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ARTICLES

The Role of Adenosine Receptors and L-Type Calcium Channels in the Regulation of the Mediator Secretion in Mouse Motor Synapses

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Abstract—The changes in spontaneous and evoked neurotransmitter release caused by agonists and antagonists of the A1- and A2A-subtypes of adenosine receptors combined with inhibition of some enzymes and voltage-dependent calcium channels of L-type were studied in mouse diaphragm motor synapses using intracellular microelectrode recordings of miniature endplate potentials (MEPPs) and evoked endplate potentials (EPPs). Simultaneous activation of presynaptic A1 and A2A receptors by endogenous adenosine during short-term rhythmic activity of motor synapses was shown for the first time. Activation of receptors A1 prevails and is followed by downregulation of the ACh release due to inhibition of intracellular cascade, which involves protein kinase A (PKA) and L-type voltage-dependent calcium channels. Activation of receptors A2A with their agonist CGS-21680 caused upregulation of the ACh secretion due to enhancement of PKA activity followed by activation of L-type voltage-dependent calcium channels. The mechanism of the evoked release potentiation, when A1 receptors are blocked or the activity of A2A receptors prevails over that of A1 receptors, involves calcium release from ryanodine-sensitive intracellular calcium stores coupled with PKA and activation of L-type voltage-dependent calcium channels. It was found that protein phosphatase calcineurin participates in downregulation of the L-type voltage-dependent calcium channels irrespective of the A1 receptors. It was shown for the first time that disinhibition of L-type voltage-dependent calcium channels caused by the calcineurin inhibition requires participation of the activity of A2A receptors and PKA. In conclusion, reciprocal interactions between presynaptic receptors A1 and A2A and their effect on the ACh release were shown in motor synapses. These interactions are mediated by the following cascade: PKA → L-type voltage-dependent calcium channels → ryanodine receptors of calcium stores. Final effect on the neurotransmitter release depends on conditions of coactivation of these receptors and interplay of enzymes and L-type voltage-dependent calcium channels within synaptic terminals.

Keywords: A1 and A2 adenosine receptors, protein kinase A, calcineurin, L-type calcium channels, ryanodine receptors

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INTRODUCTION

Presynaptic adenosine receptors of type A1 and A2 are found in the terminals of the neuromusclular synapses in skeletal muscles [1, 2]. Receptors of both types are metabotropic and in the presence of endogenous adenosine can exert opposite effects on the acetylcholine (ACh) release: facilitating, upon activation of receptors A2A, and inhibiting, upon activation of receptors A1 [3, 4]. It is suggested that one of the target of receptors A1 and A2A is the L-type calcium channels [5, 6]. These channels are expressed in motor terminals but generally remain in the inactive latent state and do not influence the ACh release [7, 8]. However, as the inhibition of these channels is abolished, they are able to modulate both calcium signals and the ACh secretion [9–11]. Some factors and enzymes controlling functional transition of L-type calcium channels from silent to active state are known [11–13]. However, the involvement of the A1 and A2A receptors and the respective signaling cascades in the evoked ACh release in motor synapses remains poorly explored. In this connection, the goals of this study were: (i) to reveal the intracellular enzymes and targets related with the activity of A1 and A2A receptors and their engagement in the regulation of the L-type calcium channels and ACh release and (ii) to elucidate the role of the stored calcium in the ACh release facilitation mediated by the activation of adenosine receptors and L-type calcium channels.

MATERIALS AND METHODS

Nerve–muscle preparation. Experiments were carried out on nerve–muscle preparations *m. diaphragma–n. phrenicus* isolated from adult (P30) male mice

(strain BALB/c) obtained from the Laboratory of experimental animals, Biology Department, MSU (Moscow, Russia). The animals were kept according to Directive 86/609/EEC; experimental protocol was approved by the Bioethics Committee of the Biology Department at MSU. Overall, 41 animal was used in the study. Mice were sacrificed by quick decapitation.

Electrophysiology. The dissection of muscle fibers was performed according to a standard protocol [10, 11, 13]; this procedure made it possible to simultaneously record the spontaneous and evoked mediator release [10, 11, 13]. The left part of the diaphragm with the diaphragmal nerve (*n. phrenicus*) was placed into a 3-mL chamber and superfused at room temperature with an oxygenated (95% O_2, 5% CO_2) Liley solution (pH 7.2–7.4) containing (in mM): NaCl, 135; KCl, 4; NaH_2PO_4, 0.9; $CaCl_2$, 2; $MgCl_2$, 1; $NaHCO_3$, 16.3; glucose, 11 (all reagents from Sigma–Aldrich, USA). Immediately after the transverse dissection of the muscle fibers (before the registration of synaptic responses) the nerve–muscle preparation was washed thoroughly in a large volume of the Liley solution (>150 mL) for at least 1 h. Owing to this procedure, a possible blockage of the action potential conduction is prevented and membrane potential (MP) is stabilized at a level somewhat lower than in non-dissected muscle fibers. All experiments were performed at 20–22°C. Intracellular recordings of the spontaneous miniature endplate potentials (MEPP) and of the multiquantal endplate potentials (EPP) evoked by the nerve stimulation were carried out using glass microelectrodes filled with 2.5 M KCl (microelectrode tip resistance was 15–20 MOhm). To study the rhythmic activity of synapses, diaphragmal nerve was stimulated by pulse trains (50 pulses, amplitude above threshold, pulse duration 0.08–0.1 ms, frequency 50 Hz). The applied protocol of a short-term high-frequency stimulation of the nerve (50 Hz, 1 s) with 4-min intervals between the stimulation sessions was evolved in a great number of earlier experiments. This regime of the synaptic activity ensured stability of the EPP parameters and EPPs train pattern and fully prevented a possible decline of the EPP and MEPP amplitudes in different synapses of a muscle in the course of the experiment due to synaptic fatigue or desensitization of the muscle nicotinic acetylcholine receptors. In each synapse studied, MEPP were recorded for 100 s immediately before the nerve stimulation (mean value of the MEPP amplitudes recorded within this period was used for the calculation of the quantal content of EPP) and right after the pulse train. In none of the synapses used for the analysis, the difference between the mean values of the MEPP amplitudes before and after the short stimulation was statistically significant. The values of MP of the muscle fibers after their dissection and washing varied in the range from –38 to –45 mV and remained stable through the recording period of 1–1.5 h; accordingly, MP of muscle fibers did not fall down during the experiment. Signals were acquired using amplifier Axoclamp-2B (Molecular Devices, USA) or Neuroprobe Amplifier Model 1600 (A-M Systems, USA) and recorded with the aid of analog-digital converter E-154 (L-Card, Russia) with PowerGraph software onto the PC hard drive for the consecutive analysis employing MiniAnalysis software (Synaptosoft, USA). As a control, MEPP and EPP from 5 or more different synapses were recorded; after that, substances under study were added into the perfusion solution in a certain order and the activity of various synapses was recorded for 1–1.5 h. Membrane potential was continuously monitored throughout the signal recordings in each synapse; if the value of membrane potential dropped by more than 5 mV, the recording was stopped and the data acquired from this synapse were not included into the sample population for the further analysis. In each experimental series at least three nerve–muscle preparations were used.

Data processing and statistical analysis. Membrane potentials of muscle fibers were measured, as well as the MEPP and EPP amplitudes and time course and the MEPP frequency. The MEPP and EPP amplitudes were standardized to the membrane potential of –50 mV (to correct the changes of the driving force of the voltage shifts upon the MP changes). Quantal content of EPP was calculated as a ratio of the mean standardized EPP amplitude corrected to nonlinear summation [14] to the mean standardized MEPP amplitude. Statistical significance between sample means was assessed using the Student's *t*-test (in the case of normal distribution) or Mann–Whitney criterion. The difference was considered significant at $p < 0.05$ (n is a number of the synapses studied).

Materials. Antagonist of A2A receptors ZM241385, agonist of A2A receptors CGS-21680, and blocker of A1 receptors DPCPX were obtained from Sigma–Aldrich, USA; cyclosporin A (calcineurin inhibitor), nitrendipine (blocker of L-type calcium channels), ryanodine, and H-89 (PKA blocker) were purchased from Enzo Life Sciences, USA. Stock solutions of all drugs except for ryanodine were prepared in DMSO; ryanodine was dissolved in distilled water. Final concentrations of DMSO in working solutions did not exceed 0.01% (vol/vol); at these concentrations DMSO did not affect the parameters of the spontaneous and evoked activity in mouse motor synapses.

RESULTS

To reveal the contribution of each subtype of adenosine receptors in the regulation of the ACh release in mouse motor synapses, the receptors were blocked. First, receptors of subtype A2A were blocked using selective antagonist ZM241385 (10 nM). No significant changes in the mean frequency and amplitude of MEPP (Figs. 1a, 1b) were observed. The amplitude and quantal content of all 50 EPPs within the short trains (50 Hz, 1 s) did not change in the presence of ZM241385 either. The mean amplitude of the first EPP

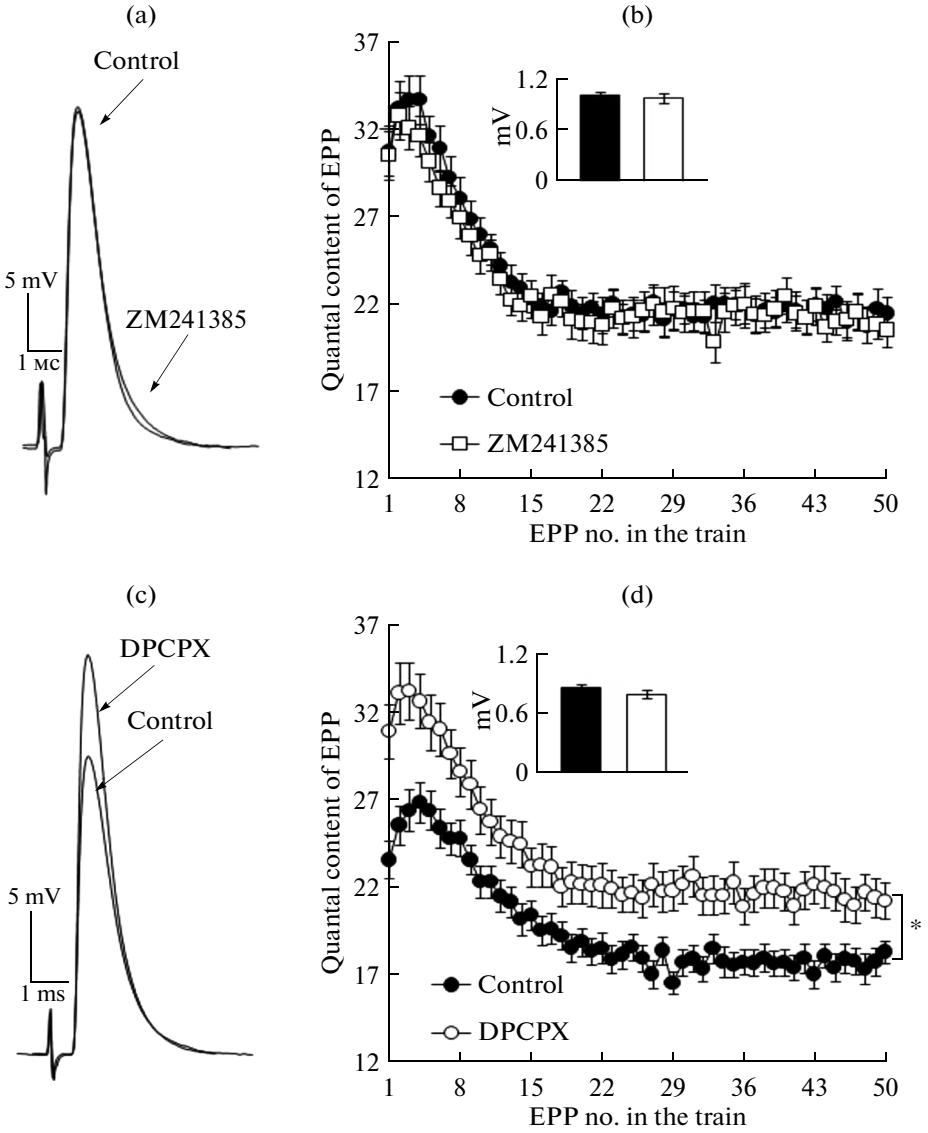

Fig. 1. Effects of selective blockers of adenosine receptors on the evoked release of ACh. (a) Averaged traces of the first EPP in the pulse train in control ($n = 21$) and in the presence of 10 nM ZM241385 ($n = 19$). (b) Changes in the quantal content of EPP in the train in control conditions and in the presence of ZM241385. (c) Averaged traces of the first EPP in the train in control conditions ($n = 24$) and in the presence of 100 nM DPCPX ($n = 24$). (d) Changes in the quantal content of EPP in the train in control conditions and in the presence of DPCPX. *Inserts*, mean amplitude of MEPP in control conditions and in the presence of blockers of the respective adenosine receptors. *The value differs significantly from the respective control value, $p < 0.05$.

in the train was 30.5 ± 2.4 mV ($n = 19$) in the presence of ZM241385 (10 nM), while in the control conditions it was 31.3 ± 2.2 mV ($n = 21$, $p > 0.05$) (Figs. 1a, 1b). Similarly, the amplitudes of the EPPs next to the first one remained unchanged. Correspondingly, the quantal content of the first and the following EPPs in the train did not change in the presence of ZM241385: in its absence (control) the quantal content of the first EPP was 30.7 ± 1.5 and in the presence of ZM241385, it was 30.5 ± 1.4 ($p > 0.05$) (Figs. 1a, 1b). This suggests that in the given mode of the synaptic activity, the A2A receptors activation by the endogenous adenosine is either absent or is not sufficient to affect the Ach release.

Blockade of A1 receptors by DPCPX (100 nM) did not alter significantly the frequency, amplitude, and temporal parameters of MEPP but considerably facilitate the synaptic transmission, which was manifested as a uniform increase of the amplitude and quantal content of the first and following EPP in the train by 26–30% on the average, as compared to control conditions (Figs. 1c, 1d). This could imply that in working synapses activation of the A1 receptors and consequent suppression of the evoked release of Ach could occur. It was important to determine, which intracel-

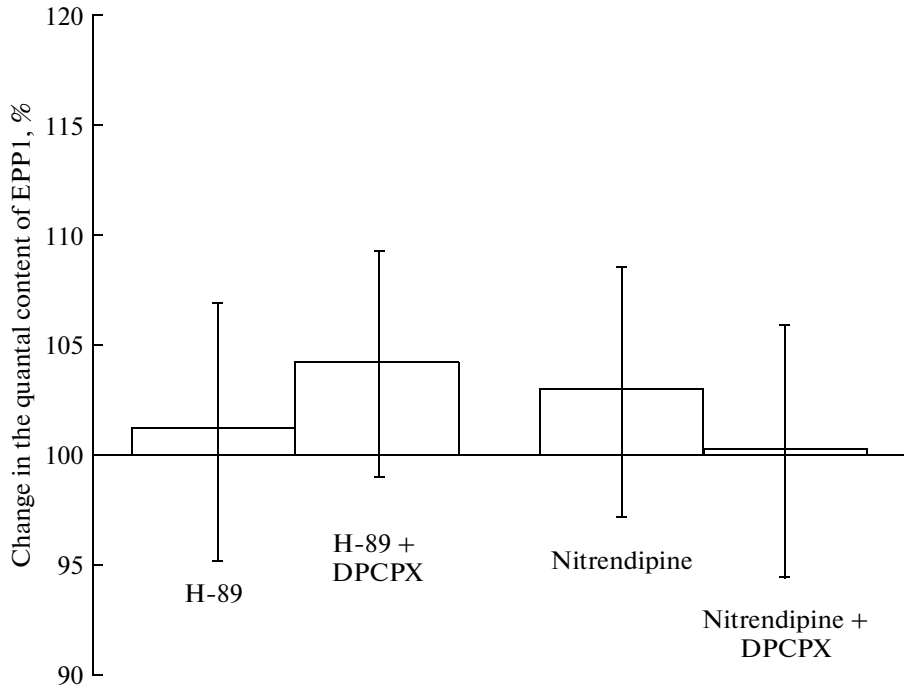

Fig. 2. Alterations in the quantal content of the first EPPs in the discharges (EPP1), normalized to the control values (taken as 100%), in the presence of 1 μM H-89 ($n = 22$) and after the addition of 100 nM DPCPX in the presence of H-89 ($n = 23$); in the presence of nitrendipine (10 μM; $n = 30$) and after the addition of DPCPX in the presence of nitrendipine ($n = 27$). Changes of the quantal content of the first EPPs in the trains (EPP1), normalized to the control value (taken as 100%), in the presence of 1 μM H-89 ($n = 22$) and after the addition of 100 nM DPCPX in the presence of H-89 ($n = 23$); in the presence of nitrendipine (10 μM) ($n = 30$) and after the addition of DPCPX in the presence of nitrendipine ($n = 27$).

lular mechanism implements this inhibitory effect observed upon activation of receptors A1 by the endogenous adenosine.

According to the published data, the A1 receptors coupled with $G_{i/o}$ proteins are able to suppress the activity of adenylate cyclase (AC) and thus decrease the PKA activity [15]. Therefore, the effects of DPCPX were further studied in the conditions of the preliminary blockade of PKA. The addition of the PKA inhibitor H-89 (1 μM) did not change significantly the quantal content of the first and subsequent EPPs in the train in comparison to the control conditions: the quantal content of the first EPP was 25.4 ± 1.4 ($n = 17$) and 25.7 ± 1.5 ($n = 22$, $p > 0.05$) in the absence and presence of H-89, respectively. The mean frequency, amplitudes and temporal parameters of the MEPPs did not change either. However, H-89 completely prevented the increase in the EPP amplitude and quantal content in the train observed upon by the blockade of the A1 receptors (Fig. 2). Thus, the inhibition of the Ach release induced by the activation of receptors A1 is indeed associated with the suppression of the PKA activity and targets of its phosphorylating activity. We suggested that a possible target of PKA in this case could be the L-type calcium channels [5]. In fact, it turn out that the preliminary blockade of the L-type calcium channels by nitrendipine (10 μM), as well as the blockade of PKA by H-89, prevented the DPCPX-induced increase of the EPP quantal content in the train; nitrendipine per se did not affect the spontaneous secretion and the EPP parameters in the train (Fig. 2). This suggests that when the activity of the A1 receptors is switched off by DPCX, not only the functional link AC → PKA is disinhibited but also the L-type calcium channels, being the PKA targets. These channels (when active) are known to considerably facilitate the Ach release.

As the applied protocols (50 Hz, 1 s) revealed only the activation of inhibitory A1 receptors, which trigger the inhibition of the intracellular cascade AC → PKA → L-type calcium channels leading to the suppression of the Ach release, we further checked whether a stronger activation of A2A receptors by an exogenous agonist could overcome this inhibition and produce a facilitation of the Ach release. Selective agonist of the A2A receptors CGS-21680 (1 μM) did enhance the evoked bursting Ach release: the amplitude and quantal content of all EPPs in the train significantly increased by 18–20% in comparison with control. For the first EPP in the train, the quantal content was 33.3 ± 1.4 ($n = 19$) in control and 39.4 ± 1.8 ($n = 28$, $p < 0.05$) in the presence of CGS-21680 (Figs. 3a, 3b). The values of the MEPP amplitude (1.05 ± 0.05 mV) and frequency (0.98 ± 0.08 Hz) in the presence of CGS-21680 did not differ significantly from the control values (0.95 ± 0.05 mV and 0.93 ± 0.09 Hz, respectively; $p > 0.05$).

It was important to check if the same intracellular cascade involving PKA and L-type calcium channels (which is inhibited when A1 receptors are activated) participates in the facilitation induced by CGS-21680 activating A2A receptors. For this purpose, in the next series of experiments we tested the effects of CGS-21680 in the conditions of preliminary inhibition of PKA or blockade of the L-type calcium channels. It was found that in the presence of H-89 (1 μM) the CGS-21680-induced activation of the A2A receptors did not lead to the increase of the amplitude and quantal content of the EPPs forming the rhythmic train. For example, in control conditions, the quantal content of the first EPP in the train was 34.8 ± 2.4 ($n = 17$); in the presence of H-89, it was 35.8 ± 1.8 ($n = 15$), and upon subsequent addition of CGS-21680, 31.9 ± 1.5 ($n = 21, p > 0.05$) (Fig. 3b). The CGS-21680-induced increase of the amplitude and quantal content of each EPP in the train (by 18–20% on the average, as compared to the control values) was abolished upon the addition of 1 μM nitrendipine (Fig. 3c).

Thus, adenosine receptors of subtypes A1 and A2A are able to exert reciprocal effects on one and the same intracellular cascade, and in both cases the ultimate target is the L-type calcium channels. It is known, however, that the activity of these channels can be modulated not only by adenosine receptors but by a wide range of regulators. In particular, we showed recently that calcium channels of L-type are subjected to an inhibitory influence of phosphatase calcineurin [11]. Therefore, we further studied how the activity of receptors A2A facilitating the functioning of calcium channels of the L-type interacts with the activity of calcineurin inhibiting these channels.

Inhibition of calcineurin by 1 μM cyclosporine A (CsA) resulted in an increase of the amplitude and quantal content of EPP by 20% on the average, equally through the whole train. Preliminary blockade of A2A receptors by 10 nM ZM241385 (which by itself did not affect the quantal content of the EPP in the train) fully prevented the facilitating effect of CsA on the evoked Ach release (Fig. 4). Moreover, in the presence of CsA blocking calcineurin and considerably increasing the quantal content of EPP throughout the whole train of 50 EPP, consequent addition of 1 μM CGS-21680 did not cause any further increase of the quantal content of EPP (Fig. 4). Thus, in the case of A2A receptors are preliminary blocked, cancellation of the inhibitory influences on the L-type calcium channels upon inactivation of calcineurin does not lead to the facilitation of the Ach release. This may indicate that when motor synapses work in the mode of short high-frequency trains, not only activation of the A1 receptors occurs (as was believed earlier) but also a concomitant and functionally significant activation of the A2A receptors by endogenous adenosine. Activation of receptors A2A (in contrast to A1) does not appreciably affect the evoked Ach release but is required for the maintenance of the refractory condition of L-type calcium channels and the possibility of their disinhibition.

It is believed that in the case of disinhibition of the L-type calcium channels in the motor terminals, their activity is sufficient for the amplification of the calcium signal in the terminals and potentiation of the calcium-dependent Ach release [6, 10, 12]. However, as it was first shown in our studies, activation of the L-type calcium channels is usually accompanied by the activation of the associated ryanodine-sensitive intracellular calcium stores and amplification of the calcium signal that upregulates the Ach release [11, 16].

To verify a possible involvement of the ryanodine receptors (RyR) and calcium release from the intracellular stores in the facilitation of the mediator secretion induced by either activation of the A2A receptors or inhibition of the A1 receptors, we studied the effects of CGS-21680 and DPCPX, respectively, in the presence of ryanodine (3 μM) that blocks RyR. Ryanodine (3 μM) by itself altered the train pattern: it suppressed the initial facilitation of the Ach release and insignificantly decreased the quantal content of the first EPP in comparison with the control values [11, 16]. When RyR were blocked, neither the activation of the A2A receptors by CGS-21680 nor the blockade of the A1 receptors by DPCPX produced any increase of the quantal content of EPP in the train (Figs. 1c, 1d, 3a, 3b, and 5). This means that activation of RyR and calcium release from the intracellular stores is a must for the potentiation of the evoked Ach secretion in the case of the activation of presynaptic adenosine receptors of the A2A subtype.

Overall, our data suggest the presence in mouse motor terminals of an intracellular cascade, which is stimulated or suppressed due to the reciprocal activity of the presynaptic adenosine receptors of subtypes A2A and A1, respectively. This cascade includes the following sequence of participants: adenylate cyclase → PKA → calcium channels of L-type → RyR (release of calcium from the intracellular stores) → modulation of the Ach secretion. Implementation of this cascade also involves phosphatase cacineurin that modulates the activity of the L-type calcium channels together with adenosine receptors.

DISCUSSION

Experiments performed here have shown that inhibition of the A1-type adenosine receptors by selective antagonist causes a considerable increase in the quantal content of the rhythmically evoked EPPs. At the same time, blockade of the A2A-type receptors by their antagonist ZM241385 does not alter the mediator release. This kind of data obtained on peripheral motor synapses [17] or central synapses [18, 19] are commonly interpreted as a confirmation of the inhibitory action of adenosine on the mediator release through the activation of receptors A1 without any involvement of receptors A2A, despite their presence

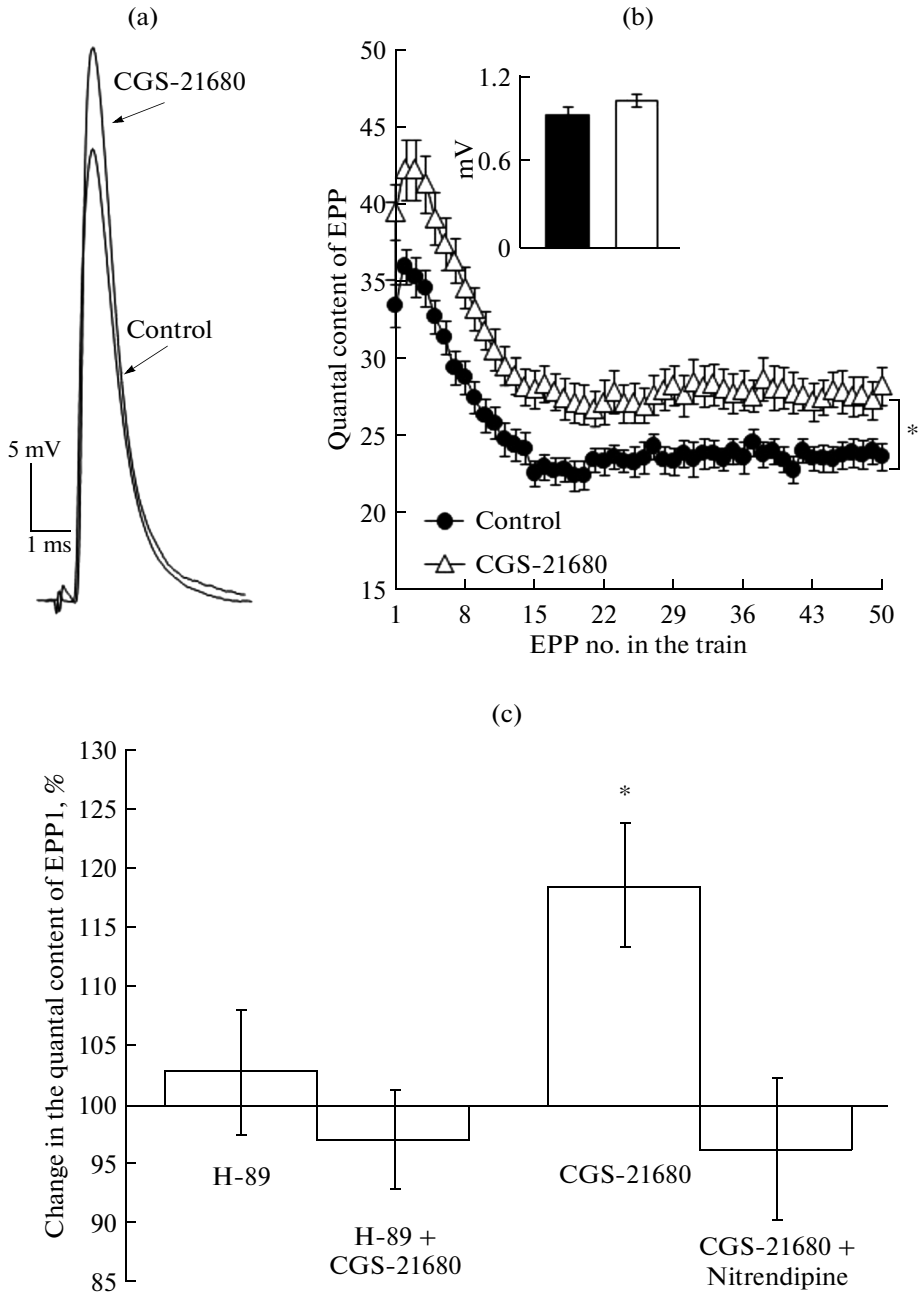

Fig. 3. (a) Averaged traces of the first EPP (EPP1) of the discharge in the absence (Control, $n = 19$) and in the presence of the A2A receptor agonist CGS-21680 (1 μM; $n = 28$). (b) Changes in the EPP quantal content throughout the train in the absence (control) and presence of CGS-21680; in the *insert*, mean amplitude of MEPP in the absence (control) and presence of CGS-21680. (c) Changes of the quantal content of the first EPPs in the trains (EPP1), normalized to the control value (taken as 100%), in the presence of 1 μM H-89 ($n = 11$) and after the addition of 1 μM CGS-21680 in the presence of H-89 ($n = 21$); in the presence of CGS-21680 ($n = 19$) and after the addition of 1 μM nitrendipine in the presence of CGS-21680 ($n = 14$). *The value differs significantly from the respective control value, $p < 0.05$.

in the terminals. However, our work shows for the first time that in intact synapses, in which only inhibitory action through the A1 receptors can be observed at first sight, but in actual fact a concomitant persisting activation of the A2A receptors by adenosine takes place, which facilitates the activity of the L-type calcium channels. In favor of such possibility testifies the fact that the removal of inhibitory influences on calcium channels of L-type upon inactivation of calcineurin does not produce facilitation of the Ach secretion, once the A2A receptors are preliminary blocked. This may suggest that in working motor syn-

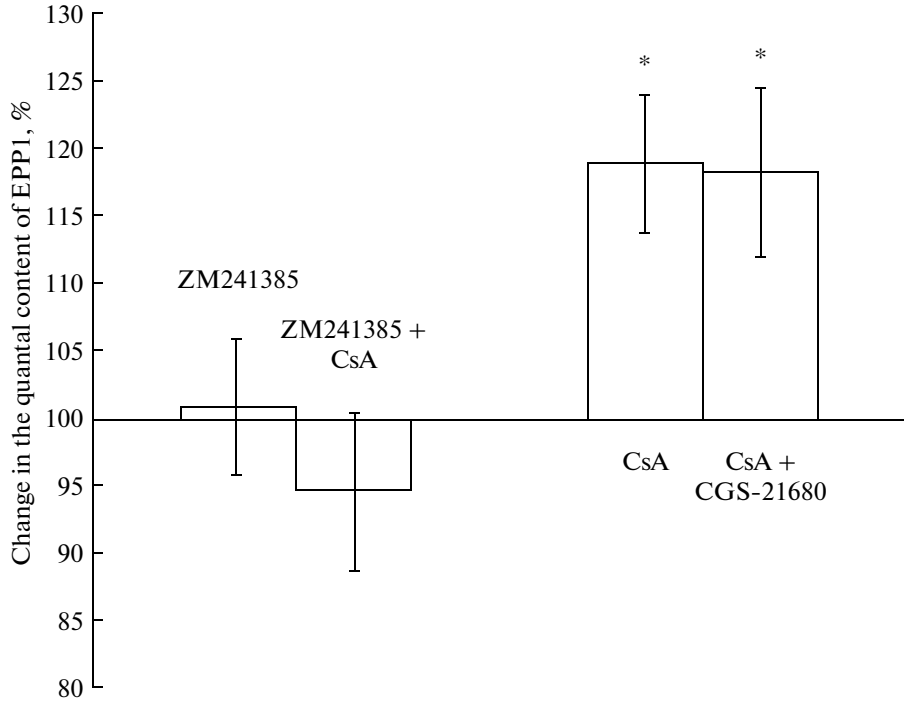

Fig. 4. Alterations in the quantal content of the first EPPs in the trains (EPP1) normalized to the control value (taken as 100%) in the presence of 10 nM ZM241385 ($n = 19$) and after the addition of 1 μM CsA in the presence of ZM241385 ($n = 18$); in the presence of 1 μM CsA ($n = 34$) and after the addition of 1 μM CGS-21680 in the presence of CsA ($n = 27$). *The value differs significantly from the respective control value, $p < 0.05$.

apses, along with the activation of A1 receptors, a persisting and functionally significant activation of A2A receptors by the endogenous adenosine takes place. This activation does not notably affect the evoked release of Ach but is required for the maintenance of the functional status of the latent L-type calcium channels and the possibility of their disinhibition upon removal of the inhibitory influences on these channels.

Reciprocal changes in the evoked release of Ach upon modulation of A1 and/or A2A receptors by the selective agonists, antagonists, and adenosine have been described in a number of works assessing the release of [^{3}H]-Ach from the terminals of motor synapses of the mammalians [3–6, 20, 21]. The lack of such changes reported recently by other authors in the conditions of electrophysiological recordings of the Ach release in motor synapses was ascribed to the differences in the initial pharmacological state of the terminals and the Ach release level in respective experiments, which needs additional analysis and revalidation [2, 22].

The revealed predominance in the functioning synapses of the activity of the inhibitory A1 receptors over A2A receptors facilitating the Ach release in the presence of the endogenous adenosine in the terminals is commonly interpreted either by lower density or accessibility of A2A receptors or by insufficient amount of the endogenous adenosine for the activation of the A2A receptors due to a low activity of the synapses [18, 19, 23]. Indeed, both in central and peripheral synapses, high concentrations of exogenous adenosine mostly induce activation of the A2A receptors and facilitation of the mediator release [4, 5, 18, 24, 25]. Our studies have shown that an additional activation of A2A receptors by means of CGS-21680 overcomes an inhibitory action produced by A1 receptors on the Ach release and leads to the facilitation of the secretion. Our data indicate the involvement of the cascade with PKA and L-type calcium channels in this process. Thus, A1 and A2A receptors exert reciprocal effects on the PKA activity in the motor nerve terminals studied. This corresponds to the generally accepted notion presuming that of the adenosine receptors, subtype A2A is coupled with G_s proteins and activates adenylate cyclase, while subtype A1, coupled with $G_{i/o}$ proteins, may, in contrast, inhibit the PKA activity [15, 19, 25]. As regards the ultimate targets of the intracellular cascades triggered upon activation of A1 and A2A receptors, in nerve terminals of the central nervous system these targets could be the N-type calcium channels [18] and in postsynaptic structures and neurons, calcium channels of L-type [15, 26].

The role of the L-type calcium channels as a target for the potentiating action of presynaptic A2A receptors in the peripheral motor synapses was earlier discussed only in connection with dominating activity of the A2A receptors revealed by means of the [^{3}H]-Ach

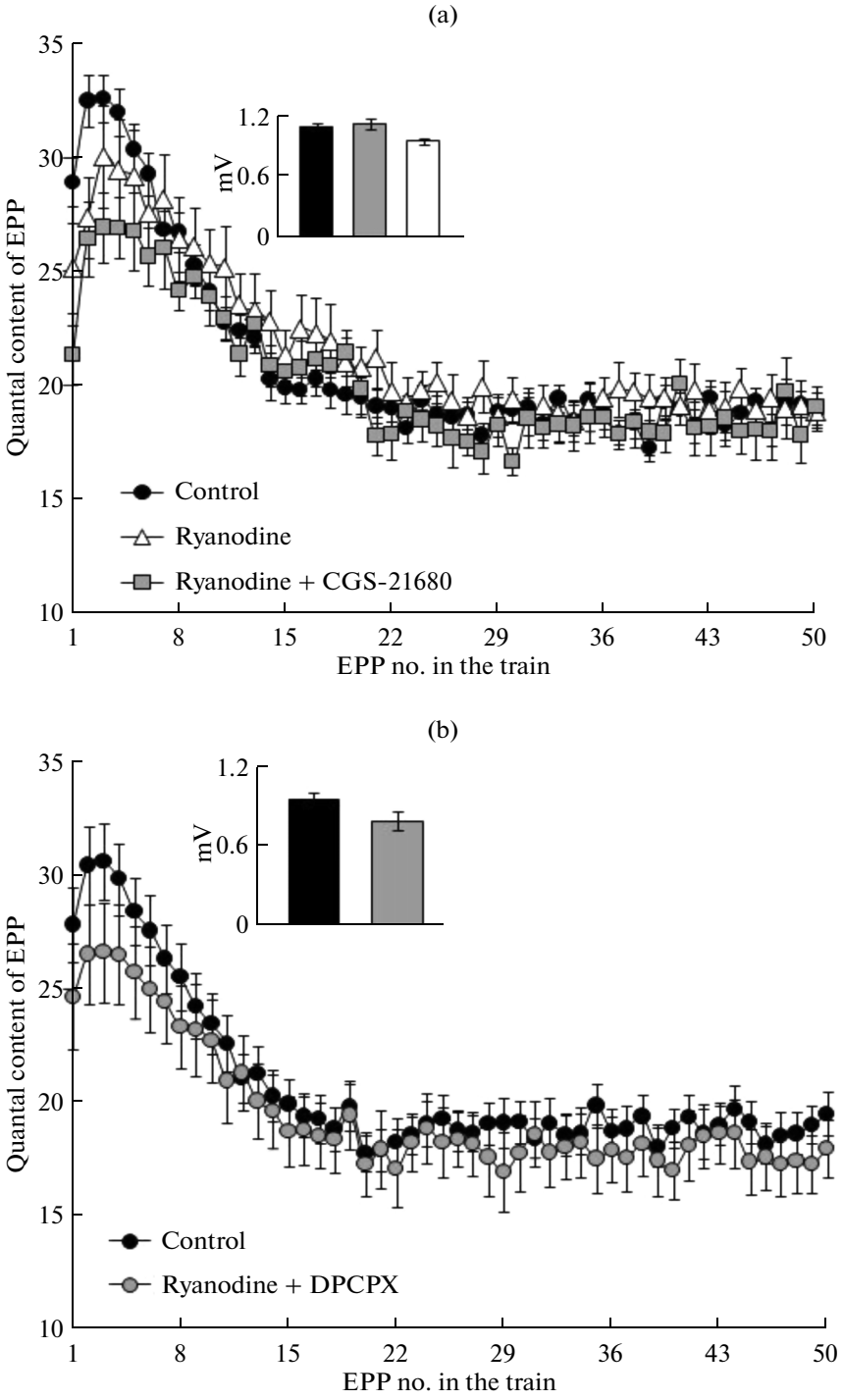

Fig. 5. (a) Alterations in the quantal content of EPP along the train in the control conditions ($n = 24$), in the presence of 3 μM ryanodine ($n = 16$), and after the addition of CGS-21680 in the presence of ryanodine ($n = 11$). (b) Alterations in the quantal content of EPP along the train in the control conditions ($n = 18$) and in the presence of 3 μM ryanodine and 100 nM DPCPX ($n = 10$); in the *inserts*, mean amplitude of MEPP in the control conditions, in the presence of ryanodine and after the addition of CGS-21680 or DPCPX in the presence of ryanodine. *The value differs significantly from the respective control value, $p < 0.05$.

release monitoring upon high-frequency and long-term stimulation of the motor synapses [5, 6]. Here, using electrophysiological registration of the evoked quantal secretion of Ach, we established for the first time that the state of the L-type calcium channels is reciprocally controlled by A1 and A2A receptors even during a short-term train-like synaptic activity (50 Hz, 1 s). It seems likely that the cascades triggered by the metabo-

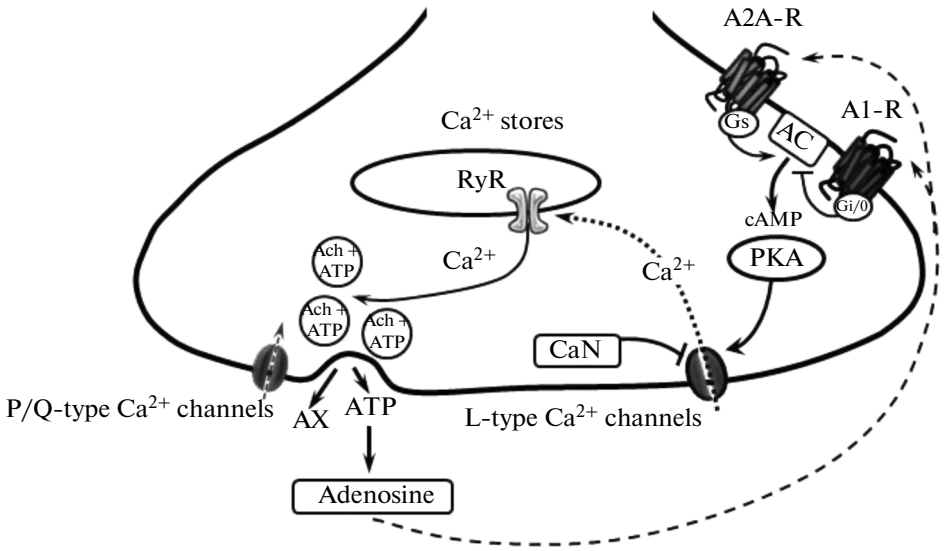

Fig. 6. Scheme illustrating the involvement of adenosine receptors of subtypes A1 and A2A (A1-R and A2A-R), adenylate cyclase (AC), protein kinase A (PKA), L-type calcium channels, calcineurin (CaN), and ryanodine receptors (RyR) in the regulatory influences on the secretion of mediator acetylcholine (Ach) in the nerve terminal of mouse motor synapses.

tropic A1 and A2A receptors oppositely modulate the PKA activity, which is directed to the L-type calcium channels. As we have shown, normally dominates the inhibitory influence of the A1 receptors on PKA.

The importance of the phosphorylating activity of PKA for the maintenance of the functional status of the L-type calcium channels was described for almost all sites of the expression of the L-type calcium channels: muscle cells [27], neuronal soma, and postsynaptic dendritic structures of the central synapses [28]. Localization and functioning of the L-type calcium channels at the presynaptic level was found only in the synaptic terminals of the skeletal muscles. Our data indicate that even here PKA participates in the phosphorylation of calcium channels, and an increased activity of PKA is associated with the activity of A2A receptors.

We first showed here that functioning or silent state of the L-type calcium channels depends not only on the reciprocal influences of A1 and A2A receptors and subsequent cascades involving PKA but also on the effects of other enzymes, phosphatase calcineurin in particular. As we showed previously, the activity of this calcium-dependent phosphatase is tonic and is directed to the inhibition of the L-type calcium channels [11]. We found that the inhibition of calcineurin by CsA leads to the disinhibition of the L-type calcium channels but only on the condition that A2A receptors and associated PKA are concurrently activated. Thus, we revealed two pathways of the inhibitory influence on the L-type calcium channels in motor nerve terminals coming from adenosine A1 receptors or from calcineurin, both of which interact with facilitating influences exerted by A2A receptors and PKA. The balance is typically shifted towards the inhibition and keeps these channels in a silent, inactive state; however, as our experiments show, weakening of at least one inhibitory factor or an increase of the facilitating effect of A2A receptors on PKA can shift this balance towards the activation of the channels and subsequent increase of the Ach secretion. An intricate kinase–phosphatase regulation of the activity of the L-type calcium channels, which involves PKA, calcineurin, and protein kinase C incorporated into the submembrane macromolecular signaling complex by means of protein AKAP anchoring A-kinase, was recently described in cardiomyocytes and smooth muscle cells [29]. It cannot be ruled out that multifactor regulation of the L-type calcium channels in motor nerve terminals of synapses studied here bears similar features.

So far, voltage-gated calcium channels were regarded an ultimate target of the intracellular cascades triggered by adenosine receptors [18, 19, 25]. Our data first show, however, that in motor nerve terminals the L-type calcium channels are functionally coupled with ryanodine-sensitive intracellular calcium stores and calcium release from the intracellular stores. The coupling of the L-type calcium channels with RyR calcium stores and the release of the intracellular calcium was described not only for muscle but also for nerve cells [30]. The RyR activation and the release of the stored calcium upon entry of the external calcium through the N-type calcium channels triggering the vesicle exocytosis was described in frog motor nerve terminals [31, 32]. We have shown that active L-type calcium channels, by passing calcium into the terminal, can also activate RyR and trigger the release of the stored calcium. This occurs upon the disinhibi-

tion of the L-type calcium channels by several methods, not related with the adenosine receptor modulation [11, 16]. Here we found that disinhibition of calcium channels due to selective activation of A2A receptors is followed by activation of RyR and calcium release from the intracellular stores. Apparently, this is a final step in the reaction sequence starting from the activation of A2A receptors and leading to the stimulation of the Ach release in motor nerve terminals. A summarizing scheme of the revealed interactions is given in Fig. 6.

The data obtained indicate that the direction of the effect of the activated presynaptic A1 and A2A receptors is determined by the modulation of the intracellular signaling cascade involving adenylate cyclase, PKA, L-type calcium channels, and RyR. The ultimate effect (as a change in the ACh release) results from the interactions at various levels: (i) relative (predominant) activation of A1 or A2A receptors by the endogenous adenosine; (ii) balance between the activation and suppression of the intracellular cascades triggered by A1 and A2A receptors with the participation of L-type calcium channels; (iii) downstream interaction of the enzymes regulating the state of the L-type calcium channels in the adenosine-dependent or independent manner, and (iv) coupling of calcium channels and RyR, calcium release from the intracellular stores, and Ach secretion.

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