Effect of PAR1 Agonist on Acetylcholine Secretion in a Newly Formed Neuromuscular Synapse in Mice

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Peptide agonist of PAR1 in a concentration of 10 μM significantly facilitated neuromuscular transmission in newly formed synapses in mice. The absence of changes in the amplitude of miniature end-plate potentials attests to presynaptic mechanism of the effect of PAR1 agonist. The effect of the peptide was blocked by protein kinase A inhibitor H89 (1 μM). Blockade of inositol-1,4,5-trisphosphate receptors with 2-aminoethoxydiphenylborate (30 μM) did not prevent the effects of PAR1 agonist. Inhibition of protein kinase C with bisindolylmaleimide (1 μM) facilitated neuromuscular transmission in newly formed synapses. Protein kinase C inhibition was associated with reversal of the object of PAR1 agonist: transmission inhibition instead of facilitation.

Key Words: *protease-activated receptors-1; newly formed motor synapses; protein kinase A; protein kinase C*

The role of serine proteases and their receptors (protease-activated receptors, PAR) in the central and peripheral nervous systems is intensely studied [9]. It was demonstrated that low thrombin concentrations protect neural and glial cells from injuries and these effects are mediated mainly by PAR1 [2,13]. PAR1 were detected on axons of developing motoneurons and on the postsynaptic membrane of newly formed neuromuscular contacts in mammals [6,12]. Activation of PAR1 during the early postnatal development promotes elimination of excessive nerve terminals and maturation of acetylcholine receptor clusters [6,7]. Trauma of a peripheral nerve is paralleled by enhanced expression of prothrombin and PAR1 mRNA in the damaged nerve and denervated muscle [5,8]. In addition to delayed effects of thrombin, its acute effects on PAR were described. Application of thrombin on developing neurons and myocytes in culture increases intracellular concentration of Ca^{2+} [4,13].

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Thrombin and PAR1 agonist peptide (PAR1-AP) facilitate regeneration of mouse peripheral nerve after its crushing [1]. Thrombin capacity to facilitate synaptic transmission in regenerating synapses has not been studied.

We studied possible acute pre- and postsynaptic effects of PAR1-AP in mouse newly formed motor synapses.

MATERIALS AND METHODS

Experiments were carried out on male albino mice (20-25 g). The mice were narcotized with ether and *n. peroneus communis* of the left hind paw near the knee joint was crushed with ophthalmic forceps with fine (1 mm) flat branches protected with plastic end-pieces. The length of the crushed nerve fragment was 1 mm.

On day 12 after surgery, the animals were decapitated and *m. extensor digitorum longus* and *n. peroneus communis* were isolated. The resultant neuromuscular preparation was placed into a box with normal Lilly solution (pH 7.2-7.4) of the following composition: 135 mM NaCl, 1 mM $MgCl_2$,

4 mM KCl, 0.9 mM NaH_2PO_4, 2 mM $CaCl_2$, 11 mM glucose, and 16 mM $NaHCO_3$, oxygenated with carbogen (96% O_2, 4% CO_2). Modified high-potassium solution Lilly (for depolarization of nerve terminals) contained 7.8 mM NaCl, 1 mM $MgCl_2$, 20 mM KCl, 0.9 mM NaH_2PO_4, 2 mM $CaCl_2$, 11 mM glucose, 16 mM $NaHCO_3$, 61.7 mM sucrose, and 55.6 mM Na_2SO_4.

Miniature end-plate potentials (MEPP; at least 100 per synapse) and end-plate potentials (EPP; at least 50) were recorded intracellularly with glass microelectrodes (5-10 MΩ resistance) filled with 2.5 M KCl. Muscle contractions were blocked by adding 0.4-1.2 μM d-tubocurarine to the solution. For registration of solitary EPP the nerve was stimulated with 1-3-V pulses 0.1-0.2 msec duration at a frequency of 0.3 Hz.

The records were processed using Axotape and MiniAnalysis software. The significance of differences between linked samples was evaluated by Wilcoxon test using Statistica software.

The following reagents were used: PAR1-AP (Ser-Phe-Phe-Leu-Arg-Asn-NH_2) and control peptide devoid of the first amino acid and incapable of PAR1 activation (Russia), bisindolylmaleimide (BIM) and H-89 (Calbiochem), 2-aminoethoxydiphenylborate (2-APB; Tocris) and d-tubocurarine (Sigma).

RESULTS

Study of the effect of PAR1-AP on EPP in mature neuromuscular synapse showed that application of PAR1-AP in a concentration of 10 μM did not change the amplitude and the time of EPP increase and half-decay (3.75±0.23 mV, 1.27±0.06 msec, and 1.75±0.05 msec in the control; 3.68±0.18 mV, 1.25±0.05 msec, and 1.68±0.04 msec, respectively; m=5, n=31, where m is the number of studied animals and n number of studied synapses). Hence, activation of thrombin PAR1 in mature neuromuscular synapse virtually did not modify the parameters of evoked activity.

Effects of PAR1-AP application on spontaneous and evoked activities of newly formed neuromuscular synapses were studied on day 12 after nerve crushing. The mean frequency of MEPP in the reinnervated muscle on day 12 after nerve crushing was 0.29±0.02 Hz (m=5, n=20). In order to simplify MEPP analysis, the terminal was depolarized by increasing $[K^+]_{HAP}$ to 20 mM. This led to an increase of MEPP frequency from 0.29±0.02 Hz (in normal Laily's solution) to 1.93±0.32 Hz (in solution with high $[K^+]_{HAP}$) (m=5, n=25). Addition of PAR1-AP in a concentration of 10 μM under these conditions significantly reduced MEPP frequency to 1.13±0.09 Hz (m=5, n=69, p<0.01). Application of PAR1-AP caused no changes in MEPP amplitude: the mean MEPP amplitude in the control was 0.42±0.03 mV, in the experiment 0.46±0.01 mV. Changed frequency of MEPP in the absence of changes in MEPP amplitude suggests presynaptic mechanism of PAR1-AP effect on spontaneous secretion of the transmitter in newly formed neuromuscular synapse.

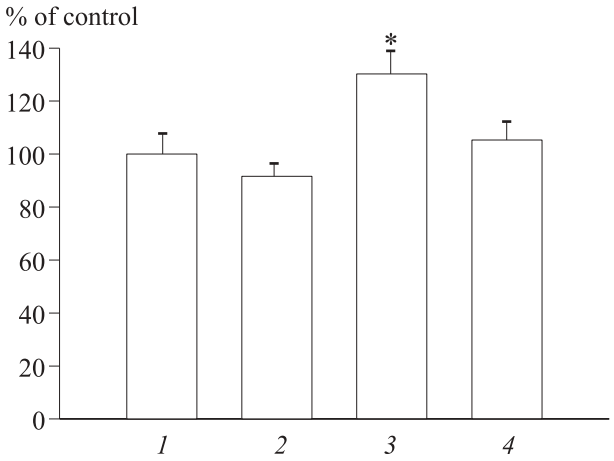

Fig. 1. Changed amplitude of EPP on day 12 after nerve crushing in control (*1*), after application of 1 μM (*2*), 10 μM PAR1-AP (*3*), and "control" peptide in a concentration of 10 μM (*4*). *p<0.05 compared to the control.

In the next experimental series, we studied the effects of PAR1-AP on parameters of evoked activity of newly formed neuromuscular synapses. Solitary EPP in the new synapses differed significantly from EPP in intact mature synapses: normally the mean EPP amplitude for curare-treated preparations was 3.75±0.23 mV, with the increment time 1.41±

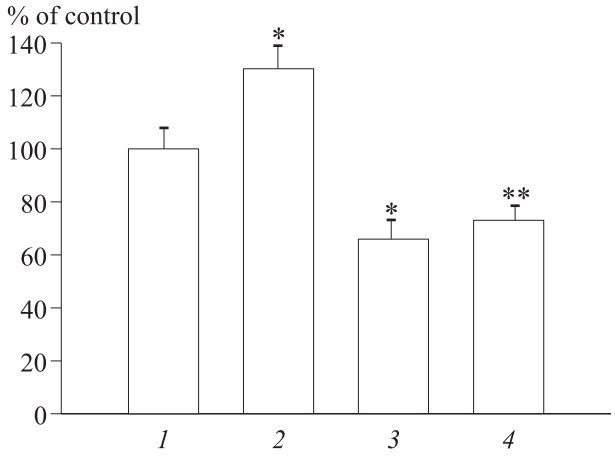

Fig. 2. Changed amplitude of EPP in new synapses in control (*1*), after application of 10 μM PAR1-AP (*2*), 1 μM H89 (*3*), and 10 μM PAR1-AP in the presence of 1 μM H89 (*4*). *p<0.05, **p<0.01 compared to the control.

0.06 and half-decay time 1.94±0.06 msec. On day 12 after nerve crushing, the EPP amplitude was 1.46±0.11 mV, the increment and half-decay times for solitary EPP were 2.01±0.13 msec and 2.68± 0.18 msec, respectively. Application of PAR1-AP in a concentration of 1 μM did not change the parameters of solitary EPP in new synapse (m=5, n=37), but increase in agonist concentration to 10 μM significantly increased EPP amplitude, the summary increment was 30% from the control (m=5, n=53, p<0.05; Fig. 1), while time parameters did not change. The effect of PAR1-AP on EPP developed slowly: the EPP amplitude increased over 2 h and was 1.70±0.14 mV during the first 30-60 min and 2.09±0.20 mV during the interval of 60-120 min. The peptide lacking the first amino acid and incapable of activating thrombin PAR1 in the same concentration caused no changes in EPP amplitude (m=4, n=29). Hence, PAR1-AP in a concentration of 10 μM facilitates neuromuscular transmission in mouse newly formed synapses. Presumably, this effect is mediated by activation of thrombin PAR1, because "control" peptide exhibited no effect of this kind.

Blockers of different stages of intracellular signal transmission were used for studying possible mechanisms of the facilitating presynaptic effect mediated via PAR1 activation.

Protein kinase A blocker H89 in a concentration of 1 μM significantly reduced the amplitude of solitary EPP by 44% in comparison with the control (m=3, n=16, p<0.05), this effect developed very slowly and manifested only 60 min after application. The time parameters of EPP did not change. In the next experimental series, H89 application was followed (after 1 h) by application of 10 μM PAR1-AP. Application of PAR1-AP in the presence of H89 caused no additional changes in the amplitude: the amplitude of solitary EPP decreased by 37% in comparison with the control (m=4, n=40, p<0.01; Fig. 2). Hence, the facilitating effect of PAR1-AP did not manifest under conditions of protein kinase A blockade.

Thrombin PAR1 in hippocampal and other neurons can react with G_q protein activating phospholipase C with the formation of inositol-1,4,5-triphosphate and diacylglycerol. Inositol triphosphate induces Ca^{2+} release from intracellular depot via a receptor-mediated mechanism, while diacylglycerol together with Ca^{2+} ions participates in activation of protein kinase C [9]. Inositol triphosphate receptor blocker 2-APB (30 μM) significantly reduces the amplitude of solitary EPP within 60 min after application. The amplitude of EPP decreased by 29.7% in comparison with the control (m=4, n=21, p<0.01). The time parameters of EPP (increase and half-decay time) did not change under the effect of 2-APB. Application of PAR1-AP after 2-APB led to a significant increase in EPP amplitude, similar to its increment after application of PAR1-AP alone: the amplitude of a solitary EPP in a newly formed synapse increased by 26.3% (m=3, n=20, p<0.05; Fig. 3, *a*). Hence, blockade of inositol triphosphate receptors inhibited neuromuscular transmission in new synapses, but did not prevent the development of facilitating effects of thrombin PAR1 activation.

It was shown that blockade of protein kinase C in intact synapses significantly reduced evoked secretion of the transmitter [10]. However, blockade of protein kinase C in new synapse with BIM (1 μM) caused a rapid increase in the amplitude of solitary EPP by 27% in comparison with the control

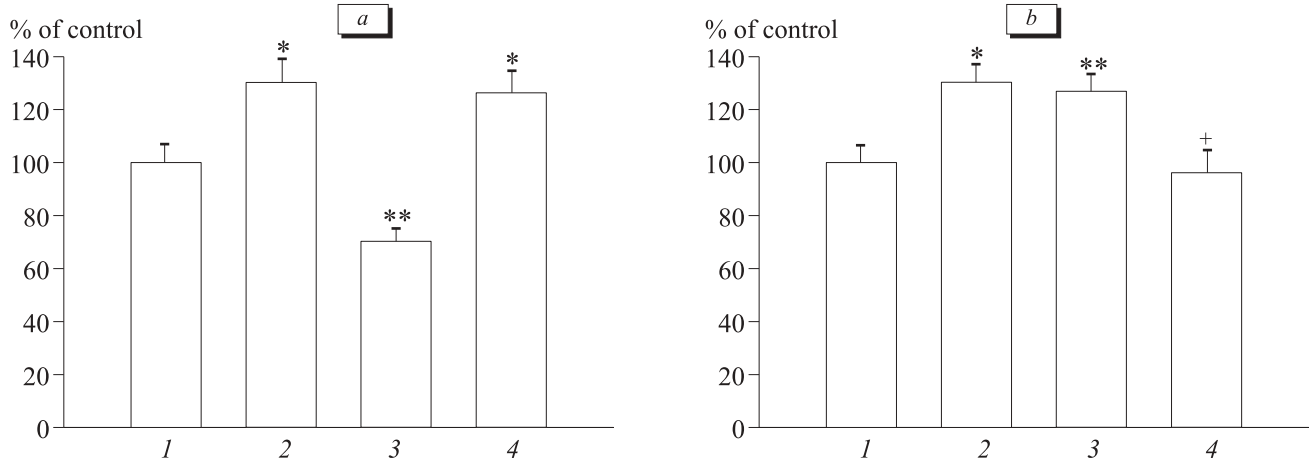

Fig. 3. Changed amplitude of EPP in new synapses. *a*) EPP amplitude in control (*1*), after application of 10 μM PAR1-AP (*2*), 30 μM 2-APB (*3*), and 10 μM PAR1-AP in the presence of 30 μM 2-APB (*4*); *b*) EPP amplitude in control (*1*), after application of 10 μM PAR1-AP (*2*), 1 μM BIM (*3*), and 10 μM PAR1-AP in the presence of 1 μM BIM (*4*). *p<0.05, **p<0.01 compared to the control, ^{+}p<0.05 compared to EPP amplitude during BIM treatment (1 μM).

(m=6, n=32, p<0.01), while time parameters of EPP remained unchanged. Under these conditions PAR1-AP (10 μM) exhibited an unexpected inhibitory effect: the amplitude of solitary EPP decreased to the initial control level and was lower than after BIM application (m=3, n=26, p<0.05; Fig. 3, *b*).

Hence, PAR1-AP in a concentration of 10 μM does not modify synaptic transmission in mature intact synapses and significantly (30%) increased the amplitude of solitary EPP in mouse new synapses. This is not paralleled by changes in the amplitude of MEPP, which suggests a presynaptic mechanism of PAR1-AP effect, when the increment of EPP amplitude results from increased quantum content of EPP and level of evoked secretion of the transmitter (acetylcholine).

It was found that inhibitors of two enzymes (protein kinases A and C) had reciprocal effects on acetylcholine secretion: inhibition of protein kinase A inhibited acetylcholine release, while inhibition of protein kinase C facilitated it. Both enzymes modulate acetylcholine release in intact mammalian motor synapses [11]. Our findings suggest that protein kinases A and C in newly formed synapses also participate in the regulation of acetylcholine secretion and, in addition, are involved in PAR-AP effect on transmitter release. Inhibition of protein kinase A activity by H89 prevented the increment of EPP amplitude induced by PAR1-AP. This could result from blockade of processes regulated by protein kinase A (such as modulation of endothelial NO-synthase, cAMP, Rho A kinase) participating in the realization of the effects of PAR1 in axon growth cone and in other cell systems [3,14].

Inhibition of protein kinase C with BIM facilitated acetylcholine secretion. This is in line with the data on reduction of EPP amplitude in new synapses during protein kinase C activation [7]. Blockade of protein kinase C and subsequent facilitation of acetylcholine secretion led to reversion of the presynaptic effect of PAR1-AP, which suppressed acetylcholine secretion in the presence of BIM. It was shown that the effect of thrombin on PAR1 is paralleled by activation of tyrosinkinases and subunits of G-proteins in cells [9]. Cascades of reactions (with participation of protein kinase C and other kinases), induced by this treatment, can regulate the same function (for example, platelet secretion), but be in intricate, including reciprocal, relations [15]. Presumably, replacement of the facilitating effect of PAR1-AP by the inhibitory effect in the presence of protein kinase C inhibition reflects similar situation in newly formed terminals of synapses characterized by high plasticity.

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