

Ovalbumin-induced sensitization affects non-quantal acetylcholine release from motor nerve terminals and alters contractility of skeletal muscles in mice

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Skeletal muscles play key roles in the development of various pathologies, including bronchial asthma and several types of auto-immune disorders, e.g. polymyositis. Since most of these maladies have an immunological/allergic element, this paper is devoted to assessing the impact of immunobiological reorganization on the functional properties of isolated skeletal muscles in mice. A combination of two methods (myography and electrophysiology) was used to evaluate extensor digitorum longus (EDL) and diaphragmatic muscle (DM) in this regard. Conventional myographic technique showed that ovalbumin-induced sensitization (OS) produced different changes in the contractile properties of EDL and DM. The amplitudes of carbachol (CCh)-induced contractions increased in DM but decreased in EDL. Those changes were inversely related to OS-mediated changes of non-quantal acetylcholine (ACh) release intensity within the muscle endplate, as shown by the electrophysiologically measured H-effect. These results clearly show that OS-mediated changes of non-quantal ACh release alter the functional properties of postjunctional ACh receptors and therefore contribute to the disturbance of CCh-induced contractility of skeletal muscles. Other mechanisms of OS-mediated changes of skeletal muscle contractility are also proposed and discussed.

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Skeletal muscle (SM) fibres of adult vertebrates differ markedly with respect to their contractile and metabolic properties. Slow-twitch (or type I) myofibres exhibit an oxidative metabolism, are rich in mitochondria, heavily vascularized and resistant to fatigue. In contrast, fast-twitch (or type II) fibres exhibit glycolytic metabolism, are involved in rapid bursts of contraction and fatigue rapidly. Myofibres can be further classified as type I, IIA, IIX/D or IIB, depending on the type of myosin heavy chain (MHC) isoform expressed (Pette & Staron, 2000). The heterogeneity of skeletal myofibres is reflected at the molecular level in that almost every protein involved in contraction (MHC, myosin light chain, troponin I, troponin T, troponin C, actin, etc.) has at least two isoforms expressed discretely in slow (type I) and fast (type II) fibres (Schiaffino & Reggiani, 1996). In adult animals, specialized myofibre phenotypes remain plastic

and vary in response to contractile load, hormonal milieu and systemic diseases (Baldwin & Haddad, 2001). Functional overload or exercise training results in transformation of pre-existing fast-twitch fibres to a slow-twitch, oxidative phenotype (Sugiura *et al.* 1993). Conversely, decreased neuromuscular activity induced by spinal cord injury, limb immobilization, space flight or blockade of action potential conduction causes a slow-to-fast myofibre conversion (Talmadge, 2000).

Interestingly, the immunobiological reorganization of homeothermic organisms is also accompanied by changes in morphofunctional condition of SM (Ado *et al.* 1984). It has previously been shown that ovalbumin-induced sensitization (OS), which is a widely accepted model of allergy, induced changes of contractile properties of slow and 'mixed-type' SM in guinea-pigs (Akhmedzyanov & Filippov, 1986). These data are in line with previous

Table 1. Biometric parameters of EDL and DM in non-sensitized and OS mice

	Extensor digitorum longus		Diaphragmatic muscle	
	Length (mm)	Weight (mg)	Length (mm)	Weight (mg)
Control	8.21 ± 0.14	7.57 ± 0.21	6.50 ± 0.27	7.00 ± 0.39
OS	9.00 ± 0.25	10.08 ± 0.40	6.14 ± 0.26	7.79 ± 0.53

findings that electrogenic properties of the plasma membrane of muscle fibers and histochemical profile of SM are altered at allergic conditions (Gushchin, 1973); however, the exact mechanism of altered SM contractility under OS remains unknown.

The pathophysiology underlying the disturbed SM contractile properties in allergy is of great interest, and SM is also the therapeutic target in some autoimmune diseases (Cognot *et al.* 1994). The mechanisms of impaired respiratory SM function (e.g. diaphragm) in different forms of chronic obstructive pulmonary disease and bronchial asthma (Barbarito *et al.* 2001) remain unclear. Furthermore, an allergic component is implicated in many SM disorders (e.g. polymyositis, myopathy, myodystrophy). Prospective studies on the pathophysiological mechanisms of these diseases are closely associated with the development of experimental models that permit evaluation of SM functional state in experimental pathology.

Here we used a combination of two methods (myography and electrophysiology) to study the influence of OS on the functions of isolated mouse SM. This paper delineates that OS differentially affects the carbachol (CCh)-induced contractility of the extensor digitorum longus (EDL, 'fast' muscle) and diaphragmatic muscle (DM, 'mixed-type' muscle). We also found that these alterations correspond to opposite effects of OS at the level of non-quantal (NQ) acetylcholine (ACh) release at the endplate region of SM fibres. The possible mechanisms underlying the effects of OS on SM functions are discussed.

Methods

Ovalbumin-induced sensitization

The experiments were carried out on white outbred mice of both sexes (weight 17–22 g). The ovalbumin sensitization of rodents (so-called OS-mice) was performed using a mixture of ovalbumin and Al(OH)₃ gel (2 µg of dry gel + 150 µg of ovalbumin in 0.5 ml of physiological saline solution as described in the following subsection) injected twice intraperitoneally (Gushchin *et al.* 1986). The second injection was performed 14 days after the first dose. The main experiment was carried out at the peak of the animals' sensitization, i.e. between 7 and 10 days after the second injection.

Preparation and solutions

Animals were euthanatized by decapitation under inhalational ether anesthesia in accordance with the European Communities Council Directive (86/609/EEC: November 24, 1986). Preparations were dissected and mounted in a recording chamber. The biometric parameters of the studied SM are presented in Table 1. The preparation was continuously perfused with physiological saline, containing (mM): 125 NaCl, 5 KCl, 2 CaCl₂, 1 MgSO₄, 1.25 NaH₂PO₄, 25 NaHCO₃ and 11 glucose. The solution was gassed with 95% oxygen and 5% CO₂, and pH was adjusted to 7.3 at 20°C. All reagents were obtained commercially from Sigma-Aldrich (St Louis, MO, USA).

Mechano-myography

Mechano-myographic experiments were carried out on isolated SM preparations under isometric conditions, which had been achieved by preliminary stretching of the SM for 40 min under 0.5 g force. The preparation was placed in a bath of volume 5 ml. Contractions were induced by application of the nicotinic ACh receptor (nAChR) agonist carbachol (CCh; 2×10^{-5} to 3×10^{-3} M) and registered using standard myographic techniques, as described previously (Grishin *et al.* 2006). For each contraction curve, the absolute force (Psc; in mg), relative force [$Rsc^* = Psc$ (in mg)/mass of preparation (in mg)], the speed (Vsc; in mg s⁻¹), shortening period (SP; in s), and half-relaxation period (RP/2; in s) of the SM contraction to submaximal and maximal concentrations of CCh were calculated.

Electrophysiology

For acetylcholinesterase inhibition, the preparations were treated with the irreversible cholinesterase blocker armin (10^{-5} M, 30 min; Tathimfarmpreparati, Kazan, Russia). The NQ ACh release, which causes depolarization of muscle fibres at the endplate zone, was estimated statistically by measuring membrane potentials (V_m) with glass micro-electrodes (filled with 2.5 M KCl; resistance 8–12 MΩ) in 20 or more fibres during a 5–7 min period before, and another 20 or more fibres 8–12 min after the addition of 10^{-5} M *d*-tubocurarine (dTC) to the solution (Galkin *et al.* 2001). The difference between the mean

resting V_m in these two sets of conditions is considered to be due to the NQ ACh release, and is termed the 'H-effect' (Katz & Miledi, 1977; Vyskocil & Illés, 1977).

Statistical analysis

The software package Microcal Origin 5.0 (OriginLab Corp., Northampton, MA, USA) was used for statistical analyses. All data are presented as means $\pm$ S.E.M., with significance assessed by Student's *t* test. A *P* value of less than 0.05 was considered as statistically significant.

Results

Ovalbumin-induced sensitization affects the contractility of fast and 'mixed-type' SM in an opposing manner

Application of CCh induced prolonged (i.e. tens of seconds of) contraction of myofibres (EDL, Fig. 1A; and DM, Fig. 1B). The contraction curve was characterized by a relatively fast shortening period (5–15 s) and much longer relaxation period (a few tens of seconds; Fig. 1).

The absolute (Psc) and relative contraction forces (Psc*) for fast SM (EDL) from non-sensitized mice in response to a submaximal CCh concentration (7×10^{-4} M) were 76.6 ± 6.5 mg and 9.9 ± 0.4 , respectively, with Vsc being

14.3 ± 1.6 mg s⁻¹ (Table 2). With OS, the Psc* decreased to 57.6% compared with non-sensitized muscle, without significant changes of contraction speed (Table 2). With a maximal CCh concentration (4×10^{-3} M), Psc, Psc* and Vsc in the control group were 103.8 ± 15.7 mg, 9.8 ± 1.1 and 11.2 ± 2 mg s⁻¹, respectively. The EDL muscles from OS mice showed decreased Rsc* (52% of control) and acceleration of contraction (Table 2).

In 'mixed-type' SM (DM) of non-sensitized mice, a submaximal CCh concentration (2×10^{-4} M) induced contraction with Psc of 342.8 ± 18.5 mg, Rsc* of 49.2 ± 1.8 and Vsc of 31.0 ± 1.7 mg s⁻¹ (Table 3). Ovalbumin-induced sensitization led to an increase of Psc* up to 119.3% of that seen with control muscles. The Vsc of DM contraction was practically the same (Table 3).

In the control group, the Psc, Psc* and Vsc of DM contractile responses to a maximal CCh concentration (1×10^{-3} M) were 544.7 ± 24.1 mg, 78.5 ± 2.6 and 36.8 ± 1.8 mg s⁻¹, respectively. Ovalbumin-induced sensitization caused a moderate increase in Psc* and Vsc (Table 3).

The contractile properties of fast-twitch fibres relative to 'mixed-type' muscles in mice are therefore differentially affected, in that contraction force is decreased in EDL (fast) but increased in DM ('mixed') as a result of OS. Interestingly, OS causes similar, but less prominent influences on the speed of contraction in EDL (deceleration) and DM (acceleration).

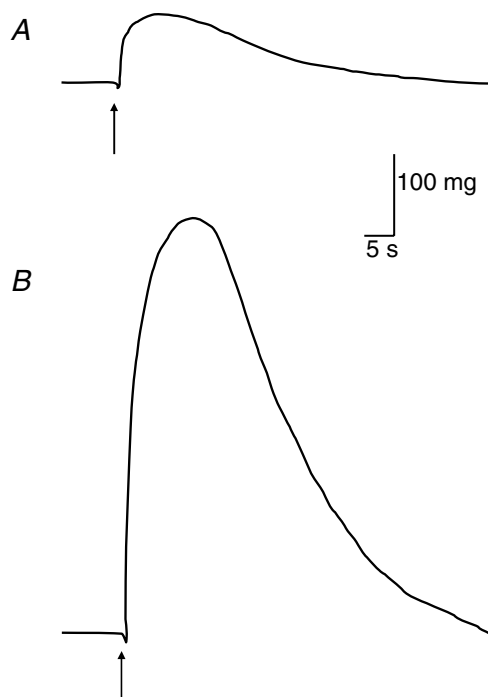


Figure 1. Carbachol-induced skeletal muscle contractions
The mechanograms represent the contraction curves of EDL (A) and DM (B) in response to the maximal CCh concentration (data of individual experiments). Arrows indicate the timing of CCh application.

Ovalbumin-induced sensitization differentially influences the intensity of NQ ACh release in neuromuscular junctions of fast and 'mixed-type' SM

The resting V_m of EDL of non-sensitized mice was 72.3 ± 0.6 mV ($n = 154$). Application of dTC increased V_m to 77.4 ± 0.9 mV; therefore, the H-effect was 5.1 ± 0.2 mV ($n = 154$). The EDL muscles from OS mice showed a resting V_m of 73.9 ± 0.5 mV ($n = 150$), which increased to 79.7 ± 1.1 mV ($n = 150$) in the presence of dTC. Thus, the H-effect in OS mice increased to 5.8 ± 0.2 mV ($n = 150$, $P < 0.05$), i.e. up to 113.7% of that seen in control animals.

The resting V_m of DM of non-sensitized mice was 70.7 ± 1.1 mV, and application of dTC induced an elevation of V_m to 75.9 ± 0.7 mV ($n = 160$); therefore, the H-effect was 5.2 ± 0.2 mV ($n = 163$). The resting V_m in DM of OS mice was 70.0 ± 1.0 mV and increased to 74.4 ± 0.6 mV in the presence of dTC ($n = 163$), suggesting that the H-effect decreased to 4.4 ± 0.3 mV ($P < 0.05$) relative to control animals (84.6%).

Taken together, these results indicate that OS differentially influences myofibres, in that NQ ACh release is facilitated in fast and depressed in 'mixed-type' SM.

Table 2. The parameters of EDL contractions induced by submaximal and maximal CCh concentrations in the control group and with ovalbumin-induced sensitization

Concentration of CCh	Experimental conditions	Contraction parameters		
		Psc (mg)	Psc*	Vsc (mg s ⁻¹)
Submaximal (7×10^{-4} M)	Control ($n = 26$)	76.6 ± 6.5	9.9 ± 0.4	14.3 ± 1.6
	OS ($n = 5$)	61.9 ± 12.4	$5.7 \pm 0.8^{\dagger\dagger\dagger}$	13.6 ± 4.1
Maximal (4×10^{-3} M)	Control ($n = 10$)	103.8 ± 15.7	9.8 ± 1.1	11.2 ± 2
	OS ($n = 5$)	$52.1 \pm 14.7^{\dagger\dagger\dagger}$	$5.1 \pm 0.8^{\dagger\dagger}$	$4.6 \pm 1.7^{\dagger}$

$^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$ compared with control conditions. Abbreviations: Psc, absolute force; Psc*, relative force; SP, shortening period; RP/2, half-relaxation period; Vsc, speed of contraction; and n is the number of experiments for each series.

Table 3. The parameters of DM contractions induced by submaximal and maximal CCh concentrations in the control group and with ovalbumin-induced sensitization

Concentration of CCh	Experimental conditions	Contraction parameters		
		Psc (mg)	Psc*	Vsc (mg s ⁻¹)
Submaximal (2×10^{-4} M)	Control ($n = 10$)	342.8 ± 18.5	49.2 ± 1.8	31.0 ± 1.7
	OS ($n = 7$)	$448.3 \pm 19.2^{\dagger\dagger\dagger}$	$58.7 \pm 4^{\dagger}$	32.1 ± 0.9
Maximal (1×10^{-3} M)	Control ($n = 10$)	544.7 ± 24.1	78.5 ± 2.6	36.8 ± 1.8
	OS ($n = 7$)	$641.1 \pm 17.9^{\dagger\dagger\dagger}$	85.21 ± 4.3	$40.1 \pm 0.8^{\dagger}$

$^{\dagger}P < 0.05$ and $^{\dagger\dagger\dagger}P < 0.001$ compared with control conditions. Abbreviations are as in the footnote to Table 1.

Discussion

Functional differences between fast and 'mixed-type' muscle fibres in non-sensitized mice

The absolute and relative forces of maximal CCh-induced contractions are around eight times higher in DM than in EDL of non-sensitized mice. This difference can be explained by the myosin composition of SM fibres, inasmuch as EDL contains 97–100% (Florendo *et al.* 1983) whereas DM is characterized by only 88.6% of 'fast' myosin (Blank *et al.* 1988). Another possible explanation stems from the peculiarities of the ultrastructure of the neuromuscular junction; the area of neuromuscular junction in fast SM is several times smaller than that seen in slow or 'mixed-type' SM (Fahim *et al.* 1984). Therefore, the higher sensitivity of DM to the cholinomimetic CCh could result from a larger number of nAChRs in the postjunctional membrane. This suggestion is also supported by a lower maximal CCh concentration in DM (1×10^{-3} M) than in EDL (4×10^{-3} M).

Ovalbumin-induced sensitization-induced alterations in skeletal muscles: contribution of synaptic mechanisms

We also revealed that OS alters the contractile properties of the isolated SM of mice (Tables 2 and 3); however, these changes differ in fast (EDL) from 'mixed-type' (DM) SM, which can be explained by: (i) differences in the initial morphofunctional status of these SM; and (ii) differences

in the processes of immunobiological reorganization in EDL and DM.

We found that the OS-mediated changes of force parameters in the two different SM types have opposite directions. The fast SM (EDL) showed decreased contraction force (Table 2), whereas in 'mixed-type' SM (DM), on the contrary, the absolute and relative forces were increased (Table 3).

Ovalbumin-induced sensitization also alters EDL sensitivity to CCh, in that the submaximal CCh concentration in non-sensitized mice triggers a maximal contraction in muscle from OS mice. Thus, OS leads to increased EDL sensitivity to the cholinomimetic CCh, presumably due to potentiation of nAChR activity.

All the changes occurring in SM during OS could theoretically affect the plasma membrane, including postjunctional membrane with a high density of nAChRs (Ado *et al.* 1984), electromechanical coupling (Gushchin, 1973) or the system of contractile proteins (Deviatev & Valiullin, 1994). The overwhelming majority (about 90%) of ACh is released from motor nerve terminals of mammals in an NQ form (Malomouzh & Nikol'skii, 2007). Continuous exposure of postsynaptic nAChRs to ACh induces their desensitization. Thus, NQ ACh release regulates the functional state of nAChRs, in that the more NQ-ACh is released, the more nAChRs are desensitized.

In our studies, the H-effect, which reflects the intensity of NQ ACh release, was measured electrophysiologically. We found that OS differentially affects the intensity of NQ ACh release in different SM types; it increases in fast and decreases in slow SM. Considering the influence of

NQ ACh release on the functional state of nAChRs, we suggest that altered NQ ACh release intensity contributes to changes of contractility. Indeed, increased NQ ACh release in EDL enhances nAChR desensitization, and therefore reduces the amplitude of the CCh-induced endplate potential and force of contraction. In comparison, decreased NQ ACh release occurring in DM reduces the degree of nAChR desensitization, and therefore increases the amplitude of endplate potential and force of contraction in response to CCh.

It is impossible to provide the quantitative correlation between the changes of H-effect and the resulting alterations of CCh-induced contractility; however a relatively small change of NQ ACh release intensity (within 20%) suggests the presence of other mechanisms that also contribute to contractility impairments in OS. Possibly, the alterations of NQ ACh release in OS serve as a negative feedback mechanism for regulation of nAChR activity in OS. Based on the above explanations, EDL sensitivity to CCh is increased in OS. In this case, increased NQ ACh release decreases nAChR activity.

Conclusion

We revealed that OS produces opposite changes of EDL and DM contractile properties; the amplitudes of CCh-induced contractions were increased in DM and decreased in EDL. These changes were inversely related to OS-mediated changes of NQ ACh release intensity within the muscle endplate, as shown by electrophysiological experiments. We concluded that OS-mediated changes of NQ ACh release alter the functional properties of postjunctional ACh receptors, and therefore contribute to disturbed SM contractility. We suggest that alterations of NQ ACh release in OS may serve as a negative feedback mechanism for regulation of nAChR activity in OS. These results broaden our understanding of the pathogenesis of allergic diseases.

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