Patch-clamp characterization of nicotinic receptors in a subpopulation of lamina X neurones in rat spinal cord slices

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1. Nicotinic acetylcholine receptors (nAChRs) on lamina X neurones in neonate (P1–P12) rat transverse thoracolumbar spinal cord slices were studied using the whole-cell patch-clamp technique. These visually selected neurones are located dorsal to the central canal, mainly in the ventral half of the dorsal commissure.

2. Pressure application of the nicotinic agonist 1,1-dimethyl-4-phenyl-piperazinium (DMPP) (1 mM) induced a rapid depolarization on which action potentials are superimposed.

3. At −50 mV, DMPP (1 mM), pressure ejected for 100 ms, induced a fast inward current with a mean amplitude of −280 pA ($n = 28$) in 90% of the neurones recorded. Superfusion of tetrodotoxin (TTX), a solution containing 0 Ca^{2+}–high Mg^{2+}, $CdCl_2$ or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) did not abolish the DMPP-induced current, which confirmed a direct postsynaptic effect of DMPP on recorded neurones.

3. The current–voltage (I–V) relationship for DMPP-induced current exhibited a reversal potential of 0 mV (NaCl outside, potassium gluconate inside) and a strong inward rectification.

4. The DMPP-induced responses were blocked by mecamylamine, hexamethonium and *d*-tubocurarine (dTC) but were insensitive to α-bungarotoxin and methyllycaconitine (MLA).

5. We conclude that lamina X neurones located dorsally to the central canal possess nicotinic acetylcholine receptors. Activation of these nicotinic receptors results in depolarization and generation of action potentials. These receptors may be involved in the modulation of the somato- and viscerosensory transmission.

The central grey region (lamina X) of the spinal cord is involved in somatosensation, particularly in nociception (Nahin, Madsen & Giesler, 1983). This region receives primary afferents and supraspinal inputs, and possesses many types of neurones: interneurones (Lamotte, 1988), including intersegmentally projecting neurones; neurones projecting to higher brain centres (Nahin *et al.* 1983); and sympathetic preganglionic neurones (SPNs) of the intermediomedial cell column (IMM), an autonomic area dorsal to the central canal (Hosoya, Nadelhaft, Wang & Khono, 1994). A striking feature of this region is the widespread distribution of cholinergic neurones in comparison with other areas of the spinal cord (Barber, Phelps, Houser, Crawford, Salvaterra & Vaughn, 1984). These cholinergic neurones, shown to be intrinsic to the spinal cord, are composed of partition cells, central canal cluster cells, and SPNs of the IMM (Borges & Iversen, 1986).

The wide distribution of cholinergic neurones suggests the presence of nicotinic and/or muscarinic acetylcholine receptors. As regards the nicotinic acetylcholine receptor (nAChR), a recent binding study (Khan, Yaksh & Taylor, 1994*b*) and *in situ* hybridization studies (Wada *et al.* 1989; Dineley-Miller & Patrick, 1992; Séguéla, Wadiche, Dineley-Miller, Dani & Patrick, 1993) revealed binding sites for nicotinic agonists and detected different subunits entering into the composition of nAChRs, respectively. Conventional extra- and intracellular recordings have been performed to study nAChR activation on spinal cord neurones of the Clarke's column and dorsal horn (Myslinski & Randic, 1977), on motoneurones (Perrins & Roberts, 1994) and on Renshaw cells (Curtis & Ryall, 1966). Recently, an *in vivo* study has shown that nAChRs mediate cardiovascular and nociceptive responses (Khan, Taylor & Yaksh, 1994*a*). However, none of these studies revealed that nicotinic receptors mediated responses in neurones of the central grey region.

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In this paper, we report nicotinic agonist-induced responses on neurones in lamina X by using whole-cell patch-clamp recordings in neonate rat spinal cord slices.

METHODS

Preparation of slices

Rats were decapitated under deep diethylether anaesthesia. Methods for the preparation of thin spinal cord slices used in this study were previously described by Krupp & Feltz (1993). Slices (200–250 μm) were transferred to a storing chamber filled with oxygenated sucrose-based artificial cerebrospinal fluid (ACSF, see composition below) at room temperature (20–22 °C). After recovery for at least 1 h, one slice was transferred to a recording chamber and held by a nylon mesh glued to a U-shaped silver wire. This chamber was superfused at a rate of 2 ml min^{-1} with warm (33 °C) oxygenated ACSF.

Solutions

The ACSF contained (mM): NaCl, 113; KCl, 3; NaH_2PO_4, 1; $NaHCO_3$, 25; glucose, 11; $CaCl_2$, 2; $MgCl_2$, 1. The sucrose-based ACSF contained (mM): sucrose, 248; KCl, 2; KH_2PO_4, 1·25; $NaHCO_3$, 26; glucose, 11; $CaCl_2$, 2; $MgSO_4$, 2. Both ACSFs had pH equilibrated to 7·4 with 95% CO_2 and 5% O_2. In some experiments, NaCl was reduced to 56·5 mM and D-mannitol was added to a concentration of 113 mM. In other experiments, calcium was omitted in the ACSF and replaced with $MgCl_2$. The intracellular solution used contained the following (mM): potassium gluconate, 140; $MgCl_2$, 1; MgATP, 1; Hepes, 10; EGTA, 10; $CaCl_2$, 1; pH 7·3 (adjusted with NaOH).

Application of drugs

All drugs were purchased from Sigma except methyllycaconitine (MLA), which was from RBI Biochemicals. Antagonists were applied to the slice by superfusion driven by gravity. Nicotinic agonists 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP) and cytisine were applied by a computer-controlled pressure-ejection system. The pressure pipette was a high resistance patch pipette (6–8 MΩ). The pressure-ejection pipette was located 20–30 μm from the recorded cell just above the slice. Pressure ejection of ACSF (without DMPP) produced no response (data not shown). When two nicotinic agonists were tested on the same cell, we used two pressure pipettes located at the same distance from the recorded cell. Under these conditions, applications of the same

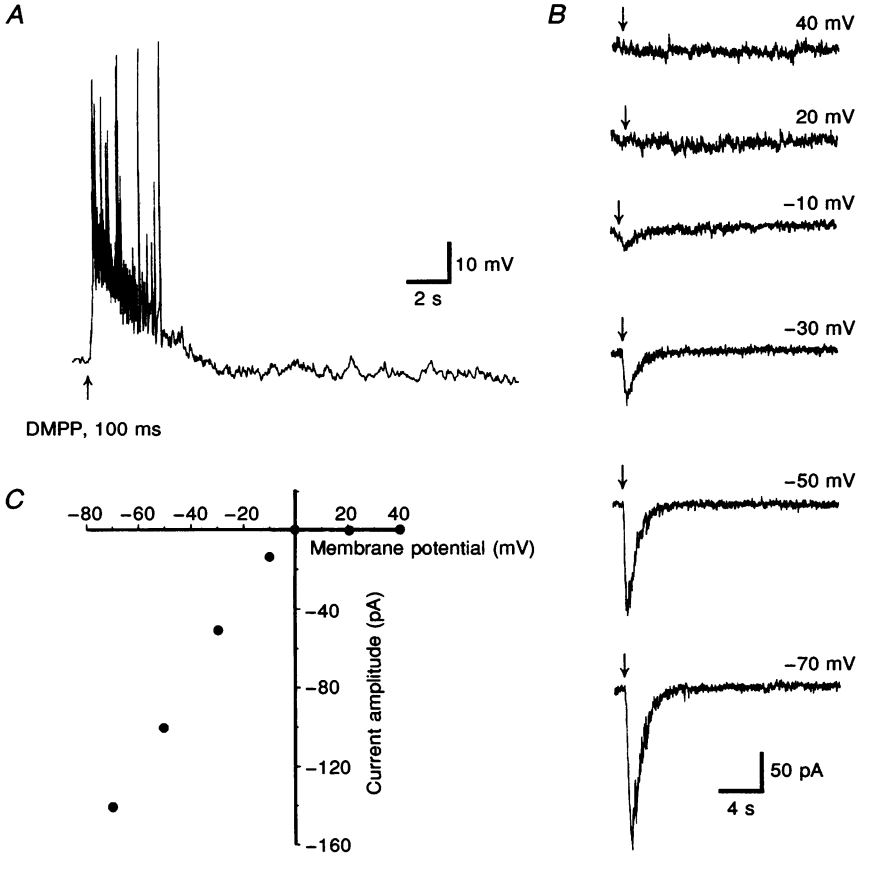

Figure 1. DMPP responses in lamina X neurones

A, under current-clamp conditions, pressure application of DMPP (1 mM) for 100 ms (indicated by an arrow) induced a depolarization on which spikes are superimposed. Resting potential, −60 mV. *B*, under voltage-clamp conditions in another cell, pressure application of DMPP (1 mM, 100 ms) elicited an inward current at different holding membrane potentials as indicated on each trace. The peak amplitude of the current was measured and plotted against the membrane potential in panel *C*. *C*, current–voltage relationship of the peak amplitude of the DMPP-induced current from the cell illustrated in *B*. The zero current is close to 0 mV; the *I–V* relationship shows a strong inward rectification of the DMPP response.

agonist from the two pipettes produced similar responses in the recorded cell.

Recording conditions and data analysis

Slices were observed under Nomarski optics using an upright microscope (Zeiss Axioskop FS) equipped with a ×40 water-immersion lens (Zeiss). Such a visualization before and during recordings allowed us to choose cells and properly position the pressure pipette. Patch pipettes for whole-cell recordings (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) were pulled on an L/M-3P-A puller (List Electronics, Darmstadt, Germany) from microfilament-filled borosilicate glass (o.d., 1·55 mm; wall thickness, 0·35 mm). When filled with intracellular solution (see above), electrodes had resistances of 4–6 MΩ. Whole-cell voltage clamp was achieved using an Axopatch-2A amplifier (Axon Instruments). The capacitance and membrane resistance were obtained by applying negative rectangular potential steps (−20 mV). The membrane voltage charging curves were fitted with the sum of two exponentials (Llano, Marty, Armstrong & Konnerth, 1991), which allowed calculation of the pipette access resistance, R_1, of the somatic and proximal dendritic capacitance, C_1, of the lumped resistance, R_2, linking the main dendrites to each distal dendrite, and of the distal dendrite capacitance, C_2. R_1 was 9·6 ± 1·6 MΩ ($n = 21$) and R_2 was 8·9 ± 3·2 MΩ ($n = 21$). Recordings of membrane currents were stored in a Hewlett-Packard Vectra computer in combination with pCLAMP 5.5 software (Axon Instruments) operating a data acquisition board (Labmaster, 40 kHz DMA). These data were acquired at 5 or 10 kHz (capacitance measurements) and digitized at 2–4 kHz with pCLAMP 5.5. Off-line analysis was performed by using CLAMPFIT 6.

Results are expressed as means ± S.D., with n being the number of cells tested.

RESULTS

Recorded neurones had a mean resting membrane potential of −59·9 ± 8·4 mV (means ± S.D., $n = 15$), an input resistance of 300·7 ± 149·7 MΩ ($n = 26$), a somatic and proximal dendritic capacitance (C_1) of 87·6 ± 32·4 pF ($n = 21$) and a dendritic tree capacitance (C_2) of 147·3 ± 51·8 pF ($n = 21$). These neurones are located dorsal to the central canal mainly in the ventral half of the dorsal commissure and also in the pericanal area. They are fusiform in shape with the soma dorsoventrally orientated in transversal slices. They are observed through the entire extent of the thoracolumbar spinal cord.

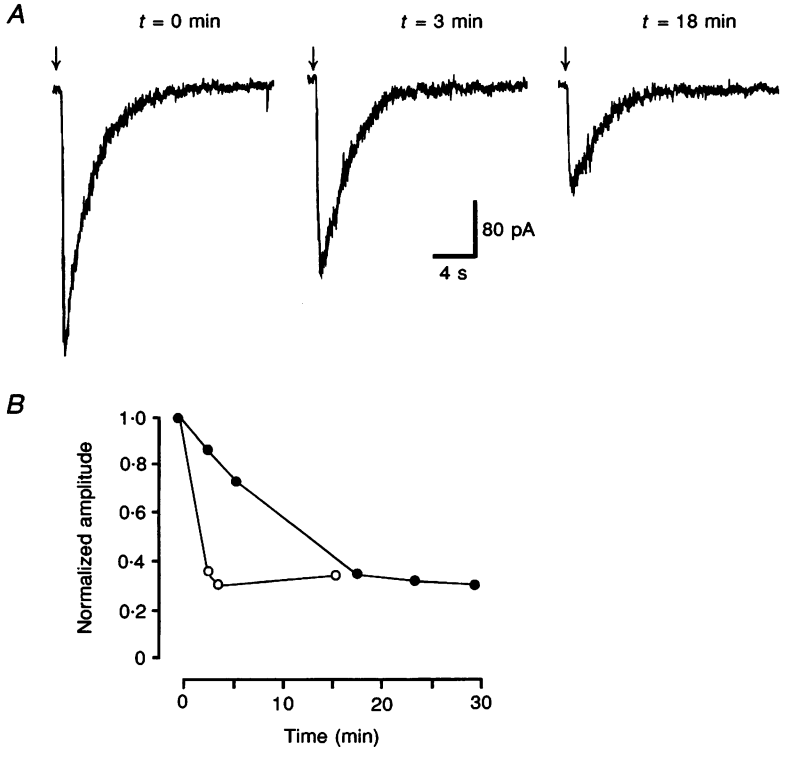

Figure 2. Desensitization of the DMPP response

A, repetitive pressure applications of DMPP (1 mM, 100 ms) induced inward currents of decreasing amplitude. The traces show control current in response to the first application of DMPP ($t = 0$) (left trace), response induced at 3 min (middle trace) and at 18 min (right trace). Peak current amplitudes were measured, normalized with respect to the first DMPP response and plotted against the time of recording in *B* (●). Holding potential (V_h) = −50 mV. *B*, normalized amplitude of the DMPP-induced currents plotted against time for two cells. ●, DMPP applications of 100 ms in the cell illustrated in *A*. ○, DMPP applications of 5 s in another cell. Desensitization observed for a 5 s DMPP application is faster than desensitization for an application of 100 ms.

In this subpopulation of neurones of lamina X, under current-clamp mode, brief pressure application of DMPP (1 mM, 100 ms) induced a depolarization crested with action potentials (Fig. 1*A*). Under voltage-clamp conditions, at −50 mV, DMPP evoked a transient inward current with an increase in membrane current noise (Fig. 1*B*).

The membrane current response followed a 100 ms pressure pulse of DMPP with an averaged latency of 204·3 ± 257·5 ms ($n = 28$) and reached its peak amplitude in 565·9 ± 522·3 ms ($n = 27$). The mean peak amplitude of the current induced by a 100 ms pressure pulse of DMPP was −280·0 ± 207·7 pA (ranging from −84 to 750 pA; $n = 28$). We did not find any correlation between peak amplitude of the current and latency or with base-to-peak rise time. At −50 mV, the mean amplitude of cytisine-induced current (100 ms, 1 mM) was −241 ± 124 pA ($n = 5$). In three cells challenged with both DMPP and cytisine (see Methods), cytisine induced an inward current of similar amplitude to the DMPP-elicited current (data not shown). TTX (1 μM, $n = 4$), a zero calcium solution ($n = 5$, see Methods) or $CdCl_2$ (100 μM, $n = 3$) did not abolish the DMPP-induced current, which confirmed a direct postsynaptic action of DMPP on recorded neurones. In zero calcium solution, the peak amplitude of the current was −260 ± 291 pA ($n = 4$), at − 50 mV. We noted that $CdCl_2$ reduced the amplitude of the DMPP-induced current (data not shown). This effect may be due to a channel block property of divalent cations on nAChRs.

Desensitization of the response to DMPP was apparent from the reduction of current amplitude observed in response to repeated agonist applications spaced by 3 min (Fig. 2*A* and *B*). At a holding potential of −50 mV, the percentage amplitude reduction of two DMPP-induced currents spaced by 2 or 3 min was 32·7 ± 23·1 % ($n = 15$). For a DMPP application of 5 s, a reduction of the amplitude of the subsequent response to DMPP was drastic and no recovery of the current was obtained for at least 15 min (Fig. 2*B*).

The response to DMPP was tested at different membrane potentials. A typical current–voltage (*I–V*) relationship for the DMPP-induced response is shown in Fig. 1*C*. This *I–V*

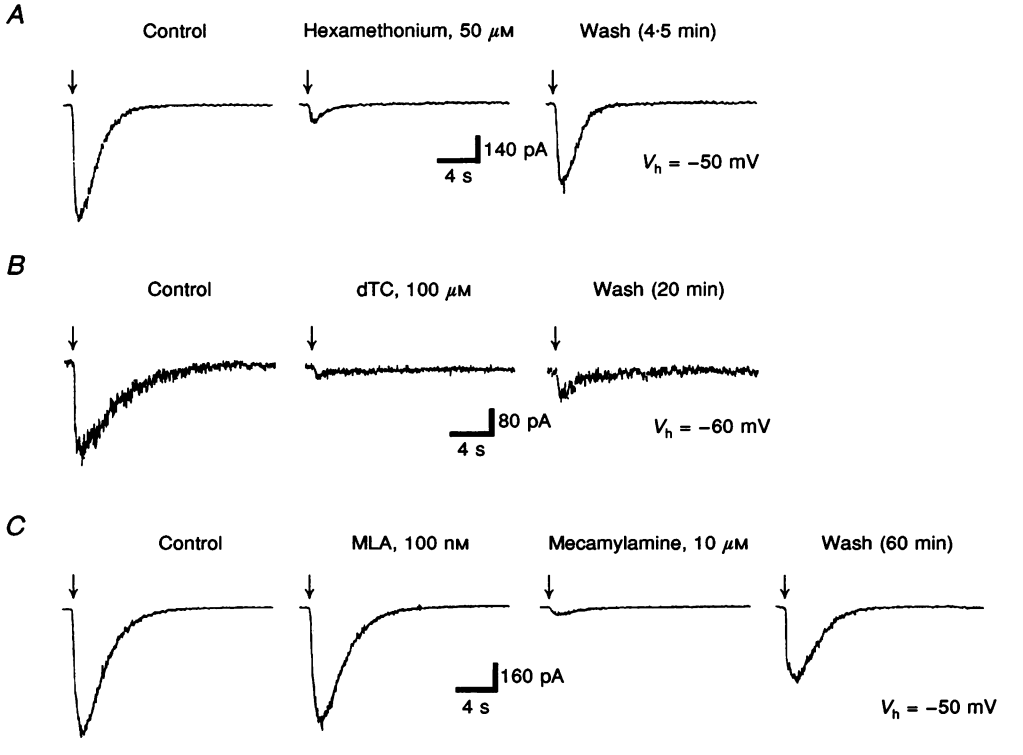

Figure 3. Antagonism of the DMPP-induced responses

A, DMPP-induced currents in control conditions (left), in presence of hexamethonium (1 min, 50 μM; middle trace) and after 4·5 min washout of hexamethonium. Holding potential was −50 mV. *B*, bath application of *d*-tubocurarine (dTC) (4 min, 100 μM) suppressed the DMPP-induced current evoked at −60 mV. The effect partially reversed after 20 min washout of dTC. *C*, DMPP-induced current was not affected by bath application of 100 nM methyllycaconitine (MLA) for 10 min. MLA was washed out for 20 min and mecamylamine (10 μM) was applied for 4 min. Mecamylamine blocked the current in a reversible manner after 60 min washout. Holding potential was −50 mV. DMPP (1 mM) was pressure applied for 100 ms at the times indicated by the arrows.

relationship has been performed in six cells showing little or no desensitization of the response as we used short applications (100 ms) of DMPP. The extrapolated reversal potential obtained from the results at negative potentials was around 0 mV. At positive potentials, no responses were obtained. Thus the DMPP-induced conductance exhibits very strong inward rectification. After a pressure ejection of DMPP at positive holding potentials, a consecutive application was performed at −50 mV to be sure of obtaining a response identical to the first response recorded at −50 mV. The value of the apparent reversal potential was compatible with a non-selective cationic current. Moreover, half-reduction of external sodium by substitution with mannitol decreased the response by 67% ($n = 3$) at a holding potential of −50 mV (data not shown).

The DMPP-induced current was almost completely blocked by various classical ganglionic antagonists: hexamethonium (50 μM, $n = 3$) (Fig. 3*A*), *d*-tubocurarine (dTC, 100 μM, $n = 3$) (Fig. 3*B*) and mecamylamine (10 μM, $n = 4$) (Fig. 3*C*) applied by bath perfusion (Fig. 3). α-Bungarotoxin (α-BGT), a potent nicotinic blocker at the neuromuscular junction, was tested after preincubation of the slice with the toxin for 45 min. αBGT (100 nM, $n = 3$) and methyllycaconitine (MLA, 100 nM, 10 min, $n = 3$) (Fig. 3*C*), a specific antagonist of the α-BGT-sensitive nAChR (Alkondon, Pereira, Wonnacott & Albuquerque 1992), did not affect the response to DMPP application.

DISCUSSION

The results of the present report indicate that neurones of the lamina X possess nicotinic acetylcholine receptors. These neurones are different from sympathetic preganglionic neurones of the IMM (Hosoya *et al.* 1994) on the basis of their different location in the spinal cord. SPNs are located at a more dorsal position to the central canal and are found in clusters along the spinal cord (Hosoya *et al.* 1994) whereas our recorded neurones are mainly in the ventral half of the dorsal commissure and are not restricted to discrete spinal levels.

Nicotinic receptor activation

The DMPP-induced current displayed classical characteristics of nicotinic receptors as regards its fast activation and its *I–V* relationship (see Role, 1992, for review). No attempt has been made to characterize the inactivation process further in this study since the kinetics of the response are determined by both the accessibility of the drug to cells and the washout of the drug in a slice. We observed a wide range of amplitudes of the DMPP-induced current. Such a variability in the current amplitude is unlikely to be explained by the accessibility of the drug to cells or by differences in the cell size since amplitude did not correlate with latency or with capacitance. This variability is likely to be due to a difference in the density of nAChR expression or by the expression of different subunits in the same subset of neurones (Alkondon & Albuquerque, 1993; see Role, 1992, for review). The presence of DMPP-induced current in these neurones is consistent with binding studies (Khan *et al.* 1994*b*) and *in situ* hybridization studies (Wada *et al.* 1989; Dineley-Miller & Patrick, 1992; Séguéla *et al.* 1993). In lamina X of the spinal cord, according to *in situ* hybridization studies, we can expect the presence of α2-, α3-, α4-, α7- and β2-subunits (Wada *et al.* 1989; Séguéla *et al.* 1993). No hybridization above background was detected for β4-subunits (Dineley-Miller & Patrick, 1992). Since the DMPP-induced current is blocked by classical ganglionic antagonists but not by α-bungarotoxin and MLA, we can probably eliminate the participation of the α7-subunit with the observed current. However, an α7 component may have escaped detection because the agonist application was too slow. According to Role (1992), we can expect a combination of α2–β2, α3–β2 or α4–β2 subunits.

Lamina X possesses the most widespread distribution of cholinergic neurones and terminals in comparison with other areas of the spinal cord (Barber *et al.* 1984). Since lamina X neurones are endowed with nAChRs and are well positioned to be contacted by cholinergic interneurones, they could together constitute a functional nicotinic cholinergic synapse.

Functional implication

Lamina X has been shown to receive inputs from dorsal horn and to be involved in the transmission and modulation of nociception (Nahin *et al.* 1983). Interneurones, including propriospinal neurones of lamina X, which are involved in such a modulation, are well positioned to influence SPNs of the IMM (Borges & Iversen, 1986; Lamotte, 1988; Hosoya *et al.* 1994) or motoneurones (Hochman, Jordan & MacDonald, 1994). It seems unlikely that our recorded neurones are the same as those involved in the production of the rhythmic motor activity and described by Hochman *et al.* (1994), as we never observed the oscillatory behaviour reported. We think instead that these neurones contribute to pain transmission and to the modulation of the sympathetic outflow, which have recently been reported to be modulated by nicotinic agonists (Khan *et al.* 1994*a*).

In conclusion, we have shown that a subpopulation of neurones of lamina X possesses functional nicotinic acetylcholine receptors. These neurones are good candidates to ensure a neuromodulatory function in the processing of the somato- and viscerosensory transmission. Moreover, they are well positioned to be the postsynaptic target of intraspinal cholinergic neurones. This arrangement is a promising model for studying a cholinergic synapse *in vivo*.

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