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Two distinct modalities of NMDA-receptor inactivation induced by calcium influx in cultured rat hippocampal neurons

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Repetitive stimulation of glutamate (glu) receptors elicits increasingly smaller ionic currents in hippocampal neurons. To investigate mechanisms underlying this phenomenon, voltage clamp whole-cell currents evoked by glu (100 μM) were recorded from hippocampal neurons in culture. These currents were primarily carried by *N*-methyl-D-aspartate-receptor (NMDA-R) channels, as shown by the voltage-dependent sensitivity to extracellular Mg^{2+} blockade, and inhibition by the specific antagonist MK-801. In the presence of 2.2 mM extracellular Ca^{2+} ($[Ca^{2+}]_e$), repetitive glu applications (15 episodes of 4 s/min) elicited progressively smaller currents that stabilized at 45% of their initial peak value. Replacement of $[Ca^{2+}]_e$ by Ba^{2+} produced similar effects. This phenomenon, defined as *interepisode inactivation*, was exacerbated by elevating $[Ca^{2+}]_e$ to 11 mM, attenuated by reducing $[Ca^{2+}]_e$ to 0.22 mM, and further diminished by shortening the length of the glu pulse to 2 s. Current decay exhibited during individual stimuli, or *intraepisode inactivation*, was dependent on $[Ca^{2+}]_e$ yet remained stable during repetitive stimulation. We conclude that interepisode and intraepisode inactivations of NMDA-R currents are the expression of two distinct processes triggered by Ca^{2+}. These modalities of inactivation may arise from Ca^{2+} binding either to the receptor or to closely associated regulatory proteins.

Glutamate receptor; Ionic channel; Receptor modulation; Synaptic transmission; Signal transduction

1. INTRODUCTION

Glutamate receptors (glu-R) have a key role in excitatory synaptic transmission in the central nervous system, especially in long term potentiation (LTP) and excitotoxicity [1,2]. Among the different classes of glu-R, the *N*-methyl-D-aspartate receptor (NMDA-R) has received special attention because of its high Ca^{2+} permeability [3–5]. It is well known that Ca^{2+} influx through the NMDA-R in the post-synaptic neuron is necessary to induce, although in very different ways, both LTP [1] and excitotoxicity [2]. However, little is known about regulatory mechanisms for NMDA-R and whether Ca^{2+} is involved in receptor modulation. Mayer and Westbrook [6] suggested that Ca^{2+} might exert an inhibitory effect on NMDA-R currents. Indeed, an increase in intracellular Ca^{2+} concentration caused by membrane depolarization reduced the current elicited by NMDA [4], and repeated applications of NMDA in cultured neurons produced a decrease in the evoked current [7–9]. Recently, Legendre et al. [10] showed that long stimulation pulses elicit NMDA-R inactivation triggered by intracellular Ca^{2+}.

Here we present a systematic electrophysiological study of the use-dependent inactivation of NMDA currents in hippocampal neurons. We report that inactivation occurring during a single episode (*intraepisode inactivation*) and that arising as a consequence of repetitive stimulation (*interepisode inactivation*) are both triggered by intracellular Ca^{2+}, yet are clearly discerned from each other. Presumably, Ca^{2+} ions exert their effect either via direct binding to the channel or to closely associated proteins.

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Abbreviations: BSS, balanced salt solution; $[Ca^{2+}]_e$, extracellular calcium concentration; $[Ca^{2+}]_i$, intracellular calcium concentration; FUDR, 5-fluoro-2′-deoxyuridine; glu, glutamate; *I*, current; *i*, inactivation; LTP, long term potentiation; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclohepten-5,10-im ine maleate; n, number of experiments; NMDA, *N*-methyl-D-aspartate; *P*, peak current; R, receptor; *S*, steady state current; *V*, voltage.

2. MATERIALS AND METHODS

2.1. *Cell cultures*

Mixed glial/neuronal cultures were prepared using a modification of the protocol described by Goslin and Banker [11]. Briefly, pregnant rats (E17 to E19) were anesthetized with CO_2 and sacrificed by cervical dislocation. Embryos were dissected out and decapitated. Brains were immediately removed and kept on ice in a divalent cation-free balanced saline solution (BSS, in mM): 137 NaCl, 3.5 KCl, 0.4 KH_2PO_4, 0.33 Na_2HPO_4, 10 glucose, 5 2-[tris(hydroxymethyl)methyl-amino]-1-ethanesulfonic acid (TES), pH 7.3, traces of Phenol red. Hippocampi were dissected and incubated in BSS supplemented with 0.25% trypsin (15 min, 37°C). Trypsin was diluted-out by rinsing the tissue 3 times for 5 min with BSS. Tissue was dissociated by repeated passings through a Pasteur pipette (tip initially unpolished, then fire-polished) until few cell clumps were seen. Cells were counted in presence of 0.04% Trypan blue, and plated (10^5 viable cells/cm^2) in minimal essential medium (Earle's salts) supplemented with 10% horse serum (Hyclone, Logan, UT), 10% fetal bovine serum (Hyclone), 1 mM glutamine, and 22 mM glucose. Tissue culture dishes (Corning 25000)

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were pretreated with 0.5 mg/ml poly-D-lysine (Sigma, St. Louis, MO) for 18 h and washed twice for 2 h. Teflon rings were used to limit the plating surface to 10 mm diameter. No antibiotics were necessary.

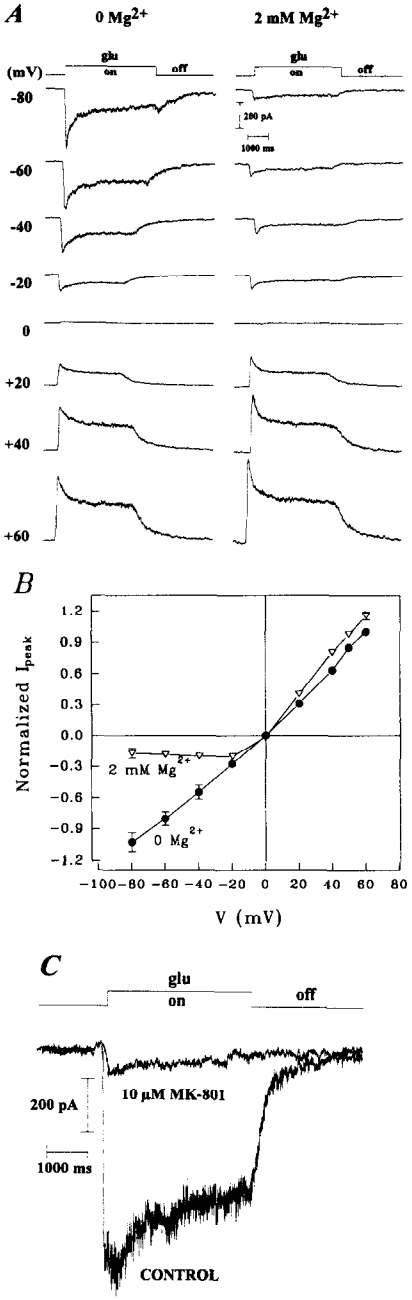

Fig. 1. Glutamate activates NMDA-R currents in hippocampal neurons. (A) Glu (100 μM) plus gly (10 μM) were applied to the cells, in the absence (left panel) and presence (right panel) of 2 mM Mg^{2+} in the extracellular compartment. Whole-cell recordings were carried out at the indicated V_m. Inward (downward deflections) and outward (upward deflections) currents are coincident with application of neurotransmitter, depicted as a pulse in the top of the figure; on and off denote initiation and cessation of the perfusion pulse. Currents were digitally filtered at 100 Hz. (B) Normalized peak currents vs. membrane potential relationships. Each point was normalized with respect to the current evoked at +60 mV in the absence of extracellular Mg^{2+} ($n = 3$, triangles; $n = 4$, circles). (C) Superimposed current traces obtained by perfusion of glu and gly, in absence (CONTROL) and presence of 10 μM of MK-801. Currents were recorded at $V_m = -80$ mV.

Cultures were maintained at 37°C in a humidified atmosphere containing 6% CO_2. Teflon rings were removed after 24 h, and the medium was replaced. After 3 to 4 days, glial cell division was halted by addition of 80 μM 5-fluoro-2'-deoxyuridine (FUDR) and 200 μM uridine, and culture medium was switched to one without fetal bovine serum. One third of the medium was changed biweekly. FUDR-uridine solution was added as needed (every 10 days) to keep glial cells from growing. Under these conditions, hippocampal neurons grew for 4–5 weeks.

2.2. *Electrophysiological recordings*

Cells cultured 7 to 21 days in vitro (DIV) were used. Neurons were removed from the incubator and the medium was replaced with extracellular recording solution, consisting of BSS supplemented with: 0.22, 2.2 or 11.0 mM $CaCl_2$ (as indicated), 600 nM tetrodotoxin (Sigma), and 10 μM glycine (gly). Mg^{2+} was omitted to avoid blockade of NMDA currents. The 35 mm dish was mounted on an inverted microscope stage (Nikon Diaphot) equipped with phase-contrast optics. Neurons with clear pyramidal shape and bright bodies were selected for recording [12]. Agonist application and extracellular solutions were controlled through a manually driven, multibarreled perfusion system. To insure the reliability of the initial conditions, a new dish was used for each experiment. Glu (Sigma) and MK-801 [(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclohepten-5,10-i mine maleate] were prepared as 1000 × stock solutions in distilled water, and added to the external solution. Experiments were performed at room temperature (22 ± 2°C).

Whole-cell currents were recorded using the patch clamp technique [13]. Patch microelectrodes were filled with the following solution (mM): 140 CsCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 5 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*',*N*'-tetraacetic acid (BAPTA), 2 Mg-ATP (added a few hours before use), 5 TES, pH adjusted to 7.3 with CsOH. Under these conditions, the estimated intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) was ≈ 12 nM, and that of unbound BAPTA ≈ 4.11 mM [14,15]. Where indicated, the free Ca^{2+} concentration was increased to 1 μM by modifying the microelectrode solution as follows (mM): 1.75 $MgCl_2$, 11 $CaCl_2$, 10 BAPTA; the estimated concentration of free BAPTA was ≈ 1 mM. Phalloidin (Sigma) was dissolved in methanol (6.34 mM), and added to the microelectrode solution to a final concentration of 1 μM, as indicated. Recordings were started 4–5 min after achieving the whole-cell configuration to allow for diffusion of reagents from the pipette to the cytoplasm. Pipette tip resistance was 1–3 MΩ. Access resistance (typically 3 to 6 MΩ) was compensated 50% to 70% through a built-in analog circuit of the patch clamp amplifier (List electronic, Model EPC-7, Darmstadt/Eberstadt, Germany).

Inactivation of glutamate-elicited currents was studied by repetitive stimulation of the neuron using the following pulse protocol: agonist was applied for 2 or 4 s (as indicated) and immediately washed out; 60 s after the episode onset, a new pulse was initiated. This protocol was repeated 15 times. Not every neuron resisted all 15 pulses: some became leaky during the experiment and, therefore, the number of data points compiled in Fig. 3 is higher for the initial episodes than for the final ones. Steady-state currents were measured at the end of the stimulation pulse. Glutamate-activated currents were in the range of 1–5 nA.

Data were filtered at 500 Hz (8 pole Bessel filter, Frequency Devices, Haverhill, MA), and acquired (4 ms/point) on line using a DMA interface (Labmaster TL-1, Axon Instruments, Burlingame, CA) connected to a 486-based computer. Acquisition and analysis were performed with the software PClamp 5.5.1 (Axon Instruments). All processed data are reported as mean ± S.E.M.; *n* represents the number of experiments.

3. RESULTS AND DISCUSSION

3.1. *Glutamate activates primarily NMDA-receptor currents in hippocampal neuronal cultures*

Glu displays mixed agonist properties for both

NMDA and non-NMDA receptors in mouse spinal cord neurons in culture [16], but activates only NMDA-receptors in cultured mouse striatal neurons [17]. In cultured hippocampal neurons, glu evokes NMDA-R currents, as shown in Fig. 1. Cells were stimulated by perfusion with 100 μM glu. A family of currents recorded at the indicated voltages is shown in the absence or presence of 2 mM Mg^{2+}. Glu induces slow inactivating currents, inward at negative membrane potentials (V_m) and outward at positive V_m, with a reversal potential at $\approx$ 0 mV (Fig. 1A,B). In presence of Mg^{2+}, inward currents are blocked at hyperpolarized potentials. The voltage-dependent blockade of glu-activated currents by Mg^{2+} is summarized in Fig. 1B. Peak currents vs. V_m relationships illustrate the switch from ohmic behavior to an outward rectifying profile in presence of Mg^{2+} (Fig. 1B): at −80 mV the current amplitude is reduced 83 ± 5% (n = 3). This rectification is characteristic of the NMDA subtype of glu receptors [6,18]. To confirm the identity of the receptor, MK-801, a specific open channel blocker for NMDA-R [19], was tested in the absence of extracellular Mg^{2+}. MK-801 (10 μM) blocked 89.0 ± 2.4% (n = 4) of the current elicited by glu at V_m=−80 mV (Fig. 1C). Hippocampal neurons express functional glu-receptors of the kainate subtype: application of kainate elicited non-inactivating ohmic currents in a dose-dependent manner (data not shown). Taken together, these results indicate that, under these recording conditions, approximately 90% of the current activated by glu is carried through NMDA-R channels.

3.2 *NMDA-receptor currents inactivate upon repetitive stimulation by glutamate*

NMDA-receptor inactivation in hippocampal neu-

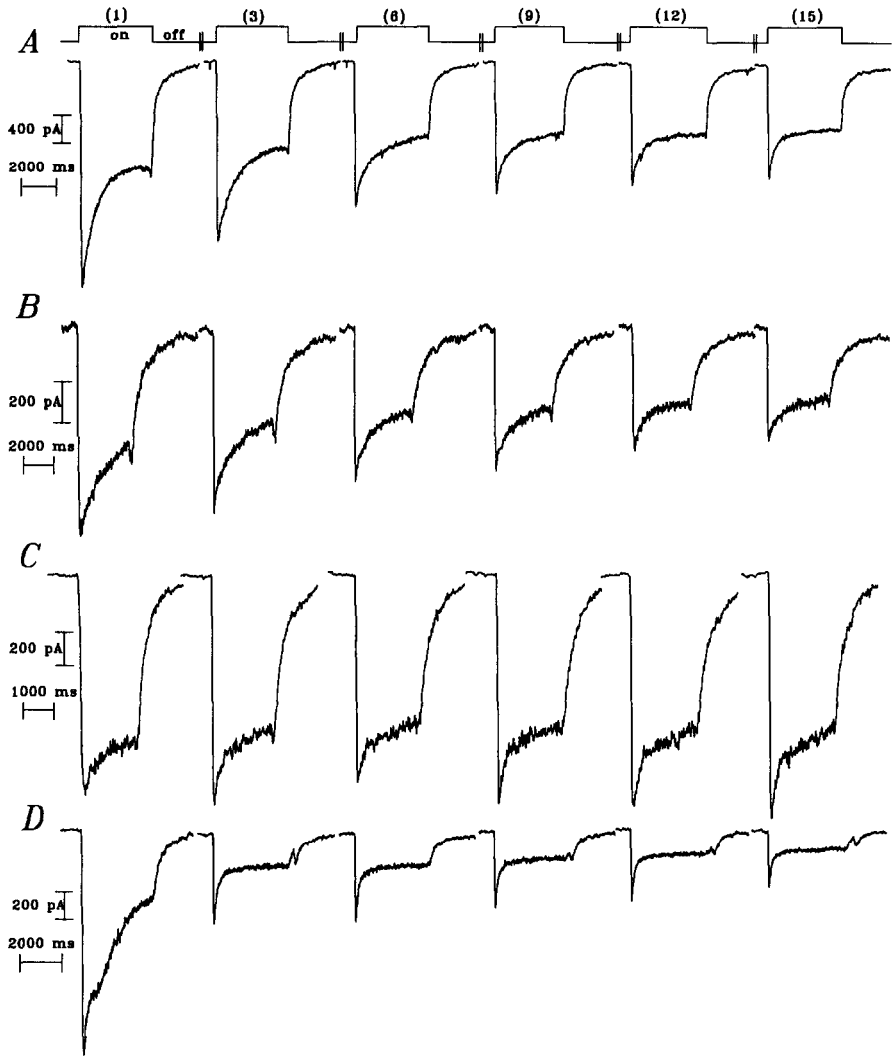

Fig. 2. NMDA-receptors display use-dependent inactivation mediated by Ca^{2+} influx. Whole cell currents recorded at V_m = −80 mV were evoked by repetitive pulses of glu (15 pulses; 2 s or 4 s/min, as indicated). The stimulation protocol is displayed in the top trace: six representative episodes are plotted. Episode number is indicated in parentheses. (A) $[Ca^{2+}]_e$ = 2.2 mM; pulse length = 4 s. (B) $[Ca^{2+}]_e$ = 0.22 mM; pulse length = 4 s. (C) $[Ca^{2+}]_e$ = 0.22 mM; pulse length = 2 s. (D) $[Ca^{2+}]_e$ = 11.0 mM; pulse length = 4 s. Traces were digitally filtered at 30 Hz.

rons was studied using a repetitive stimulation protocol. A series of 15 pulses (4 s/min) of 100 μM glu in presence of 2.2 mM Ca^{2+} in the extracellular solution ($[Ca^{2+}]_e$), evoked increasingly smaller inward currents. This is shown in Fig. 2A: both peak and steady state currents diminish with consecutive pulses, as clearly discerned by comparing the selected six episodes in the sequence. This slow inactivation phenomenon occurred regardless of whether the stimulation protocol started immediately after perforating the membrane to enter the whole-cell configuration, or after a delay (10–20 min). This suggests that inactivation is not related to a time-dependent run-down arising from the wash-out of high-energy phosphates [8]. Fig. 3A (filled circles) displays the progress of inactivation with stimulation. Peak currents were normalized with respect to the first response and plotted vs. the episode number. Note that there is a dramatic reduction in current amplitude during the first three episodes, and then inactivation asymptotically reaches a plateau at ≈ 45%. We define this phenomenon as interepisode inactivation.

3.3. *Interepisode inactivation is mediated by Ca^{2+} influx into the neuron*

To test the role of Ca^{2+} influx in the use-dependent inactivation, the same repetitive stimulation protocol was applied in presence of different $[Ca^{2+}]_e$. As shown in Fig. 2B, lowering the external Ca^{2+} concentration to 0.22 mM decreased the extent of interepisode inactivation. The averaged peak currents from 16 neurons (Fig. 3A, open triangles) clearly illustrates this effect. If Ca^{2+} ions mediate inactivation, shortening the pulse length should attenuate it. Fig. 2C shows that this is indeed the case for pulses of 2 s. As summarized in Fig. 3A (filled triangles) interepisode inactivation was < 20%, and stabilized after the sixth episode. In contrast, when the short pulse protocol was applied in 2.2 mM $[Ca^{2+}]_e$, inactivation was only partially prevented (data not shown). Elevating $[Ca^{2+}]_e$ to 11 mM (Figs. 2D and 3A, open squares) dramatically increased inactivation. These results demonstrate that NMDA-receptors ex-

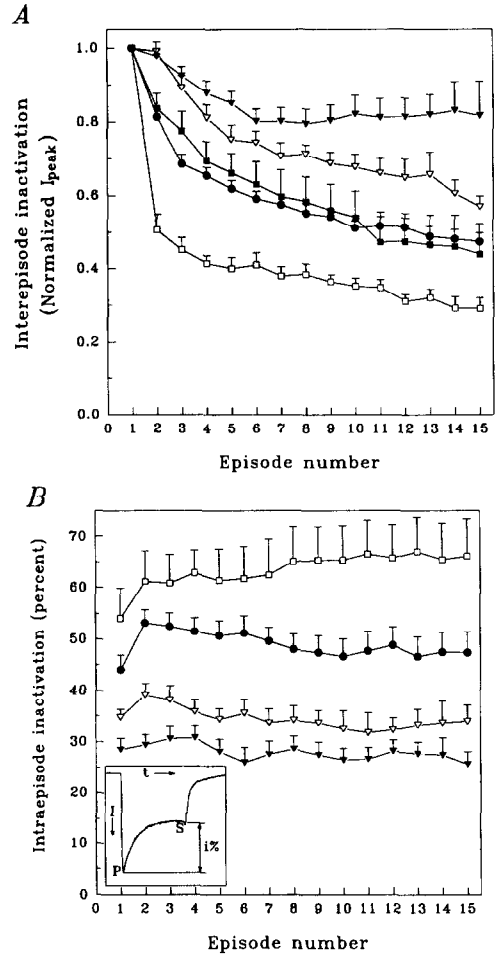

Fig. 3. Interepisode and intraepisode inactivations are both modulated by Ca^{2+} influx. (A) Interepisode inactivation, expressed as normalized peak currents with respect to the first episode vs. episode number. □: $[Ca^{2+}]_e$ = 11.0 mM, pulse length = 4 s ($7 \leq n \leq 9$); ●: $[Ca^{2+}]_e$ = 2.2 mM, pulse length = 4 s ($10 \leq n \leq 17$); ■: $[Ba^{2+}]_e$ = 2.2 mM, pulse length = 4 s ($7 \leq n \leq 10$); ▽: $[Ca^{2+}]_e$ = 0.22 mM, pulse length = 4 s ($5 \leq n \leq 16$); ▼: $[Ca^{2+}]_e$ = 0.22 mM, pulse length = 2 s ($8 \leq n \leq 18$). Each point represents the mean+S.E.M. (B) Intraepisode inactivation, calculated with the expression i (%) = $(1 - S/P) \times 100$, where P and S denote peak and steady state currents, as indicated in the inset. Other conditions were as described in Fig. 2.

Table I

Modification of the intracellular conditions

Intracellular conditions	Interepisode inactivation		Intraepisode inactivation (%)	
	Episode no. 5	Episode no. 10	Episode no. 5	Episode no. 10
Control (12 nM Ca^{2+})	0.619 ± 0.023 (n = 14)	0.511 ± 0.019 (n = 12)	50.7 ± 2.9 (n = 14)	46.6 ± 3.5 (n = 12)
1 μM Ca^{2+}	0.629 ± 0.031 (n = 9)	0.612 ± 0.048* (n = 5)	54.5 ± 3.5 (n = 9)	58.3 ± 7.7 (n = 5)
1 μM phalloidin	0.638 ± 0.030 (n = 11)	0.555 ± 0.059 (n = 6)	58.8 ± 4.6 (n = 11)	58.5 ± 7.3 (n = 6)

Neurons were stimulated 15 times in presence of $[Ca^{2+}]_e$ = 2.2 mM during 4 s. Tabulated values for inter- and intraepisode inactivations correspond to those of episodes number 5 and 10. Recordings were obtained as described in Fig. 2, except that $[Ca^{2+}]_i$ was set at 1 μM, or 1 μM phalloidin was included in the pipette solution. Results for test and control conditions were evaluated using a two-tailed t-test; * indicates that the difference is significant, with $P < 0.05$.

hibit use-dependent inactivation, and that this phenomenon is dependent on Ca^{2+} influx.

3.4. *Inactivation during individual stimuli is also dependent on Ca^{2+} influx*

Ca^{2+} also affects the amplitude of the current decay during individual pulses (Fig. 2). We define this phenomenon as intraepisode inactivation, and quantitate it, as shown in the inset to Fig. 3B, with the use of the formula: $i\,(\%) = (1 - S/P) \times 100$, where i denotes inactivation, and P and S the amplitudes of the peak and steady state currents, respectively. Accordingly, a non-inactivating current will have an $i = 0$, and a current that inactivates completely will have $i = 100\%$ (Fig. 3B, inset). Fig. 3B summarizes the effect of Ca^{2+} on intraepisode inactivation. For $[Ca^{2+}]_e = 0.22$ mM, both 4 s and 2 s pulses (filled and open triangles, respectively), inactivation is $\approx 30\%$, basically unaltered by repetitive stimulation. Intraepisode inactivation increases at $[Ca^{2+}]_e = 2.2$ mM (filled circles) and 11 mM (open squares), and is not modified by repetitive stimulation. Currents appear to inactivate less during the first episode than during subsequent pulses. This arises from the fact that currents evoked during the first episode do not invariably reach a plateau in 4 s, thereby yelding an underestimated value. Thus, for all $[Ca^{2+}]_e$ tested, intraepisode inactivation is practically constant during repetitive stimulation, independent of previous events. In contrast, interepisode inactivation is dependent on the pulse history, displaying a cumulative effect (Fig. 3A). This phenomenological analysis, therefore identifies different processes underlying each modality of inactivation.

The effect of cytoplasmic Ca^{2+} on inactivation was tested by increasing $[Ca^{2+}]_i$ to 1 μM. As shown in Table I, this change in $[Ca^{2+}]_i$ was not sufficient to interfere with the effect of Ca^{2+} influx on inter- or intraepisode inactivations.

3.5. *Ba^{2+} mimics the effect of Ca^{2+} in use-dependent inactivation*

As shown in Fig. 3A (open squares), perfusion of glu during 4 s in presence of 2.2 mM Ba^{2+} in the extracellular solution induces a similar pattern of interepisode inactivation. The intraepisode inactivation displayed a variable pattern, fluctuating between 30 and 40% throughout the stimulation protocol (data not shown). These results show that both Ba^{2+} and Ca^{2+} induce use-dependent inactivation. This lack of specificity may provide a clue to the underlying mechanism.

3.6. *Conclusions*

Our results show that, in cultured hippocampal neurons, glu activates receptors of the NMDA- but not of the kainate-subtype. NMDA-receptors activated by glu exhibit two distinct types of inactivation: interepisode and intraepisode. Both modalities of inactivation are modulated by intracellular Ca^{2+}, yet they display different properties, therefore suggesting that the underlying molecular mechanisms are separate. Recent pharmacological evidence indeed alludes to the involvement of actin depolymerization and, therefore, to cytoskeletal-receptor interactions in interepisode but not intraepisode inactivation [20]. Under our experimental conditions, however, phalloidin had no significant effect on inactivation (Table I). In contrast, intraepisode inactivation may arise from Ca^{2+} binding either directly to the channel or to closely associated proteins.

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