

RAPID COMMUNICATION

Spontaneous Quantal and Non-Quantal Release of Acetylcholine at Mouse Endplate During Onset of Hypoxia

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Summary

At 20 °C, both quantal and non-quantal spontaneous acetylcholine release (expressed as miniature endplate potential frequency [f-MEPPs] and the H-effect, respectively) increased during the first 30 min of hypoxia in solution with normal extracellular calcium ($[Ca^{2+}]_o = 2.0$ mM). The hypoxia-induced tenfold increase of the f-MEPPs was virtually absent in low calcium solution ($[Ca^{2+}]_o = 0.4$ mM) whereas there was still a significant increment of non-quantal release. This indicates that each of these two processes of acetylcholine release is influenced by mechanisms with different oxygen sensitivity. The rise of f-MEPPs during the onset of hypoxia apparently requires Ca^{2+} entry into the nerve terminal, whereas the non-quantal release can be increased by another factors such as a lower level of ATP.

Key words

Miniature endplate potential • Acetylcholine • Quantal release • Non-quantal release • Hypoxia

Because of the high energy consumption, nerve tissue requires sizeable oxygen supply (e.g. Benešová *et al.* 2004) as compared with other tissues (Kolář and Ošťádal 2004, Jones *et al.* 2004). The risk of reduction in oxygen intake by the brain is of high clinical importance (Lischke *et al.* 2004a,b,c) can cause a rapid and complete loss of excitability and asphyxia may lead to a breakdown of brain activity and irreversible release of intracellular potassium into the interstitial space, which is the marker of the cell death (Vyskočil *et al.* 1972). Synaptic transmission at the peripheral neuromuscular endplate

also suffers by a deficiency in oxygen (Lipton 1999) and its failure is the main reason for muscular weakness during ischemia (Eccels *et al.* 1966). Oxygen deficiency studies at peripheral synapses mostly describe the action of chronic hypoxia. In contrast to central synapses (Katchman and Hershkowitz 1993), there is not much information about synaptic activity, both evoked and spontaneous, at the periphery during the onset of hypoxia. Reports about spontaneous quantal release are rather controversial. Some authors describe Ca^{2+} -dependent increase of the miniature endplate potential frequency

(Hubbard and Løyning 1966), others state that this increase cannot be removed by lack of Ca^{2+} or by increase of Mg^{2+} concentration in the medium (Nishimura *et al.* 1984, Nishimura 1986).

Nothing is known about the effect of hypoxia on another route by which acetylcholine (ACh) can leave the nerve terminal, i.e. on spontaneous non-quantal release which was demonstrated at the mammalian endplate in particular (Katz and Miledi 1977, Vyskočil and Illes 1977). Therefore, the purpose of the present report is to compare both quantal and non-quantal types of ACh release and their dependence on external calcium at the muscle endplate during the first 30 min of reduction in the partial tension of oxygen.

Diaphragm muscles with a 10-12 mm nerve stump were dissected rapidly from male adult mice (10-16 g body weight) sacrificed under ether anesthesia by cervical dislocation and immediate decapitation. The muscle strips were pinned with stainless steel needles to layer of silicone rubber on the bottom of a 3.5 ml perspex chamber and superfused at a rate of 5 ml/min with a oxygenated (95 % O_2 and 5 % CO_2) Krebs-Ringer solution containing (in mM): NaCl 120.0, KCl 5.0, CaCl_2 2.0, MgCl_2 1.0, TRIS base 10, glucose 11.0, pH 7.3. Krebs-Ringer solution was enriched with 1×10^{-5} M choline chloride (Serva, Germany) to prevent spontaneous decrease of the non-quantal release (Nikolsky *et al.* 1991). For cholinesterase inhibition, the preparations were treated with an irreversible anticholinesterase inhibitor 1×10^{-5} M armin (diethoxy-p-nitrophenyl phosphate, Institute of Organic Chemistry, Moscow, Russia) for 30 min and then rinsed several times for 15 min with normal saline. Experiments were performed at 20-21 °C.

The non-quantal release which causes depolarization of muscle fibers at the endplate zone was quantified statistically by measuring membrane potentials with glass microelectrodes (filled with 2.5 M KCl, tip resistance 8-12 M Ω) in 20 or more fibers during a 5-7 min period before, and another 20 or more fibers 8-12 min after the addition of 1×10^{-5} M (+)tubocurarine (TC, Sigma) to the medium. The differences between the mean resting membrane potentials (RMP) under these two conditions are generally considered to be due to the non-quantal release of ACh. In each group, 4-8 muscles from several mice were used (H-effect, Katz and Miledi 1977, Vyskočil and Illes 1977, Vyskočil *et al.* 1983). The amount of O_2 in the solution was measured with the aid of the oximeter ISO2 connected with OXEL-Y electrode

(Word Precision Instruments, USA). In the control, the content of O_2 was 16 % of the atmospheric pressure corrected for altitude and 20 °C. Lowering of O_2 was achieved by bubbling the perfusion solution with nitrogen (95 % N_2 and 5 % CO_2). The superfusing chamber with muscle was covered with two slide glass for averting the oxygen from the air. Only small round opening remained available for the recording microelectrode. During the replacement of control solution by the nitrogen-saturated one, the level of oxygen in the chamber decreased to 8 % within 7-8 min and remained constant for at least 60 min.

Spontaneous miniature endplate potentials (MEPPs) were recorded from one endplate before and during hypoxia, if stable microelectrode impalement made it possible and counted by a decadic counter connected with a discriminator unit (AXON Instruments, CA). In most cases, 150-200 events from the bell-shaped part of the amplitude histogram were captured from each fiber in the control and 15-20 min after application of low O_2 solution, and analyzed by a computer for frequency, amplitudes, rise times of EPCs (from 10 to 90 % of the maximal amplitude) and exponential decay constants τ_{dec} . Tukey-Kramer multiple comparisons test for one-way analysis of variance (ANOVA) was performed using GraphPadInStat program (GraphPad Software, Inc., San Diego, CA) for statistical analysis of data, and a value of $p < 0.05$ was considered to indicate a significant difference between two groups of experimental data. Data are presented as mean \pm S.E.M.

The effect of hypoxia on the resting membrane potential

First of all, it was necessary to assess whether resting membrane potential (RMP) of the small-diameter mouse muscle fibers (25-40 μm) could be influenced by hypoxia, because this parameter is fundamental for correct measurement of the postsynaptic responses. The control RMP in the solution with 16 % O_2 was 72 ± 1 mV for (inside negative, five muscles, 20 fibers each). It did not change significantly for at least one hour and was 70 ± 2 mV ($p > 0.05$) when measured 50-60 min after application of 8 % O_2 *via* nitrogen-saturated solution.

The effect of hypoxia on the spontaneous quantal release

The frequency of miniature endplate potentials (f-MEPPs *per second*) was 1.38 ± 0.30 in control oxygenated solution (16 % O_2). It increased ten times when O_2 in the solution was lowered to 8 % (Fig. 1B) and the maximum f-MEPPs (11.17 ± 0.32 ; 7 endplates) was observed in the period between 15 and 20 min after the

low oxygen reached the plateau in the solution (Fig. 1A, cf. Vyskočil *et al.* 1985). The amplitude, rise time and decay time of MEPPs was unchanged in low O₂ solution (Table 1) which means that there were no differences in

quantum size, postsynaptic sensitivity to ACh and ACh receptor-channel opening in endplates bathed in either 16 % or 8 % O₂ solution.

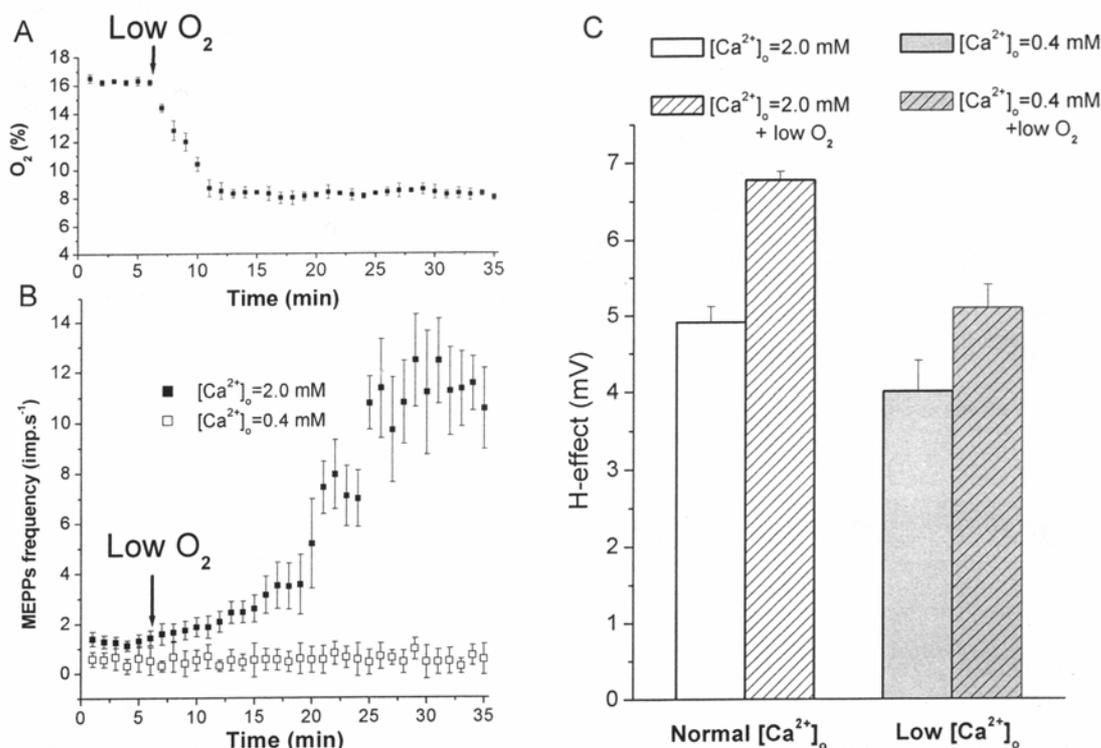


Fig. 1. Time course (abscissas in min) of effect of hypoxia (**A**, ordinate O₂ in %) on MEPPs frequency (**B**, in impulses per sec) and H-effect (**C**, in mV) at the mouse neuromuscular junction at 20 °C. In **A**, oxygen tension was measured continuously and recorded on every minute of each experiment. Two concentrations of extracellular calcium ([Ca²⁺]_o = 2.0 mM, full squares and left columns in **C**; and [Ca²⁺]_o = 0.4 mM, empty squares in **B** and right columns in **C**) were tested in control (16 % O₂) and low (8 % O₂, arrows in **A** and **B**) oxygen solutions. Vertical bars show S.E.M. of the mean of 7 determinations.

Table 1. Amplitude (A), rise time (RT) and decay time constant (τ_{dec}) of mEPPs in control solution (16 % O₂) and 15-30 min after the application of hypoxic solution (8 % O₂)

16 % O ₂			8 % O ₂		
A	RT	τ_{dec}	A	RT	τ_{dec}
0.29±0.09	0.74±0.11	3.00±0.96	0.28±0.16	0.78±0.14	3.07±0.46

Mean values ± S.E.M. are given.

Because the changes in f-MEPPs depend to a great extent on extracellular [Ca²⁺]_o (Baker 1972), following experimental series was done in low [Ca²⁺]_o solution (0.4 mM). The f-MEPPs was 0.47±0.09 (n=4) in control 16 % O₂ solution, but no rise of f-MEPPs in 8 % O₂ solution was observed, in contrast to high Ca²⁺ solution.

Effect of hypoxia on the spontaneous non-quantal release

The H-effect (which is a measure of non-quantal release) was 4.91±0.21 mV (n=4, 80 fibers) in 16 % O₂ solution and – similarly to f-MEPPs – it significantly increased to 6.78±0.11 mV (by 38 %, n=4, 80 fibers, p<0.05) during hypoxia. In contrast to f-MEPPs, which was stable in Ca²⁺-low solution, H-effect increased also in 0.4 mM [Ca²⁺]_o solution during hypoxia. In accordance

with previous observation (Vyskočil *et al.* 1983), the H-effect was smaller in low calcium (4.0 ± 0.4 mV, $n=4$, 80 fibers) and increased to 5.1 ± 0.3 mV (by 27 %, $n=4$, 80 fibres, $p < 0.05$) when N_2 -saturated solution was applied (Fig. 1C).

Both f-MEPPs and H-effect increased during the onset of hypoxia in solution with normal 2.0 mM $[Ca^{2+}]_o$. The hypoxia-induced increase of f-MEPPs was, however, absent in low 0.4 mM $[Ca^{2+}]_o$ whereas there was still significant increment of the non-quantal release. It means that the rise of f-MEPPs during onset of hypoxia apparently needs the Ca^{2+} entry into the nerve terminal. Under conditions of normal O_2 , Ca^{2+} is extruded rapidly from nerve ending and its concentration is kept low. However, the lack of oxygen might hinder the ATP supply for ion exchange and higher Ca^{2+} , which persists in hypoxic terminals, may cause the rise in quantal output (Katchman and Hershkowitz 1993).

In contrast to spontaneous quantal release which remained unchanged when less $[Ca^{2+}]_o$ is available (Samigullin *et al.* 2005), the non-quantal release increased during anoxia and this increase was present even in low $[Ca^{2+}]_o$. In low $[Ca^{2+}]_o$, absolute level of the H-effect was decreased by 18 %, as compared with the normal solution. This is in accordance with our previous report where the bell-shaped dependence of the H-effect on external Ca^{2+} concentrations was described with maximum at 2 mM Ca^{2+} (Vyskočil *et al.* 1983, Fig. 1). The increase of non-quantal release during the onset of anoxia in both normal and low $[Ca^{2+}]_o$ points to a negligible role of external calcium. However,

intracellular sources of Ca^{2+} can still be considered; the lack of oxygen would cause the lowering of proton gradient and membrane potential of the inner mitochondrial membrane which subsequently triggers the release of Ca^{2+} through Na^+ -induced Ca^{2+} release from the mitochondria (Hashimoto *et al.* 1992, Zhang and Lipton 1999). Non-quantal release could then rise just because it follows the upward part of the Ca^{2+} /H-effect curve (Vyskočil *et al.* 1983, Fig. 1). However, the increase in cytosolic Ca^{2+} from mitochondria would also affect the quantal release (Vyskočil and Moravec 1974) and one could therefore expect the rise of MEPPs frequency. As we found no such increase in the f-MEPPs, this mechanism seems to be less probable.

On the other hand, the increase of non-quantal release can be explained by lower concentration of ATP available at the endplate during hypoxia. It has recently been demonstrated that ATP inhibits – through presynaptic metabotropic P_2 receptors – the H-effect (Galkin *et al.* 2001). If ATP is progressively exhausted during hypoxia in both nerve terminals and muscle fibres (Hagberg 1985, Speriagh and Vizi 1996), its lowering can unblock the partly depressed non-quantal release, leaving the quantal release unchanged. Further experiments in this respect are in progress.

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