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ARTICLES

Epileptiform Postsynaptic Currents in Primary Culture of Rat Cortical Neurons: Calcium Mechanisms

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Abstract—In this study we demonstrate that the primary culture of rat cortical neurons is a convenient model for investigations of epileptogenesis mechanisms and specifically, of the postsynaptic epileptiform currents (EC) reflecting periodical asynchronous glutamate release. In particular, we have revealed that in primary culture of cortical neurons EC can appear spontaneously or can be triggered by the withdrawal of magnesium block of NMDA receptor channels or by shutting down GABAergic inhibition. EC were found to depend on intracellular calcium oscillations. The secondary calcium release from intracellular stores was needed for EC synchronization. EC were suppressed by the influences causing either neuronal calcium overload or decrease of intracellular calcium concentration. Calcium entry into neurons in the case of NMDA receptor hyperactivation or in the case of calcium ionophore ionomycin treatment eliminated EC. The suppression of EC also occurred after a decrease of intracellular calcium concentration induced by BAPTA loaded into the neurons or by stimulation of calcium removal from cells via Na^+/Ca^{2+} exchanger by 1 nM ouabain. Partial dependence of EC on action potential generation was found. Thus, EC in neurons are activated by intracellular periodic calcium waves within a limited concentration window.

Keywords: epileptiform currents, primary culture, neurons, cortex, calcium, ouabain

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Embryonic development of brain cortex engages significant changes of neuronal network. During this period brain is very vulnerable to generation of epileptiform activity. Even later in postnatal period epileptic focus is often located in cortex [1]. Similar state of neuronal network takes place at 12 day of in vitro culture (DIV). This is probably related to predominant expression of GluA2 subunit lacking calcium-permeable subtype of AMPA receptors at this period [2, 3]. The primary culture of cortical tissue is a convenient model to study the physiology of neuronal network. The lack of glia and flat topology of the cell culture determine specific functional features that distinguish it from brain slices [4]. Nevertheless, primary culture of cortex and hippocampus demonstrates the same type of epileptiform spiking activity of neurons as brain slices [5]. Recordings performed with extracellular microelectrode arrays from the cortical culture treated with magnesium free medium revealed recurring spontaneous epileptiform discharges lasting up to several days after the treatment [5, 6]. Short-term (few minutes) block of inhibitory synaptic transmission with bicuculline triggers self-maintained epileptiform spiking of neurons in brain slices [7] and in tissue explants [8]. Both mentioned ways of inducing epileptiform activity require periodic increase in the intracellular calcium ion concentration ($[Ca^{2+}]_i$) [9]. This increase can result either from increased $[Ca^{2+}]_i$ entry via NMDA receptor channels or from hyperexcitation of all types of calcium-permeable channels in the absence of inhibitory synaptic transmission. In neurons $[Ca^{2+}]_i$ is a key signal messenger regulated by multiple mechanisms. Moderate elevation of $[Ca^{2+}]_i$ takes place during normal physiological events like mediator release, plasticity changes, long-term potentiation, etc. Excessive irreversible increase of $[Ca^{2+}]_i$ happens during excitotoxic action of glutamate in stroke, brain traumas, or some neurodegenerative disorders. Calcium hypothesis of epileptogenesis assumes that epilepsy originates from long-term subcritical reversible elevation of $[Ca^{2+}]_i$ leading to pathological neuronal activity and increasing the probability of neuronal damage [5].

Miniature excitatory postsynaptic currents (mEPSCs) occur in response to single neurotransmitter vesicles release to synaptic cleft [10, 11]. The frequency and

amplitude of mEPSCs in primary culture of cortical neurons depend on presynaptic $[Ca^{2+}]_i$ [4], which is regulated by nonquantal glutamate secretion [12]. In magnesium-free medium the mEPSCs frequency is higher than in control conditions [13], which is related to increase of quantal content of spontaneous currents [4]. Brain parts differ by functional characteristics of excitatory synaptic transmission because of the variation of expression glutamate receptors subtypes [14]. This possibly introduces the difference in postsynaptic currents generation between different brain areas. Despite of numerous investigations of mEPSCs in hippocampus cultures, there are only few studies concerning postsynaptic currents in cortical culture. In fact epileptiform postsynaptic currents in primary culture of cortical neurons were not studied. In this study we examine the influence of intracellular calcium regulation mechanisms on epileptiform currents generation in primary culture of rat cortical neurons.

MATERIALS AND METHODS

Experiments were performed on primary culture of Wistar rat cortical neurons at room temperature 23–26°C. The procedure of culture preparation from 16-day embryos was previously described [15, 16]. Cortex was extracted in ice cooled Petri dishes. Cells were grown in culture medium on glasses coated with poly-*D*-lysine. Whole-cell patch clamp recordings of postsynaptic currents were performed from 10 to 16 days of in vitro incubation (10–16 DIV). The following extracellular medium was used for recording (in mM): 140 NaCl; 2.8 KCl; 2.0 $CaCl_2$; 1.0 $MgCl_2$; 10 HEPES, at pH 7.2–7.4. In some experiments magnesium was removed from medium to promote epileptiform currents generation (see Results). Mg^{2+}-free medium was also used for experiments with NMDA-receptors blockers. Path-pipette solution had the following content (in mM): 9 NaCl, 17.5 KCl, 121.5 K-gluconate, 1 $MgCl_2$, 10 HEPES, 0.2 EGTA, 2 MgATP, 5 NaGTP [17]. We used MultiClamp 700B patch-clamp amplifier with Digidata 1440A acquisition system controlled by pClamp v10.2 software (Molecular Devices, USA). Acquisition sample rate was 20000 s^{-1}. The signal was 8-order low-pass Butterworth filtered at 1.4 KHz to remove high frequency noise. Micropipette positioning was made by MP-85 micromanipulator (Sutter Inc, USA) under the visual control of Nikon Diaphot TMD microscope (Nikon, Japan). For fast medium exchange we used BPS-4 fast perfusion system (Ala Scientific Instruments, USA). The following concentrations of agents were used: 30 μM NMDA (N-methyl-*D*-aspartate, NMDA-receptors agonist) always coapplied with coagonist 30 μM glycine; 50 μM AP5 ((2R)-amino-5-phosphonovaleric acid, specific NMDA-receptors antagonist); 30 μM CNQX (6-cyano-7-nitroquinoxaline-2,3-dione, AMPA-kainate receptors antagonist); 20 μM bicuculline (antagonist of γ-aminobutyric acid A-type, $GABA_A$ receptors); 0.5 μM tetrodotoxin (TTX, blocks fast sodium channels), 1 nM ouabain (specific ligand for Na^+,K^+-ATPase); 2 μM ionomycin (calcium ionophore); 2–5 μM BAPTA-AM (membrane permeable form of calcium chelator BAPTA). Cell culture medium was obtained from Biolot company (Russia); other compounds were from Sigma Aldrich (USA). Postsynaptic currents detection and other data analysis was carried out using Clampfit 10.2 software.

RESULTS

Starting from 12 DIV, in some neurons spontaneous excitation waves were recorded. These waves arise from massive mediator release resulting in high amplitude inward currents (up to 5 nA) (Fig. 1) with the decay phase of up to 5 s. Similar currents can be recorded from motor neurons during stimulation of dorsal roots [18] causing synchronous activation of numerous synaptic inputs. In cultured neurons generation of such waves was possible in both magnesium-containing (Fig. 1a) and magnesium-free medium (Fig. 1b). In the magnesium-free solution the probability of generation of these currents was much higher. This epileptiform currents (EC) were periodic and resulted from overlapping of multiple mEPSCs (Fig. 1c). EC produced significant depolarization of neurons (Fig. 1d) promoting action potentials generation. In some of neurons EC were completely suppresses with 0.5 μM TTX (Fig. 2a). However, in 12 out of 24 neurons tested EC were not eliminated with TTX. Apparently, EC generation only partly depends on action potentials generation. In turn, EC can trigger action potentials. Recordings in current-clamp mode demonstrate that action potentials appear in response to strong (up to 30 mV) depolarization caused by EC.

In magnesium-free medium the block of NMDA receptors (NMDARs) with specific antagonist AP5 (50 μM) completely inhibited EC generation in all 14 cells tested (Fig. 2b). Similar effect was observed after the addition of 1 mM $MgCl_2$ ($n = 12$) (Fig. 2c). Competitive inhibitor AP5 prevents activation of NMDARs, while Mg^{2+} is a noncompetitive inhibitor and blocks NMDAR channels. Thus, both agents suppress Ca^{2+} entry into neurons via NMDARs, demonstrating that NMDAR functioning is critical for EC generation. AMPA receptors antagonist CNQX (30 μM) in magnesium-free solution decreased mEPSCs amplitude, but did not affect EC in 9 out of 11 cells tested (Fig. 2d). Thus, EC generation is mostly independent of AMPA-receptors, despite of their key role in excitatory synaptic transmission.

In one fifth of the experiments EC could be provoked by the block of $GABA_A$ receptors with bicuculline. Bicuculline (20 μM) application increased spontaneous mEPSCs frequency, which was followed by mEPSCs synchronization and appearance of EC lasting even after the bicuculline washout (Fig. 3). Bicuculline-evoked EC were completely inhibited

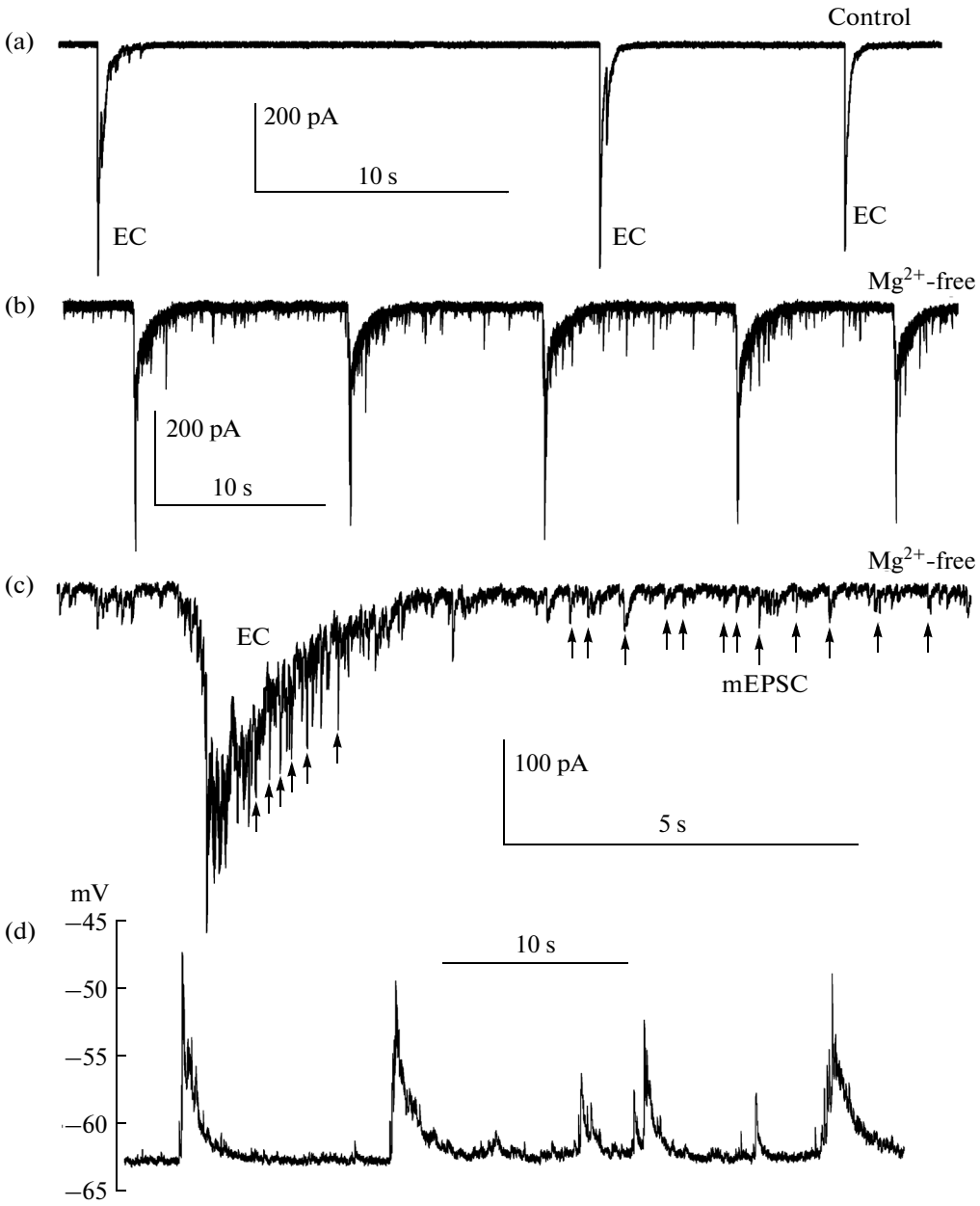

Fig. 1. Epileptiform currents (EC) in primary culture of cortical neurons. Repeated spontaneous EC in control (a) and in magnesium-free medium (b). The composition of EC (c) as an overlay of multiple mEPSCs. Cell membrane depolarization produced by EC (d).

with TTX, which distinguishes them from spontaneous EC. Taking into account that $[Ca^{2+}]_i$ controls vesicular neurotransmitter release and then generation of all types of postsynaptic currents, we have tested the possibility of EC modulation by pharmacological treatments increasing or decreasing $[Ca^{2+}]_i$ in the cytoplasm. Spontaneous EC were completely inhibited with receptor saturating concentration of NMDA (30 μM, plus 30 μM glycine as NMDAR coagonist) (Fig. 4a) causing stable inward current ($n = 22$). This current promoted $[Ca^{2+}]_i$ elevation in neurons [19], which was accompanied with mEPSC frequency increase, as it was shown previously [20]. In two experiments NMDA wash-out partially restored the EC generation. The accelerated accumulation of free calcium in cytoplasm could also be produced by calcium ionophore ionomycin (2 μM). This kind of $[Ca^{2+}]_i$ elevation desynchronized EC and gradually reduced EC amplitude until complete vanishing (Fig. 4b). At the same time ionomycin significantly increased mEPSCs frequency.

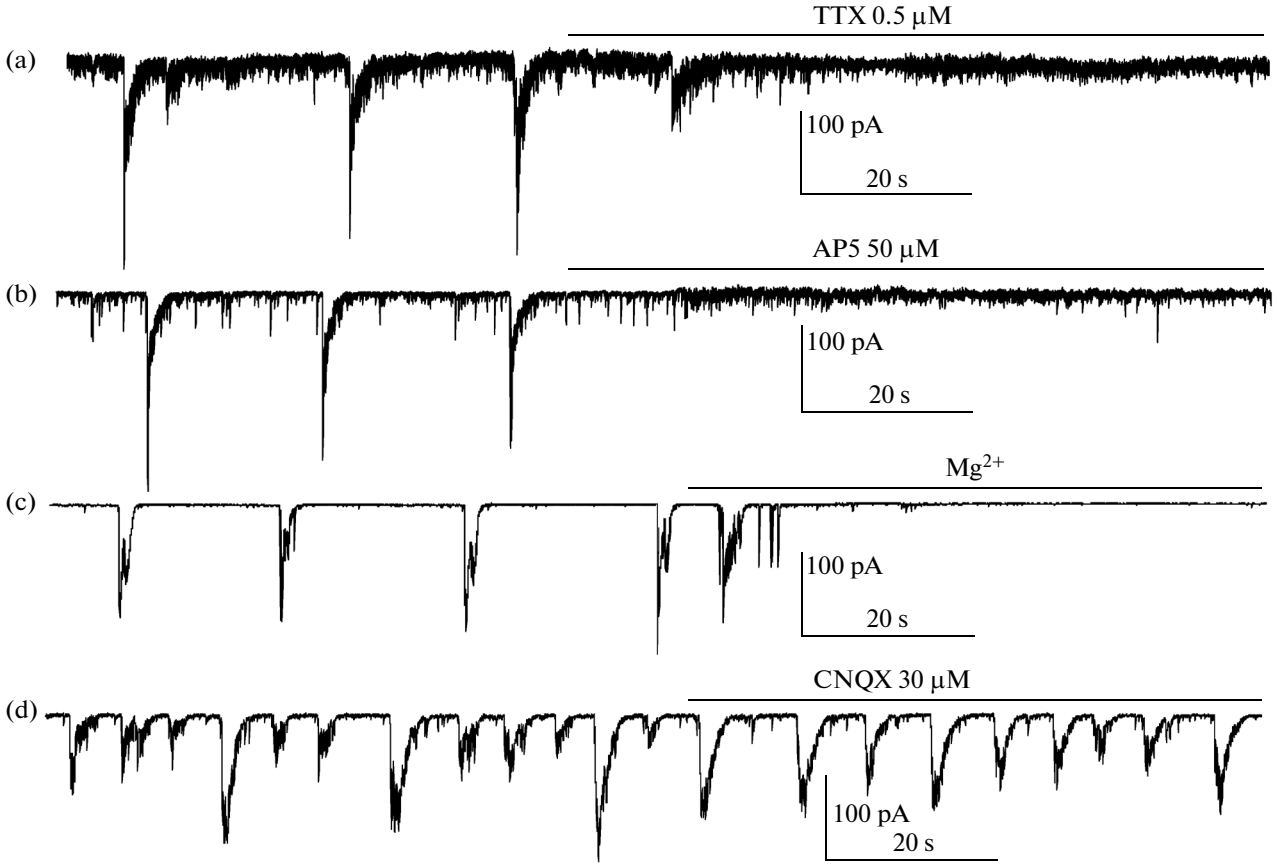

Fig. 2. The role of different types of ion channel in generation of epileptiform currents (EC) by neurons in magnesium-free medium. (a) The block of action potentials with tetrodotoxin suppresses EC. Inhibition of NMDA receptors with AP5 (b) or with magnesium ions (c) stops EC generation. (d) The block of AMPA receptors with CNQX does not affect EC.

Physiological $[Ca^{2+}]_i$ elevation can result from activation of IP_3-sensitive calcium channels located in the membrane of intracellular calcium stores and secondary release of free calcium ions [19, 20]. In neurons this process is involved in synchronization of vesicle release. The block of IP_3 receptors with membrane-permeable inhibitor 2-APB (4 μM) desynchronized EC (Fig. 4c; $n = 6$). The concentration of 2-APB used in our experiments was enough to block IP_3 receptors but insufficient to produce nonspecific effects of 2-APB, such as inhibition of TRP-channels, activation of TRPV-channels, or suppression of some calcium-permeable channels of mitochondria or endoplasmatic reticulum [22]. In the presence of 2-APB, high-amplitude EC dissipated into multiple events of smaller amplitude with further gradual decrease of amplitude to the level of mEPSCs. Thus, Ca^{2+} release from intracellular stores is required to synchronize mEPSCs and to form EC.

Previously we have shown that in primary culture of cortical neurons the chelation of intracellular Ca^{2+} with BAPTA decreases mEPSC frequency in a concentration-dependent manner [20]. Here we demonstrate that 30-min preincubation of neurons with 2 or 5 μM BAPTA-AM inhibits the EC generation (Fig. 4d).

Cytoplasmatic free calcium clearance with Na^+/Ca^{2+} exchanger is critically important mechanism of neuronal calcium homeostasis. Recently [20] we have found that ouabain in subnanomolar concentrations does not affect the Na^+,K^+-ATPase pumping function but interact with Na^+,K^+-ATPase in a ligand–receptor manner and triggers several intracellular pathways. The fastest of these pathways increases the effectiveness of calcium removal with Na^+/Ca^{2+} exchanger, which can even save neurons from glutamate receptors hyperactivation induced calcium overload [20, 23]. This effect manifests only in calcium overload states but does not affect $[Ca^{2+}]_i$ in control conditions. Ouabain at 1 nM did not influence spontaneous mEPSC frequency in control (Fig. 5a; $n = 9$). In magnesium-free medium EC with typical complex shape appeared in some neurons (Figs. 5b, 5c). In all experiments ($n = 9$) ouabain application (1 nM) decreased the number of mEPSCs inside EC within 1 min (Fig. 5d). Treatment with 1 nM ouabain for 5 min caused desynchronization and gradual fading of EC (Fig. 5e).

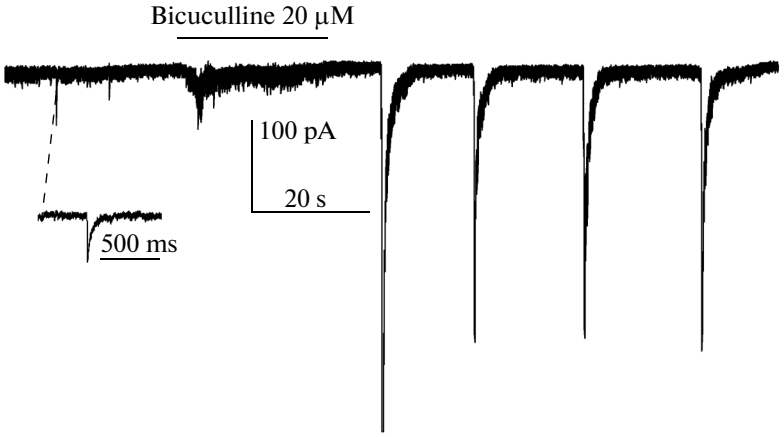

Fig. 3. Epileptiform currents (EC) caused by short-time application of bicuculline in control solution. Increase of mEPSCs frequency is followed by further synchronization into EC. The *insert* on the left shows time-stretched single mEPSC illustrating its small amplitude as compared to EC.

Thus, generation of EC in cortical neurons in primary culture can appear in a spontaneous manner or can be provoked either by removal of magnesium block of NMDA receptors, or by inhibition of GABAergic synaptic transmission. Most likely EC depend on oscillations of $[Ca^{2+}]_i$, which in turn depend on the NMDA receptor activation and secondary release of Ca^{2+} from the intracellular stores. Treatments causing calcium overload (NMDA, ionomycin) or calcium shortage (BAPTA, ouabain) suppress the EC generation.

DISCUSSION

A lot of data have been published indicating that incubation of cortical [8] or hippocampal neuronal culture [8] in magnesium-free medium [24] leads to synchronized neuronal spiking with periodic waves of abnormally high frequency of action potentials [24]. Microelectrode arrays were used to observe these waves in brain slices [25] and in neuronal cultures [26, 27]. In our experiments, the removal of magnesium block of NMDARs [28] was an effective approach to provoke postsynaptic EC in primary culture of cortical neurons after 12 DIV. The suppression of epileptiform spiking activity in brain slices and in cultures by NMDAR antagonists was described by many authors [29–31]. However, our work concerns not spiking but postsynaptic EC in cultured cortical neurons. Suppression of EC in our study with AP5 and Mg^{2+} was anticipated. On the contrary, AMPAR antagonist CNQX did not influence spontaneous EC. Thus, AMPAR activation is not required for the EC maintenance. Similar data on the absence of effective block of epileptiform spiking of neurons with AMPA and metabotropic glutamate receptor inhibitors were obtained eaarlier [5, 23, 31]. However, pretreatment of neuronal culture with CNQX prevented appearance of EC in Mg^{2+}-free solution. Therefore, we cannot exclude some possible involvement of Ca^{2+}-permeable AMPA channels in the initiation of EC, while the rhythm of EC depends predominantly on NMDARs. The reason for this difference is probably a diverse $[Ca^{2+}]_i$ dynamics observed after NMDAR or AMPAR activation [32].

In primary culture of cortical neurons EC could be successfully inhibited by returning 1 mM of Mg^{2+} back to the perfusion medium. This discerns cultured cortical neurons from hippocampal neurons that maintained epileptiform activity (burst of spiking) after returning of 1 mM of Mg^{2+} to the external medium [30]. It has long been known that in hippocampal slices [7] and in neocortex explants [8] blocking of GABAergic synaptic transmission by bicuculline results in appearance of epileptiform waves of neuronal depolarization. In our experiments bicuculline promoted an increase of mEPSC frequency followed by appearance of EC (Fig. 3), while bicuculline washout and consequent restoring of inhibitory GABAergic system did not suppress EC. It is worth mentioning that TTX successfully inhibited bicuculline-triggered EC in all experiments performed in Mg^{2+}-containing medium. In the case of EC provoked with Mg^{2+}-free solution, TTX generally but not always blocked EC. Thus, an absence of Mg^{2+} block of NMDARs allows EC to maintain during block of action potentials generation. In neuronal network an appearance of synchronized excitation waves and EC is a periodic process depending on depolarization of neurons leading to action potentials generation and simultaneous release of mediator by groups of neurons, which in turn provokes oscillations of $[Ca^{2+}]_i$ controlling neuronal excitability [9]. EC are periodic and because of that require reversibility of $[Ca^{2+}]_i$ oscillations while epileptiform activity [5]. Accordingly treatments shifting $[Ca^{2+}]_i$ to elevation or to decrease should distort epileptiform calcium waves. Forced loading of neurons with calcium by ionophore produces irreversible

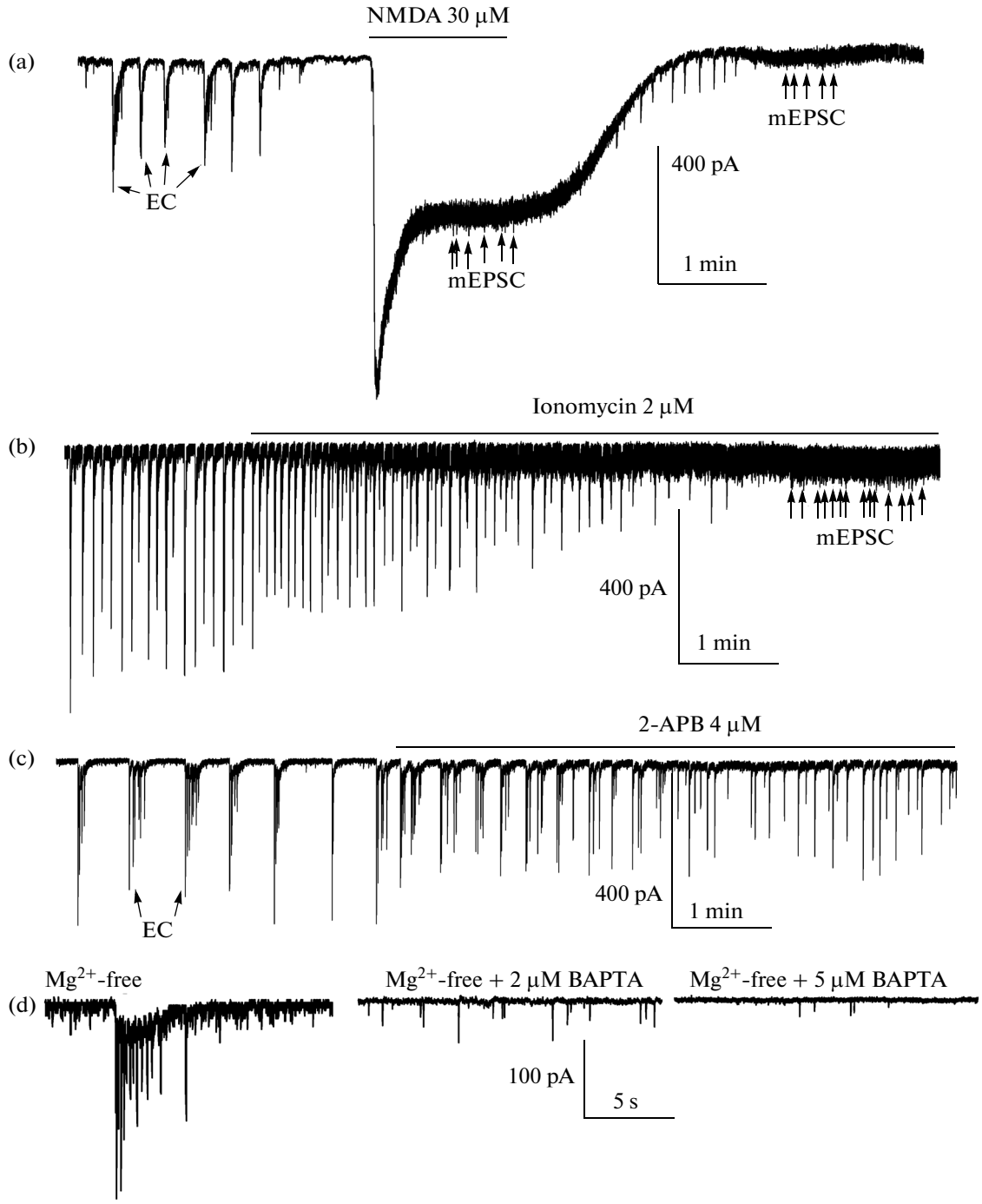

Fig. 4. Spontaneous epileptiform currents (EC) in neurons in magnesium-free medium can be suppressed with different distortions of calcium homeostasis. (a) Block of EC generation by activation of all NMDA receptors forcing calcium entry. Partial recovery of EC after NMDA washout is observed. (b) Calcium ionophore increases the mEPSCs frequency but abolishes the EC generation. (c) Inhibition of IP_3 receptors suppresses calcium release from intracellular stores and desynchronizes EC. (d) Binding of intracellular calcium decreases the mEPSC frequency and completely inhibits EC generation.

elevation of $[Ca^{2+}]_i$ and gradual fading of EC. Simultaneously the frequency of mEPSCs increased dramatically (Fig. 4b). The other condition of EC autogeneration is periodic activation of NMDARs. Hyperactivation of NMDARs with the saturating concentration of specific agonist maintained NMDARs in open state and breached reversibility of epileptiform wave causing EC disappearance (Fig. 4a). In primary culture of hippocampus it was shown [33] that block of IP_3-sensitive calcium channels with 2-APB lowers $[Ca^{2+}]_i$ and this effect is more pronounced in neurons involved in epileptiform spiking activity. IP_3 receptors were even

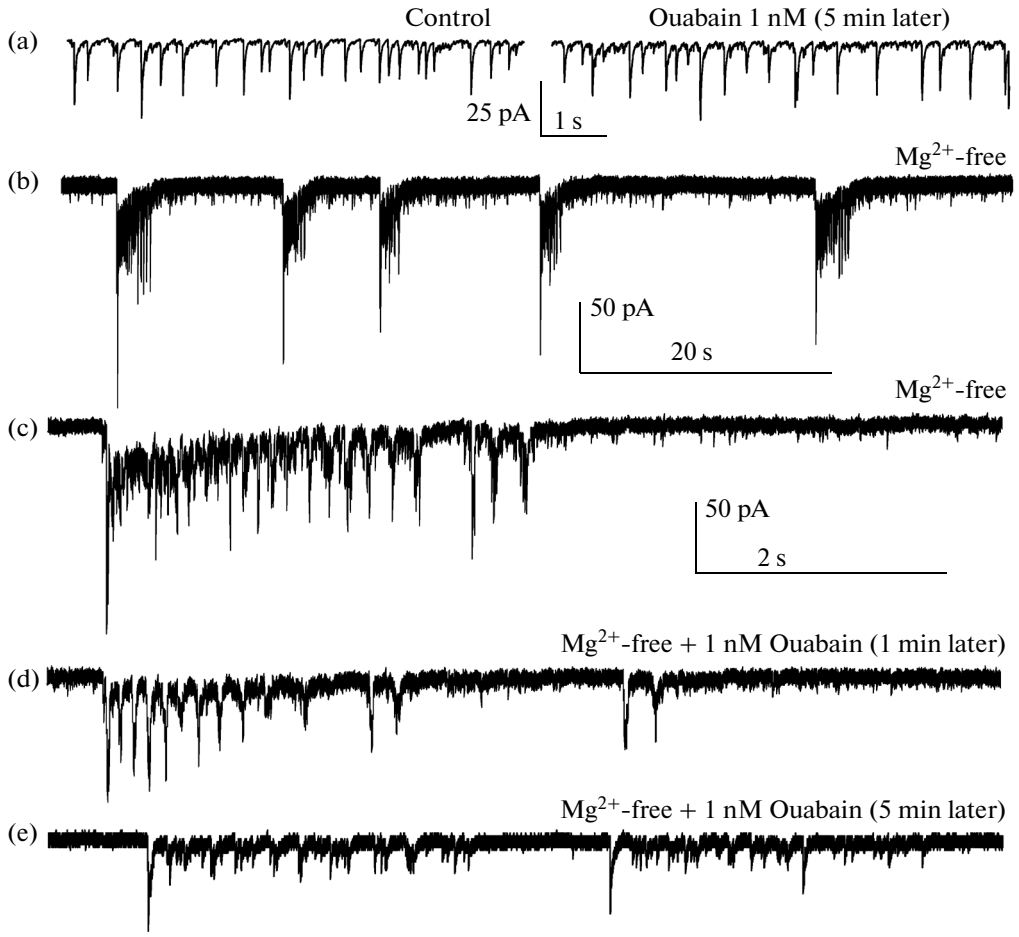

Fig. 5. Inhibition of epileptiform currents (EC) with ouabain at subnanomolar concentrations, which does not suppress the Na^+/K^+-ATPase pumping function. (a) In control conditions ouabain has no effect on the mEPSC frequency. (b) EC provoked by magnesium-free medium. (c) The structure of single EC in magnesium-free medium. (d) One min after the treatment with ouabain the amplitudes and the number of mEPSCs composing the EC are reduced. (e) Desynchronization and almost complete disappearance of EC five min after the treatment with ouabain.

expected [33] to have a key role in epileptiform spiking. IP_3-dependent release of calcium from intracellular stores is important for synchronization of multiple mediator vesicles release. In our experiments block of IP_3 receptors with 2-APB desynchronized EC (Fig. 4c). Thus, preventing secondary release of calcium from intracellular stores desynchronizes mediator vesicle release. In the presence of 2-APB postsynaptic currents are not clustered in time and does not join into giant EC, which result in gradual lowering of EC even in absence of Mg^{2+}.

Lowering of $[Ca^{2+}]_i$ was also effective to suppress the EC generation (Fig. 4d). There is no surprise that binding of intracellular calcium with BAPTA inhibited not only EC but all types of postsynaptic currents. Spontaneous vesicular neurotransmitter release decreased proportionally to the degree of calcium chelation [20]. BAPTA action resembles function of own neuronal calcium homeostasis mechanisms like calcium binding proteins. Neurons also do have another mechanism of $[Ca^{2+}]_i$ lowering by calcium removal via Na^+/Ca^{2+} exchanger. Previously we have demonstrated that subnanomolar concentrations of cardiotonic steroids, ouabain in particular, do not inhibit the Na^+,K^+-ATPase pumping function but possess neuroprotective properties realized by lowering of $[Ca^{2+}]_i$ to safe (near control) values [20, 22]. This mechanism dramatically increases the viability of neurons in excitotoxic stress [34] and depends on the activity of the Na^+/Ca^{2+} exchanger. It should be mentioned that this effect of cardiotonic steroids manifests only in the case of elevated $[Ca^{2+}]_i$, so ouabain did not change the mEPSC frequency in control (Fig. 5a). EC provoked by treating neurons with Mg^{2+}-free solution were suppressed with ouabain within minutes (Figs. 5b–5e). Interestingly, ouabain did not desynchronize EC but caused a gradual decrease of the EC amplitude. Therefore, we can expect the existence of an effective mechanism that can protect neurons from EC an calcium overload and can be triggered with subnanomolar concentrations of cardiotonic steroids. This concentra-

tions of endogenous cardiotonic steroids is characteristic of blood plasma and cerebrospinal fluid [35–37], which denotes their important role in neuronal calcium homeostasis.

EC in neuronal culture can evoke spontaneously or be provoked either by the block of inhibitory synaptic transmission or by magnesium unblock of NMDARs. The repeated activation of NMDARs is required to maintain epileptiform activity. NMDAR block or hyperactivation arrests spontaneous EC generation. The quantal release of the neurotransmitter owing to action potentials significantly contributes to EC but the block of action potentials is not always enough to stop epileptiform activity. Synchronization of mEPSCs and aggregation into EC depends on the release of calcium ions from intracellular stores. Lowering of $[Ca^{2+}]_i$ by chelation or by stimulation of its clearance also suppresses EC generation. Thus, epileptiform activity of neurons in primary culture is directly linked to periodic reversible increase of intracellular calcium concentration occurring as the result of NMDA-receptor activation.

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