric neurons were slow, and that EPSPs in S-neurons (the electrophysiological class that includes inhibitory motor neurons) are not blocked by AMPA antagonists (Ren *et al.*, 2000). One further possibility, calcitonin gene-related peptide (CGRP), has been suggested by the results of several studies of reflexes in the rat colon (Grider, 1994; Grider *et al.*, 1996) and in the guinea-pig submucous plexus (Pan & Gershon, 2000). However, this peptide was found to produce slow synaptic responses in the submucous plexus (Pan & Gershon, 2000) and is unlikely to be a transmitter mediating fast EPSPs in the descending reflex pathways. Thus, identification of the transmitter(s) acting at synapses between intrinsic sensory neurons and inhibitory motor neurons must await further studies.

Conclusions

P2X purinoceptors play a significant role in transmission from descending interneurons to inhibitory motor neurons of the descending inhibitory reflex pathways excited by distension and mucosal distortion. However, at least two other forms of excitatory transmission to the inhibitory motor neurons can be identified. One of these is via nicotinic ACh receptors, but the presynaptic neurons from which this input arises have not been identified. P2 receptors are also important for inhibitory neuromuscular transmission in the guinea-pig ileum, although these receptors may be less sensitive to PPADS than the neuronal P2X receptors. Transmission from

long anally directed axons of distension-sensitive intrinsic sensory neurons to inhibitory motor neurons is not mediated via nicotinic or muscarinic ACh receptors, via P2X receptors or via NK_1 and NK_3 tachykinin receptors. This suggests that a hitherto unidentified neurotransmitter may have an important role in transmission between some enteric neurons during intestinal reflexes.

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FIGURE LEGENDS

Figure 1. Schematic diagram illustrating the experimental design.

A. Organ bath (cross hatch) divided by a plastic wall (left hatch) into an oral stimulation chamber and an anal recording chamber. A balloon (white) was embedded in the stimulation chamber to distend the intestinal wall and a pair of silver wires (right hatch) was place above and below the sheet of intestine in the recording chamber to allow electrical stimulation of the enteric nerves. An opened segment of ileum (black) was pinned mucosa up in the divided organ bath. The anal end of the segment was folded over to allow microelectrodes to be inserted through the longitudinal muscle into circular muscle cells. *B*. The descending neural circuit inferred from anatomical and physiological studies. All intrinsic sensory neurons excite neighbouring descending interneurons, and some have long descending axons that can directly excite inhibitory motor neurons. EFS excites both local inhibitory motor neurons and the pathways that impinge on them.

Figure 2. Effects of suramin (100 μM) on IJPs evoked by distension and EFS in guinea-pig ileum.

A. IJPs recorded from circular muscle in control (top panels) and IJPs evoked in the same circular muscle cells when suramin was present in the recording chamber (bottom panels). The traces on the left show IJPs evoked by EFS and on the right are IJPs evoked by distension. The strength of each stimulus was adjusted so that the control responses were about 12 mV. *B*. Pooled data from 8 experiments showing the relative amplitudes (expressed as a percentage of the controls) of IJPs evoked by EFS and distension with suramin in the recording chamber. Error bars show the S.E.M. in this and all subsequent Figures. IJPs evoked by EFS and distension were both reduced by suramin ($P < 0.05$, Student's paired *t* test). IJPs evoked by distension were significantly more depressed than IJPs evoked by EFS ($P < 0.01$ Wilcoxon signed rank test).

Figure 3. Effect of PPADS (10 μM) or PPADS (10 μM) plus hexamethonium (200 μM) on IJPs evoked by distension and EFS.

A. IJPs recorded from a circular muscle cell in control (top) and when PPADS (middle) or PPADS and hexamethonium (HEX) (bottom) were present in the recording chamber. The left column shows IJPs evoked by EFS and the right IJPs evoked by distension. *B*. The relative amplitudes of IJPs evoked by EFS and distension with PPADS (hatched bars) or PPADS plus hexamethonium (solid bars) in the recording chamber. IJPs evoked by EFS and distension are reduced by PPADS ($P < 0.05$, Student's paired *t* test, n = 12). IJPs evoked by distension were significantly more depressed than IJPs evoked by EFS ($P < 0.01$, Wilcoxon signed rank test, n = 12). Addition of hexamethonium to the PPADS in the recording chamber significantly reduced the IJPs evoked by EFS, but did not depress IJPs evoked by distension more than PPADS alone (one-way ANOVA, n = 6).

Figure 4. Effect of PPADS (10 μM) on IJPs evoked by EFS and mucosal compression.

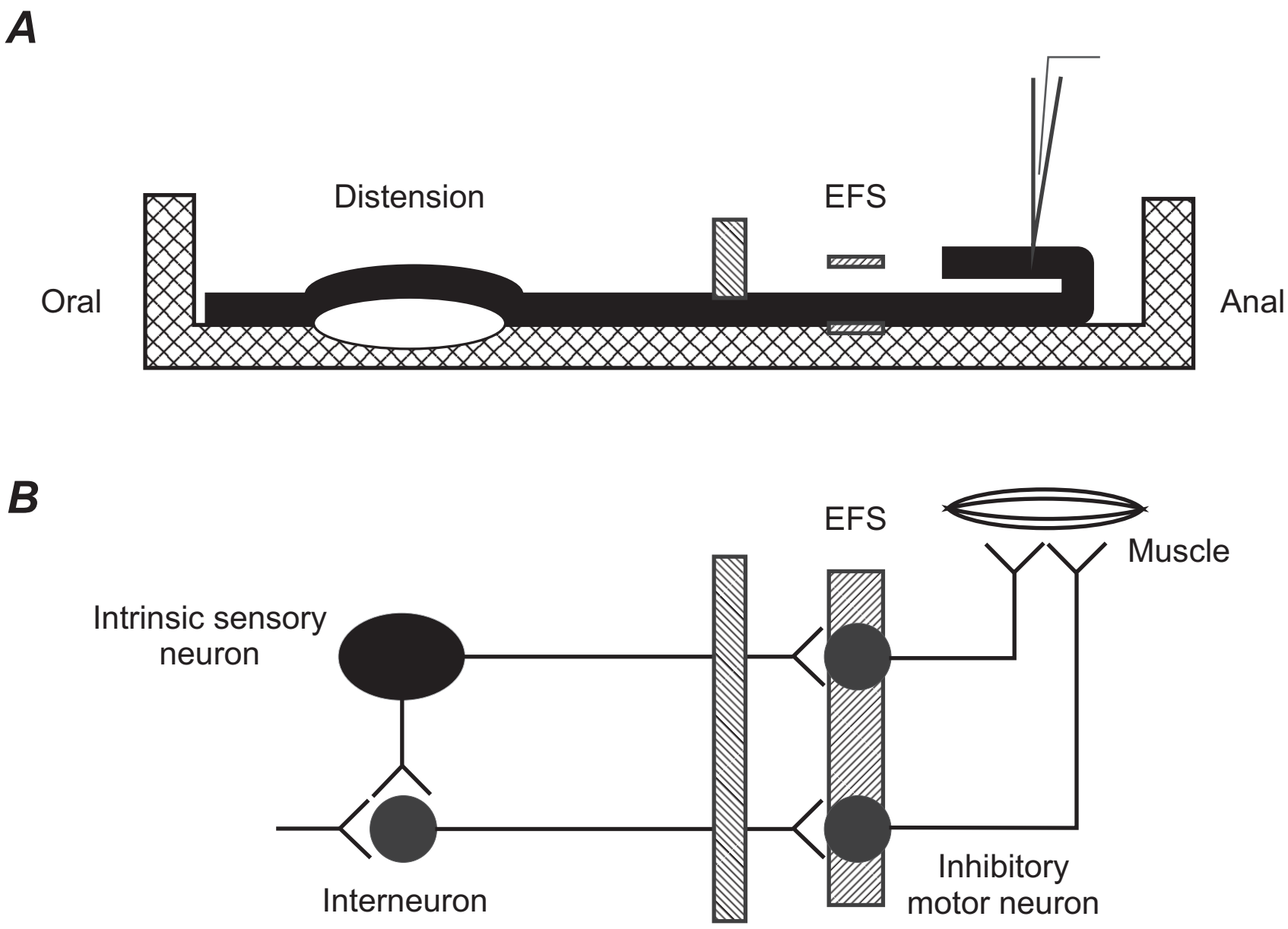
A. IJPs recorded from a circular muscle cell in control (top), with PPADS in the recording chamber (middle) and after 20 min washout of the PPADS (bottom). *B*. Relative amplitudes of IJPs evoked by EFS (left) and compression (right) with PPADS in the recording chamber. IJPs evoked by EFS and compression were both reduced by PPADS ($P < 0.05$, n = 7). IJPs evoked by compression were significantly more depressed than those evoked by EFS ($P < 0.01$, Wilcoxon signed rank test).

Figure 5. Effect of PPADS (10 μM) in the recording chamber on IJPs evoked by EFS and distension when synaptic transmission in the stimulation chamber was blocked.

A. IJPs recorded from a circular muscle cell in control (top) and when PPADS was present in the recording chamber (bottom). *B*. Relative amplitudes of IJPs evoked by EFS (left) and distension (right) with PPADS in the recording chamber (n = 6). There was no significant changes in the responses to either stimulus ($P > 0.05$, n = 6). Synaptic transmission in the stimulation chamber was blocked with a low Ca^{2+}, high Mg^{2+} solution.

Figure 6. Lack of effect of muscarinic and nicotinic antagonists in the recording chamber, when synaptic transmission is blocked in the stimulation chamber

A. IJPs recorded from a circular muscle cell in control (*a*), in hyoscine (1 μM) (*b*), in hyoscine (1 μM) plus hexamethonium (200 μM) (*c*) and in hyoscine (1 μM) plus hexamethonium (200 μM) and PPADS (10 μM) (*d*). *B*. The relative amplitudes of IJPs evoked by EFS and distension in hyoscine (empty bars), in hyoscine plus hexamethonium (hatched bars) and in hyoscine plus hexamethonium and PPADS (solid bars). Hyoscine plus hexamethonium in the recording chamber significantly reduced the IJPs evoked by EFS ($P < 0.01$, one-way ANOVA; n = 6), but not the IJPs evoked by distension. Hyoscine plus hexamethonium and PPADS did not significantly change the IJPs evoked by either EFS or distension ($P > 0.05$, one-way ANOVA, n = 5).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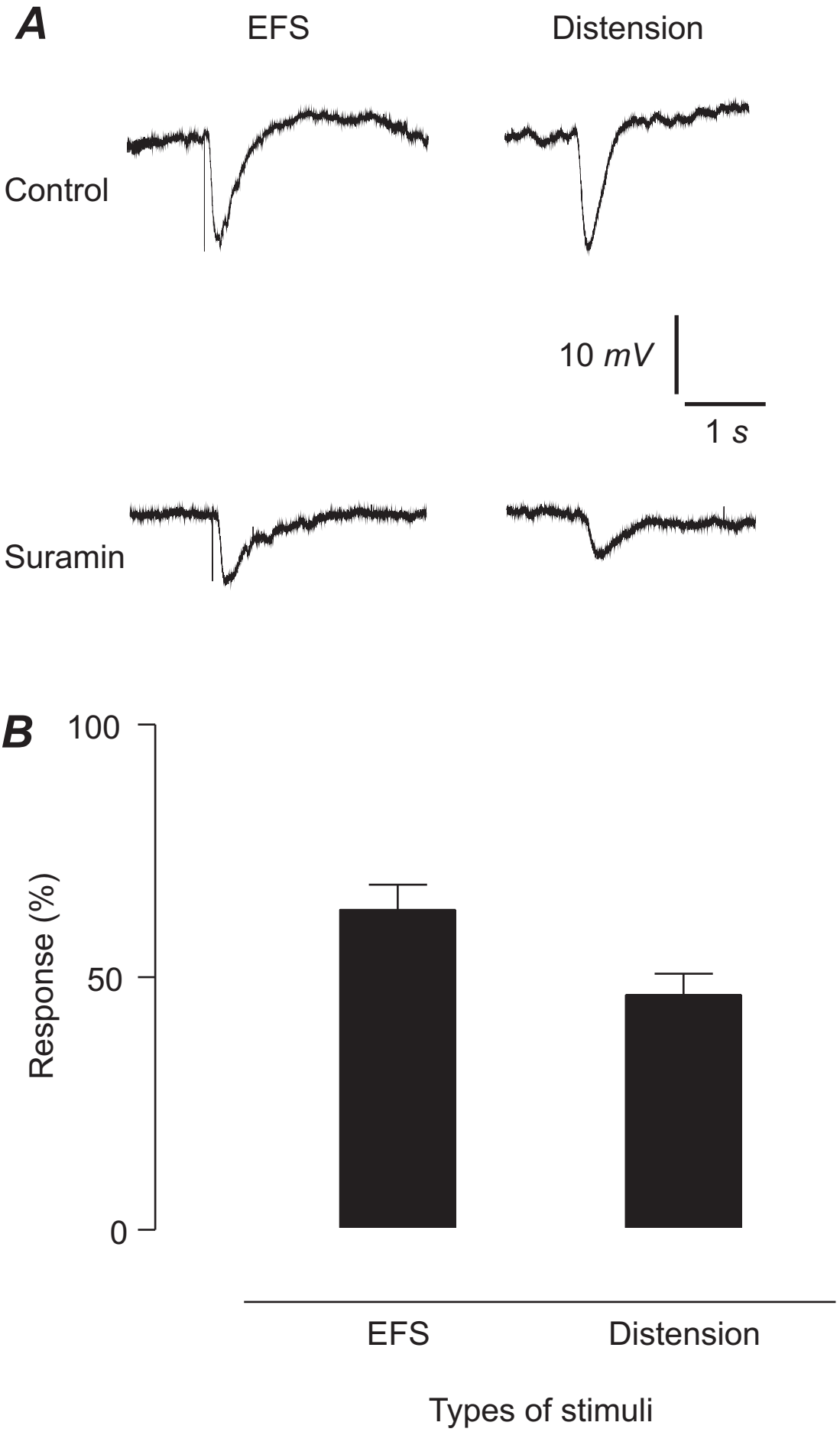
Interneuron
Inhibitory motor neuron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Distension
Control
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B
100
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Response (%)
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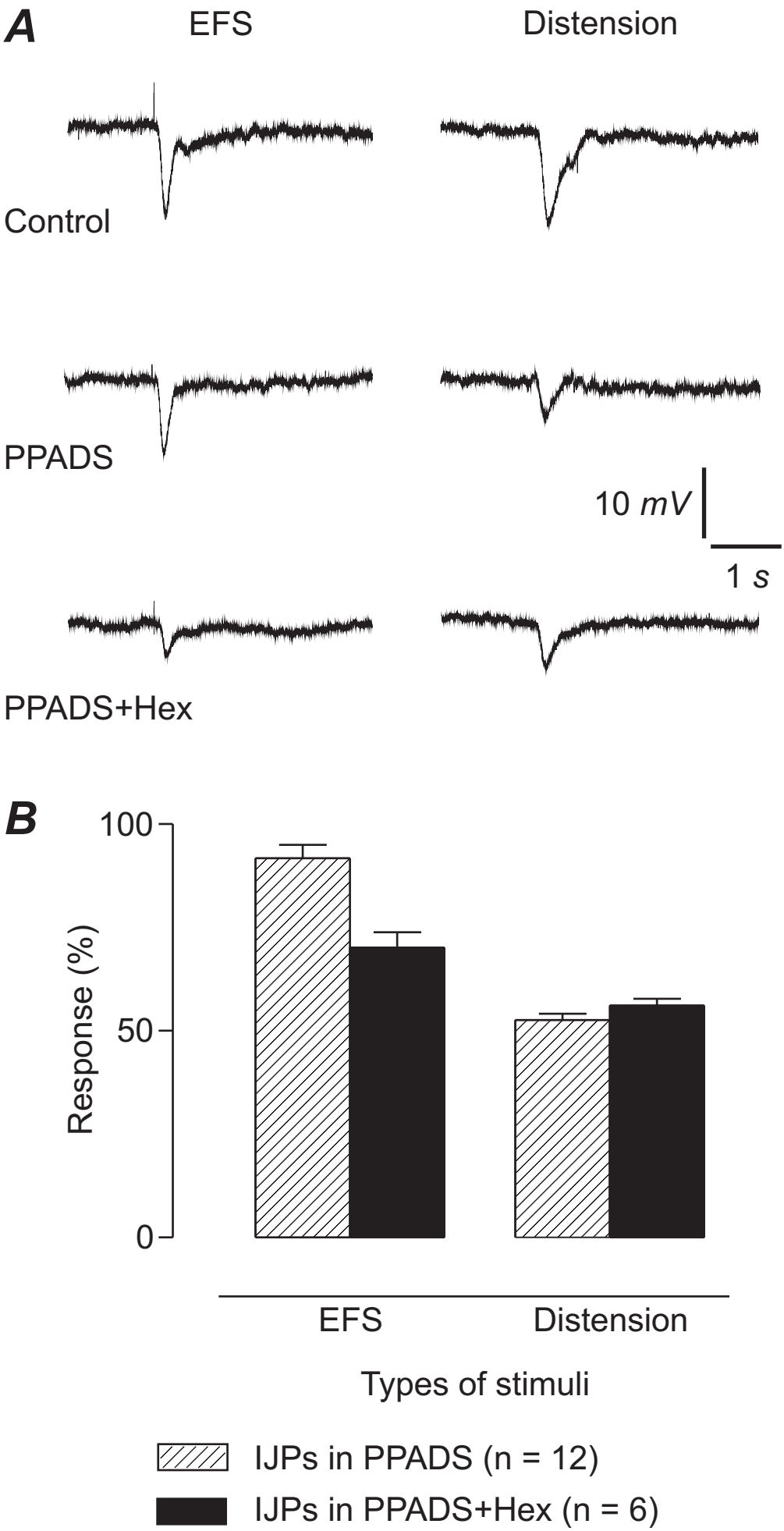
Distension
Types of stimuli


A
EFS
Distension
Control
PPADS
10 mV
1 s
PPADS+Hex
B
100
50
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Response (%)
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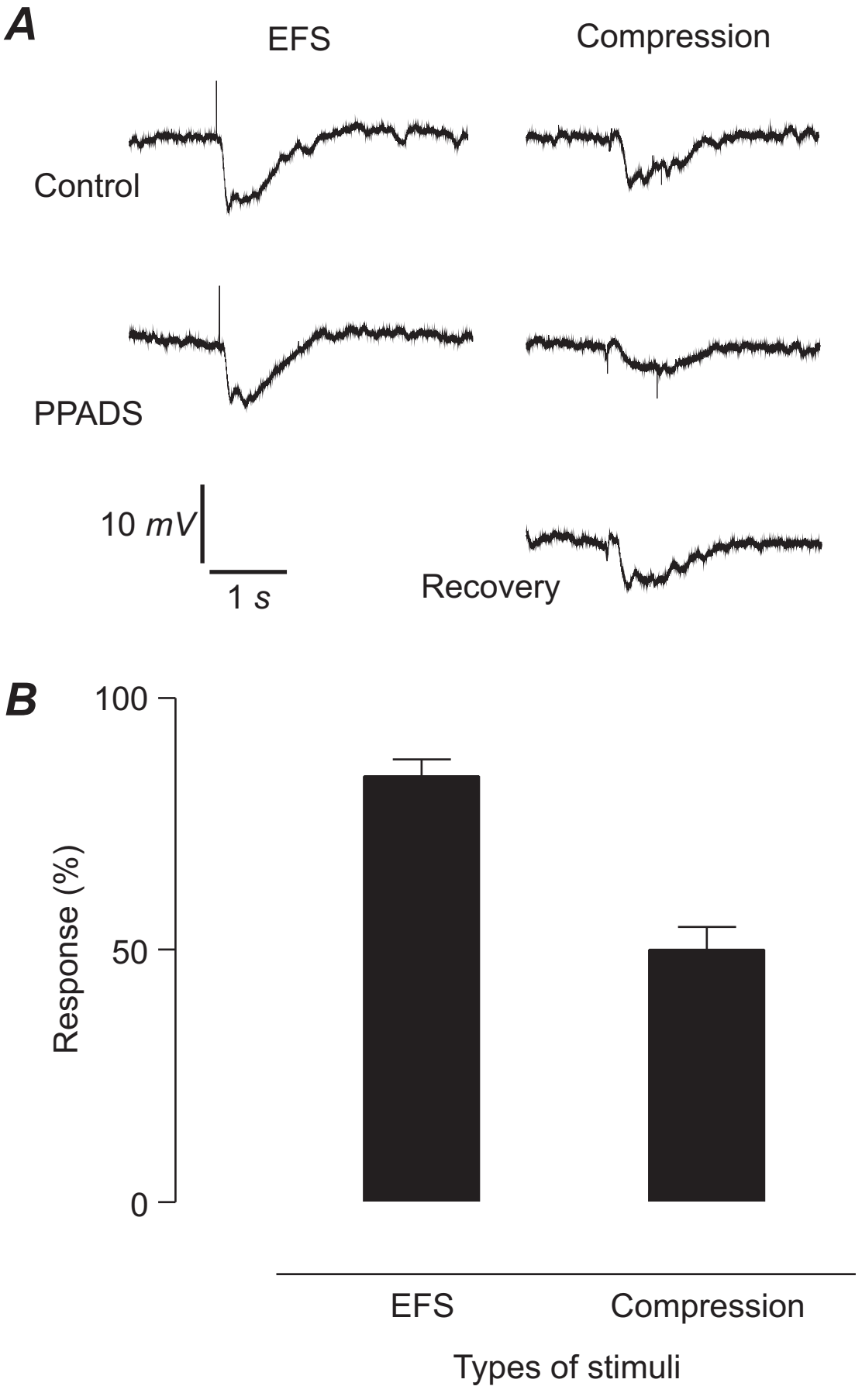
Types of stimuli
IJPs in PPADS (n = 12)
IJPs in PPADS+Hex (n = 6)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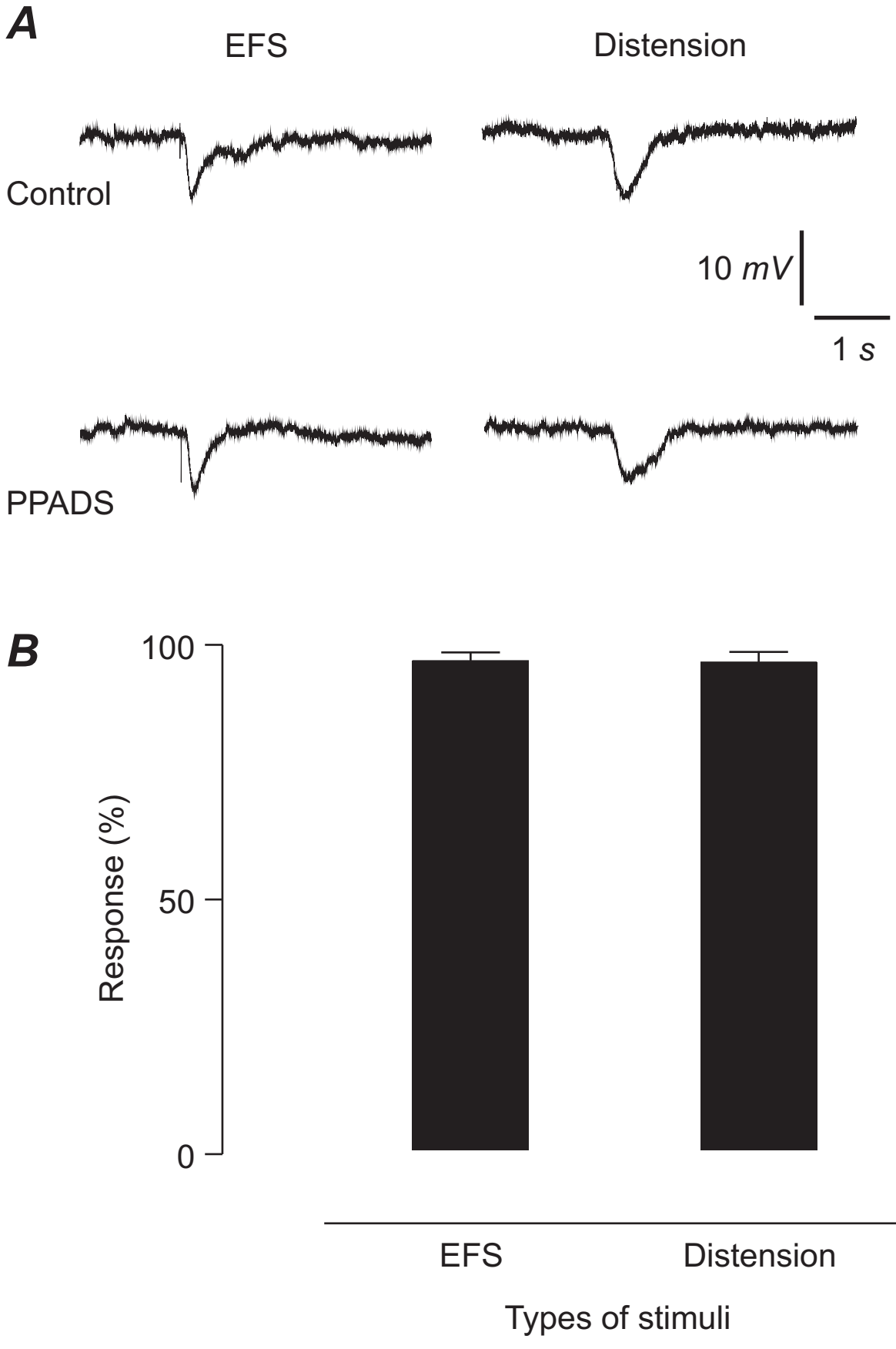
Compression
Control
PPADS
10 mV
1 s
Recovery
B
100
50
0
Response (%)
EFS
Compression
Types of stimuli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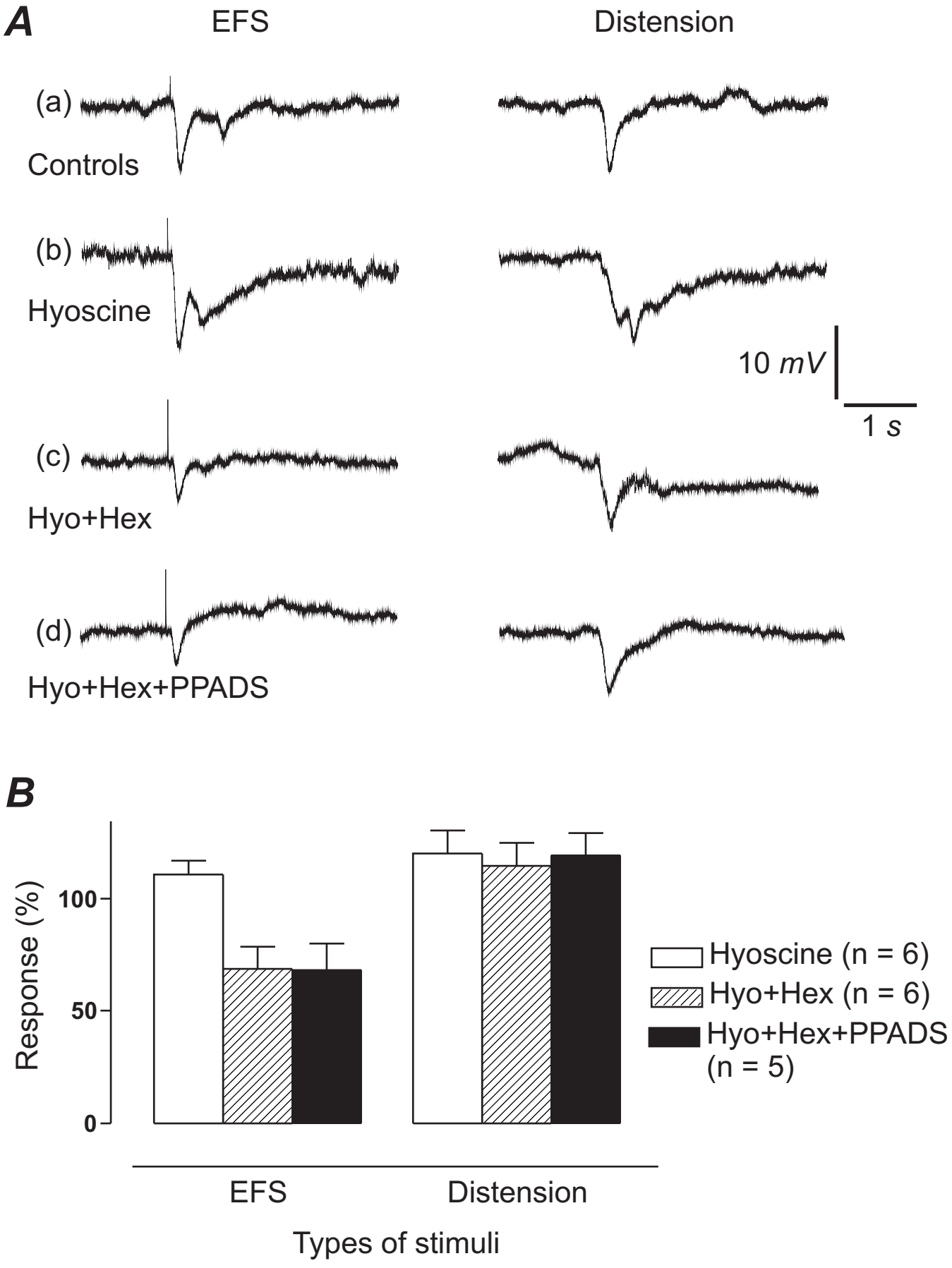
Distension
Types of stimuli


A
EFS
Distension
(a)
Controls
(b)
Hyoscine
10 mV
1 s
(c)
Hyo+Hex
(d)
Hyo+Hex+PPADS
B
Response (%)
100
50
0
Hyoscine (n = 6)
Hyo+Hex (n = 6)
Hyo+Hex+PPADS
(n = 5)
EFS
Distension
Types of stimuli