DOI 10.1007/s11055-015-0099-3
Neuroscience and Behavioral Physiology, Vol. 45, No. 5, June, 2015

The Key Role of Calcium in the Mechanism of Deprivation Potentiation of Population Responses of Neurons in Hippocampal Field CA1

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UDC 612.822.3

Translated from Zhurnal Vysshei Nervnoi Deyatel'nosti imeni I. P. Pavlova, Vol. 64, No. 1, pp. 54–63, January–February, 2014. Original article submitted August 1, 2013. Accepted September 26, 2013.

In vitro studies on living rat hippocampus slices addressed the role of Ca^{2+} in the mechanism of deprivation potentiation of population responses (pop spikes) of neurons in field CA1 induced by prolonged (60 min) interruption of low-frequency test stimulation of Schaffer collaterals. Two phases were seen in deprivation potentiation, with presumptively different origins: an initial short-lived "peak" (about 12 min) and a longer-lasting "plateau" (more than 1 h). The experiments reported here showed that the presence of a penetrating Ca^{2+} chelator (BAPTA-AM), decreasing the Ca^{2+} concentration in the solution, and depletion of the intracellular calcium depot (presence of thapsigargin/cyclopiazonic acid in the solution) led to reductions in the transient phase and blockade of the longer-lasting phase of deprivation potentiation. These studies thus demonstrate the key roles of both extracellular and stored intracellular calcium in the mechanism of development of deprivation potentiation.

Keywords: deprivation potentiation, calcium, hippocampal slices, pop spike, BAPTA-AM, thapsigargin, cyclopiazonic acid.

Current concepts of the mechanisms of learning and memory are based on the concept of synaptic plasticity – afferent activity-dependent changes in the efficiency of synaptic transmission [Bliss and Collingridge, 2010; Collingridge et al., 2010; Lynch, 2003]. Our previous in vitro experiments on living hippocampal slices showed that interruption of the stimulation of Schaffer collaterals (from 10 min to 4 h) led to significant (up to 100% of the baseline response level) and long-lasting (more than 1 h) input-specific potentiation of the population responses of neurons in field CA1, while no significant changes were seen in control experiments with constant test stimulation [Popov, 1994; Popov and Markevich, 2001]. We termed this type of potentiation deprivation potentiation (DP). We obtained analogous results in chronic in vivo experiments in rats in conditions of narcotic sleep with recording of population spikes from neurons in the dentate fascia in response to stimulation of fibers in the medial perforant pathway [Popov and Markevich, 1999]. Calcium ions (Ca^{2+}), the key second messenger in a variety of signaling pathways, are known to be involved in almost all physiological cell functions, and even small changes in calcium homeostasis lead to profound functional changes, including synaptic plasticity [Gleichmann and Mattson, 2011; Kawamoto and Camandola, 2012; Malenka et al., 1989; Nikoletopoulou and Tavernarakis, 2012]. Our previous studies demonstrated that the dynamics of the development of DP and impairments to DP both in the presence of a protein kinase C (PKC) blocker and after previous induction of LTP (both conditions for the blockade of PKC-dependent phase of LTP) [Frey et al., 1995; Reymann et al., 1988; Reymann, 1993] provide evidence of similarity in the mechanisms responsible for the development of DP and the LTP phase associated with protein phosphorylation [Popov and Markevich, 2001]. This in turn provided grounds for our suggestion that Ca^{2+} is involved in the mechanisms of development of DP.

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The aims of the present work were to verify the role of Ca^{2+} in the mechanisms of development of DP and to search for its possible sources.

Methods

Experiments were performed on living hippocampal slices from male Wistar rats weighing 150–200 g; animals were anesthetized with ether prior to decapitation. Three slices of thickness 400 μm were placed in an incubation/recording chamber with shallow immersion into solution – artificial cerebrospinal fluid (aCSF). This solution contained 124 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 1.25 mM KH_2PO_4, 2.2 mM $CaCl_2$, and 1.8 mM $MgCl_2$ (in low-calcium solution, the concentrations of $CaCl_2$ and $MgCl_2$ were 1.0 and 3.0 mM, respectively), and 10 mM D-glucose; the gas mix consisted of 95% O_2 and 5% CO_2; the pH was 7.4; the solution temperature in the chamber was 32°C. Saturation of aCSF with gas mix was by a contraflow arrangement in the initial thermostatted segment of the flow system (25 ml). The working volume of the recording chamber was 0.9 ml and the flow rate was 0.9 ml/min. The original design of the chamber and the uniform bidirectional solution flow above and below the slice allowed prolonged recording of responses without fixing the specimens. Slices were incubated for 2 h before electrode implantation and the start of stimulation.

Overall responses and population spikes (pS) were recorded in the pyramidal layer of hippocampal field CA1 with glass microelectrodes filled with 2 M NaCl solution (electrode resistance 3–5 MΩ). pS amplitudes were measured from the first positive peak to the negative peak of the response.

The monopolar stimulating electrodes (electrolytically sharpened tungsten wires, 100 μm thick, coated with viniflex lacquer) were positioned in Schaffer collaterals in the midpart of the radial layer of hippocampal field CA1.

Recording of responses to test stimuli was initiated after 30–60 min of preliminary stimulation (the response stabilization period). Test stimulation parameters were: frequency 0.05 Hz and stimulus duration 100 μsec; amplitude was selected such that response amplitude was about 30% of maximal (usually about 40–50 μA).

DP was induced after testing the baseline level of responses by terminating stimulation for 60 min, after which it was restarted with the initial test stimulus parameters; test stimulation at 0.05 Hz was continued throughout in control experiments.

The following agents were used: the selective calcium chelator (membrane-penetrating form) 1,2-bis(2-amino-5-fluorophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl)ester (BAPTA-AM, Sigma, USA) at 30 μM, the sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPase inhibitors thapsigargin (ICN, USA) at 1 μM and cyclopiazonic acid (Sigma) at 1 μM. Each of these agents was initially dissolved in dimethylsulfoxide (DMSO, Sigma), the concentration of which in aCSF was 0.05% for thapsigargin and cyclopiazonic acid and 0.2% for BAPTA-AM. Separate control samples for solvent effects were run with a DMSO concentration of 0.2%. Stock solutions were diluted to final concentrations in aCSF about 10 min before switching of the flow system.

Recorded responses were averaged for 10 stimulus presentations (for one point on plots).

Averaged responses to the last 10 pre-pause stimuli were taken as the baseline level of 100%, using the 10 responses recorded at the same time period in controls. Significant differences were identified using Student's t test run on SigmaStat 3.1.

Results

In the experimental series ($n = 14$), 60-min interruption of low-frequency (0.05 Hz) test stimuli of Schaffer collaterals led to the development of DP – an increase in population spike (pS) amplitude recorded in the pyramidal layer of field CA1 (Fig. 1, *A*). Immediately after restarting of test stimulation, response amplitude was maximal at 173.1 ± 7.0% of the baseline response level, after which it declined over a period of 10–12 min and then stabilized at a level of about 150% (values averaged over 12 responses in the interval 72–120 min ranged from 145.6 ± 6.5% to 151.2 ± 7.2%, with a mean of 148.9%). Two components could be discriminated in the pattern of DP: an initial short-term "peak" and a subsequent "plateau" phase. This initial "peak" has previously been observed to a greater or lesser extent in the suppression of DP in medium containing polymxin B (a PKC blocker) and, after prior induction of LTP [Popov and Markevich, 2001], which may be evidence that the early and late phases of DP are of different nature.

The existence of common features in the mechanisms of DP and the PKC-dependent phase of LTP, associated with protein phosphorylation, provided grounds for suggesting that Ca^{2+} is involved in the mechanism of development of DP. With the aim of verifying this hypothesis, the procedure of inducing and testing DP using 60-min deprivation (interruption of stimulation) was performed on the background of addition of a calcium chelator to bind intracellular Ca^{2+}.

Our experiments (Fig. 1, *B*) used the membrane-penetrating form of the calcium chelator BAPTA-AM (30 μM). The flow system was switched to delivery of the BAPTA-AM-containing solution 21 min before deprivation ended. Immediately after reinitiation of test stimulation, mean pS amplitude was significantly greater than that in controls (125.7 ± 3.2%, $n = 14$ and 103.7 ± 4.6%, $n = 8$, respectively; $p < 0.001$) but significantly smaller than the amplitude of the DP "peak" in normal medium (173.1 ± 7.0%, $n = 14$; $p < 0.001$); at about 10 min there were no significant differences in responses between experiments and controls (responses at 67.0–70.5 min were 109.7 ± 2.6%, $n = 14$ and 103.0 ± 5.5%, $n = 8$, respectively; $p > 0.05$). Thus, these experiments showed that binding of intracellular Ca^{2+} leads to impairments to the development of DP: a reduction in the initial "peak" and suppression of the "plateau" phase of DP.

With the aim of testing the possible involvement of intracellular Ca^{2+} in the induction of DP, we ran experiments

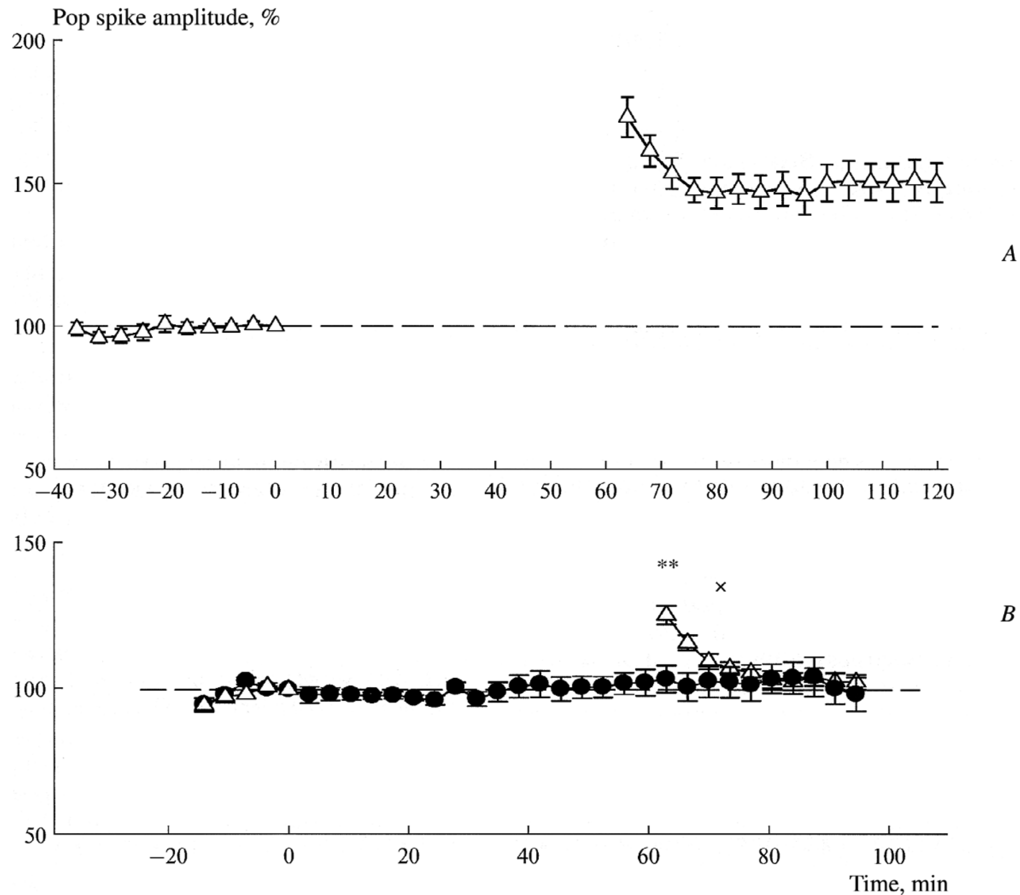

Fig. 1. The role of Ca^{2+} in the development of deprivation potentiation (DP). *A*) "Normal" development of DP: 60-min interruption of test stimulation (deprivation) leads to a prolonged increase in the amplitude of responses (pS) to test stimulation ($n = 14$); plots of DP discriminate two components: an initial phase (the transient "peak") lasting about 10–12 min and a subsequent "plateau" lasting more than 1 h. *B*) Presence of the penetrating calcium chelator BAPTA-AM (30 μM) in the medium leads to a reduction in the initial phase and suppression of the "plateau" phase; light triangles show the experiment (deprivation) ($n = 14$); dark circles show controls (constant stimulation) ($n = 8$). The abscissas show time, min; the ordinates show mean pS amplitude normalized with respect to the baseline response level (%). Dotted lines show the baseline response level: the mean pS amplitude in the last pre-deprivation 10 stimuli was taken as 100% (corresponding to the "0" point on the abscissa). Mean values ± errors of the mean are shown; statistical differences between values in experiments and controls (Student's *t* test): **significant ($p < 0.001$); $^{\times}$not significant ($p \gg 0.05$).

in low-calcium medium. In these experiments, the decrease in the Ca^{2+} concentration in solution (2.2 → 1.0 mM) was accompanied by a simultaneous increase in the Mg^{2+}+ concentration (1.8 → 3.0 mM), thus maintaining the total concentration of divalent cations and enhancing magnesium blockade of NMDA channels. These experiments showed that a low Ca^{2+} concentration in solution impaired the development of DP, with a reduction in the initial transient "peak" and suppression of the longer-lasting "plateau" phase (Fig. 2). Immediately after reinitiation of stimulation, mean pS amplitude in the experiment was significantly greater than that in controls (130.5 ± 3.9%, $n = 9$ and 100.0 ± 3.3%, $n = 8$, respectively; $p < 0.001$) but lower than that in "normal" conditions (173.1 ± 7.0%, $n = 14$; $p < 0.001$); after 15 min, there were no significant differences between the values in the experiments and controls (mean response amplitudes at 74–77.5 min were 114.2 ± 4.4%, $n = 9$ and 102.8 ± 5.0%, $n = 8$, respectively; $p > 0.05$). Thus, these experiments demonstrated the important role of extracellular Ca^{2+} for induction of DP.

One potential source of increased intracellular Ca^{2+} is provided by stored Ca^{2+}, which is known to play an important role in the induction of both LTP and LTD. To test the possible involvement of stored Ca^{2+} in inducing DP, we used thapsigargin (Tg) and cyclopiazonic acid (CPA) – penetrating inhibitors of microsomal CA1-ATPase, blocking ATP-dependent Ca^{2+} reuptake into intracellular depots and inducing depletion of the greater part of the intracellular Ca^{2+} pools [Harvey and Collingridge, 1992].

The Tg concentration in our experiments was 1 μM and normal solution was switched to Tg-containing solution 30 min before stimulation was interrupted. These experi-

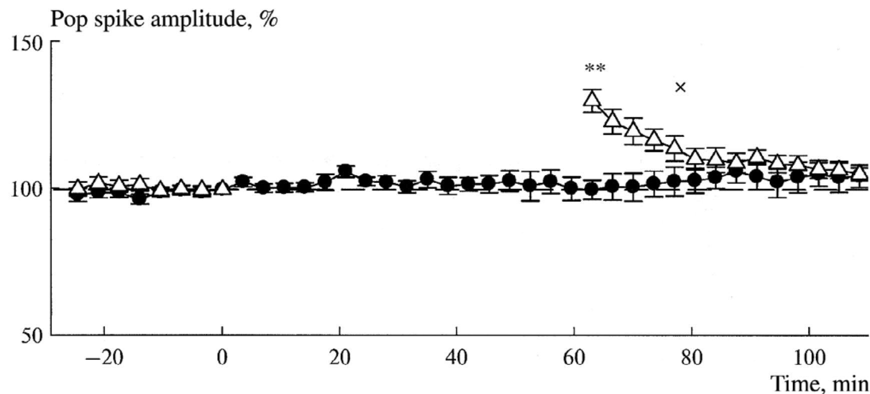

Fig. 2. Simultaneous decrease in the Ca^{2+} concentration and increase in the Mg^{2+} concentration in the medium ($[Ca^{2+}]_{ecell}$ = 1.0; $[Mg^{2+}]_{ecell}$ = 3.0 mM) leads to a reduction in the transient phase and suppression of the prolonged phase of DP; light triangles show the experiment (n = 9); dark circles show controls (n = 8); $^{**}p < 0.001$; $^{\times}p > 0.05$.

ments showed that Tg impaired the development of DP, decreasing the initial "peak" phase and suppressing the "plateau" phase (Fig. 2). The mean pS amplitude in experiments immediately after reinitiation of stimulation was significantly greater than the corresponding value in controls (132.1 ± 3.3%, n = 12 and 111.9 ± 2.4%, n = 9; $p < 0.001$) but lower than the "normal" level (173.1 ± 7.0%, n = 14; $p < 0.001$); at about 13 min, there were no significant differences between values in experiments and controls (mean pS amplitudes at 70.5–74 min were 117.3 ± 3.1%, n = 12 and 109.8 ± 3.9%, n = 9, respectively; $p > 0.05$).

CPA had a more marked effect on the development of DP than Tg. Switching of the flow system to deliver CPA (1 μM) was performed 35 min before stimulation was interrupted. After reinitiation of test stimulation, experiments showed a transient increase in pS amplitude as compared with controls, this settling over a period of 7 min (Fig. 3, *B*): immediately after the reinitiation of stimulation, mean pS amplitude in experiments was significantly greater than that in controls (122.6 ± 4.7%, n = 17 and 107.3 ± 1.9%, n = 15, respectively; $p < 0.05$) but lower than the DP "peak" in "normal" conditions (173.1 ± 7.0%, n = 14; $p < 0.001$); by the next time period (63.5–67 min), there were no significant differences between experiments and controls (113.4 ± 4.9%, n = 17 and 105.6 ± 2.2%, n = 15; $p > 0.05$). Thus, the results of experiments with both Tg and CPA provided evidence that stored calcium is involved in the mechanism of formation of DP.

Discussion

In experiments addressing the mechanisms of synaptic plasticity, the moderate-strength, low-frequency test stimuli used for assessment of baseline response levels, even on prolonged testing, do not usually elicit significant changes in response amplitude. This lack of changes in the efficiency of synaptic transmission can be termed "null plasticity." Increases in afferent activity, depending on the pattern, produce synaptic changes in two directions [Conti and Lisman, 2002; MacDonald, et al., 2006]: transient high-frequency stimulation, or tetanization, induces long-term post-tetanic potentiation (LTP) [Bliss and Lømo, 1973], while longer-lasting low-frequency stimulation leads to long-term depression of responses (LTD) [Lynch et al., 1977]. Our experiments – both on living hippocampal slices with recording of pop spikes from neurons in field CA1 during stimulation of Schaffer collateral-commissural fibers and in vivo, in anesthetized animals with recording of pop spikes from neurons in the dentate fascia during stimulation of the medial perforant pathway – showed that prolonged interruption of stimulation leads to significant changes in the amplitudes of responses to test stimulation, i.e., input-specific deprivation potentiation [Popov, 1994; Popov and Markevich, 1999]. This effect depended on the duration of the pause in stimulation (periods from 10 min to 4 h were studied); the longest pauses produced values comparable with LTP (on average to almost 200% of the initial 100% baseline response level). After reinitiation of test stimulation, DP persisted for more than an hour. In vivo experiments showed that the effect was not an artifact of the living slices method and that its occurrence was not restricted to connections between Schaffer collaterals and field CA1 neurons (in some in vitro experiments, testing of the responses (pS) of field CA1 neurons to stimulation of afferent fibers in the stratum oriens also demonstrated DP after a 60-min interruption in stimulation).

Calcium plays an important role in regulating many and varied neuronal processes. Increases in the free Ca^{2+} concentration in the postsynaptic cell trigger whole cascade systems of second messengers leading to changes in the efficiency of synaptic connections. Depending on the nature of the local Ca^{2+} signal and the magnitude and duration of the increase in the intracellular Ca^{2+} concentration, induction in different directions can be triggered, such as LTP or

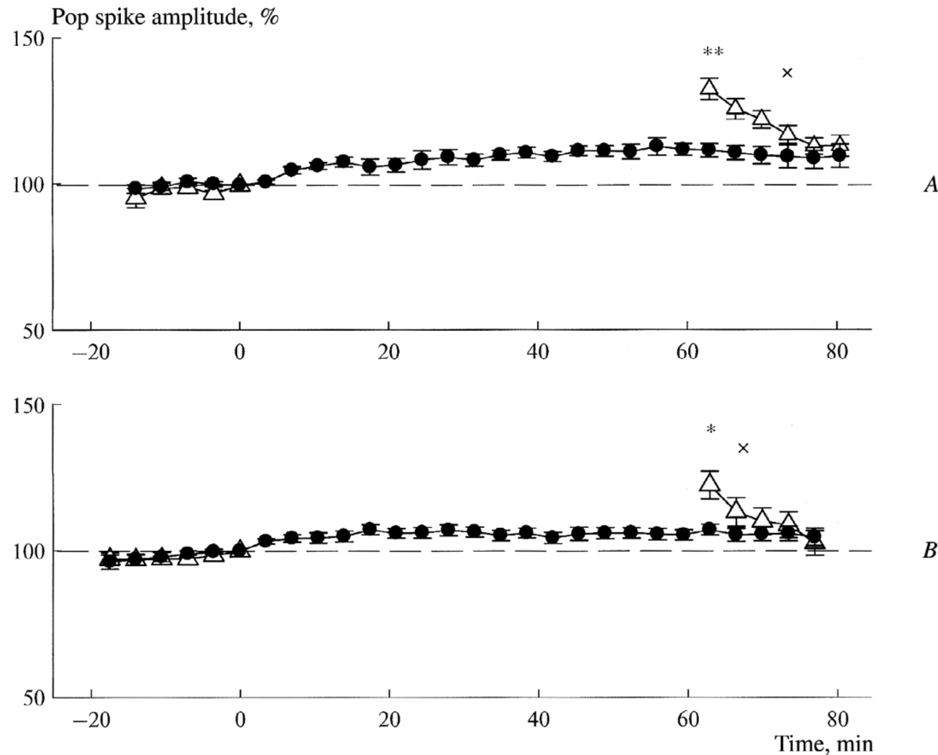

Fig. 3. Depletion of the endoplasmic Ca^{2+} depot with SERCA inhibitors (thapsigargin/cyclopiazonic acid) leads to impaired development of DP, with a reduction in the initial transient phase and suppression of the prolonged phase of DP. *A*) Experiments in the presence of thapsigargin (1 μM). Light triangles show experiments (n = 12); dark circles show controls (n = 9). *B*) Experiments in the presence of cyclopiazonic acid (1 μM) in the solution. Light triangles show experiments (n = 17); dark circles show controls (n = 15); $^{*}p < 0.05$; $^{**}p < 0.001$; $^{\times}p > 0.05$.

LTD [Conti and Lisman, 2002; Mizuno et al., 2001], while different tetanization conditions leading to different forms of LTP with different decay constants are linked with different Ca^{2+} sources [Raymond and Redman, 2002].

Our previous studies of the dynamics of the development of DP, the PKC dependence of the development of DP, and the suppression of DP by prior induction of LTP [Popov and Markevich, 2001] provide evidence supporting the suggestion that there is similarity between the mechanisms of the development of DP and the protein kinase C-dependent phase of LTP (LTP-1), linked with protein phosphorylation [Frey et al., 1995; Reymann et al., 1988; Reymann, 1993]. In turn, this provided grounds for suggesting the possibly key role of Ca^{2+} in the mechanisms of development of DP.

Administration of Ca^{2+} into cells led to induction of LTP [Malenka et al., 1988]; conversely, intracellular injection of chelators (enterosorbents) of Ca^{2+} (EGTA/BAPTA) suppressed the development of LTP/LTD [Lynch et al., 1983; Mulkey and Malenka, 1992]. Similar actions (depression of LTP) in experiments on living hippocampal slices from young animals were produced by the membrane-penetrating Ca^{2+} chelator BAPTA-AM [Tonkikh et al., 2006].

We tested the role of Ca^{2+} in the mechanism of DP without disturbing the overall scheme of the experiments using BAPTA-AM. These experiments showed that addition of BAPTA-AM (30 μM) to the solution led to impairment of the "normal" development of DP on 60-min deprivation: short-term persistence of a reduced initial "peak" was accompanied by marked suppression of the "plateau" phase. Thus, these experiments demonstrated the important role of Ca^{2+} for the development of DP regardless of its source.

One of the main sources of free cytosolic Ca^{2+} is the extracellular medium. Experiments on living hippocampal slices showed that a reduction in the Ca^{2+} concentration in the solution (from 2.5 to 1.0 mM) and an increase in the Mg^{2+} concentration (from 2.4 to 10 mM) or a simultaneous decrease in Ca^{2+} with an increase in Mg^{2+} (to 1.0 and 3.9 mM, respectively) led to impairments in long-term but not short-term post-tetanic potentiation of neuron responses in field CA1 [Dunwiddie and Lynch, 1979]. In our experiments, the possible involvement of extracellular Ca^{2+} in the induction of DP was tested using conditions with a simultaneous decrease in the Ca^{2+} concentration and an increase in the Mg^{2+} concentration in the perfusion solution with no

change in the total content of divalent cations and enhanced magnesium block in NMDA channels. These experiments showed that a decreased Ca^{2+} concentration in the solution (to 1 mM) and an increased Mg^{2+} concentration (to 3 mM), with the usual experimental scheme using a 60-min interruption of stimulation, led to suppression of the long-term phase of DP; the initial transient component of DP persisted in reduced form. Thus, the important role of extracellular Ca^{2+} in the development of DP was demonstrated, though the question of the mechanism by which Ca^{2+} entered the cells remains open, as the main Ca^{2+} gate, i.e., voltage-dependent NMDA receptors, should be inactivated with the cell in the resting state and in concentrations of enhanced magnesium block.

A very important source of free Ca^{2+} for cytosolic cellular signal transmission is provided by intracellular organelles, the main Ca^{2+} storage role being played by the endoplasmic reticulum (ER) [Camello et al., 2002]. In cells, the ER forms a single, extended space in which Ca^{2+} ions can diffuse relatively freely, creating a "calcium tunnel" [Lam and Galione, 2013]. The overall set of structural and functional characteristics of the ER provide grounds for regarding it as a "neuron within a neuron" [Berridge, 1998]. The role of the ER in forming cytosolic Ca^{2+} signals is supported by two counteracting streams: Ca^{2+} uptake mediated by pumps of the SERCA family (sarco- and endoplasmic reticulum calcium ATPases) and the release of Ca^{2+} from the ER via Ca^{2+} channels (ryanodine and inositol triphosphate receptors), as well as Ca^{2+} leakage independent of the main receptor classes, which in the presence of SERCA inhibitors can almost completely deplete the ER reserves over a period of a few minutes [Camello et al., 2002]. Studies on living hippocampal slices showed that SERCA inhibitors (including Tg and CPA) impair the differently directed induction of synaptic plasticity: Tg blocked induction of LTP [Harvey and Collingridge, 1992] and also blocked LTP induced by weak but not strong tetanization [Behnisch and Reymann, 1995]; induction of LTD was blocked in the presence of both Tg and CPA [Reyes and Stanton, 1996].

Our experiments tested the possible involvement of stored calcium in the mechanism of development of DP. These studies showed that both Tg (1 μM) and CPA (1 μM) impaired the development of DP: both suppressed the "plateau" phase of DP, while the transient initial "peak" after reinitiation of stimulation persisted in reduced form. Thus, the experiments reported here demonstrate the important role of the ER depot as a source of Ca^{2+} for the development of DP.

Overall, these experiments show that calcium plays a key role in the mechanism of development of DP, its sources being both the extracellular medium and the intracellular ER depot. At the same time, how Ca^{2+} enters from outside during induction of DP remains to be understood, and the mechanism of initiation of Ca^{2+} release from the intracellular depot is also unclear. The persistence of the initial "peak" of DP in a more or less reduced form in all cases of suppression of the long-term "plateau" phase (at reduced solution Ca^{2+} content, in the presence of BAPTA-AM, Tg, or CPK, and also after prior induction of LTP and in the presence of PKC blockers [Popov and Markevich, 2001]) provided grounds for the suggestion that the mechanisms of development of these phases are different in nature and that the "plateau" phase is linked with protein phosphorylation. One view is that Ca^{2+} release from the ER reservoir, operating as an amplifier to increase the cytosolic Ca^{2+} concentration, at least theoretically, gives this organelle the capacity to generate a short-term jump in Ca^{2+} in the absence of marked depolarization of the plasma membrane [Gleichmann and Mattson, 2011]. This provides grounds for the suggestion that that the increase in the cytosolic Ca^{2+} concentration during induction of DP may be impulsive and perhaps local in nature.

Conclusions

1. Calcium plays a key role in the mechanism of induction of deprivation potentiation developing after prolonged interruption of low-frequency test stimulation.

2. The sources of the calcium required for deprivation potentiation are both the extracellular medium and the intracellular endoplasmic reticulum reservoir.

This study was supported by the Russian Academy of Sciences and the Russian Foundation for Basic Research (Grant No. 13-04-021a).

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