Enhancement of Voltage-gated Ca^{2+} Currents Induced by Daily Stimulation of Hippocampal Neurons with Glutamate

David E. García,^a Adolfo Cavalié, and Hans D. Lux

Department of Neurophysiology, Max-Planck-Institut für Psychiatrie, D-82152 Martinsried, Germany

The regulation of calcium channel currents (I_{Ca}) induced by daily stimulation (1 hr) with 10 μM glutamate was studied in full differentiated hippocampal cells in culture. We report a specific enhancement of the high-voltage-activated current type (HVA I_{Ca}) ongoing over days. The density of HVA I_{Ca} increased about twofold after the second glutamate session, and this enhancement was still observed after the fifth day of treatment, while low-voltage-activated calcium currents (LVA I_{Ca}) remained unchanged. During glutamate application, a transient increase of intracellular calcium (Ca_i) was observed, followed by a slow decay within 2–3 min, and substantial recovery in about 10 min. Similarly, Ca_i transients induced by periodic membrane depolarization mimicked the long-term effect of glutamate on I_{Ca} . These results demonstrate for the first time an increase of I_{Ca} in a time frame of days. Since the effect of glutamate on I_{Ca} was prevented by cycloheximide, neosynthesis of channel proteins presumably supports this enhancement.

[Key words: calcium currents, intracellular calcium transients, glutamate, hippocampus]

L-Glutamate is the major excitatory neurotransmitter in mammalian nervous system, which induces Ca_i elevations directly through ionotropic (Hollmann et al., 1989; Boulter et al., 1990; Keinänen et al., 1990; Sommer et al., 1990) and metabotropic receptors (Sugiyama et al., 1987; Schoepp et al., 1990), and indirectly, through membrane depolarization that activates voltage-gated Ca channels (Kudo and Ogura, 1986; MacDermott et al., 1986; Murphy et al., 1987; Murphy and Miller, 1988). Vigorous Ca_i elevations can be also induced in nerve cells by membrane depolarization alone (Kudo and Ogura, 1986; Murphy and Miller, 1988). It has been assumed that such elevations of Ca_i are critical in inducing long-term changes in neurons (Baudry et al., 1981; DeLorenzo, 1981; Lynch and Baudry, 1984). Indeed, agents that elevate Ca_i induce differentiation and growth (Shubert et al., 1978; Kater and Mills, 1991), and trigger gene expression (Sheng and Greenberg, 1990; Zafra et al., 1990). During development, Ca channel currents undergo progressive enhancement in hippocampal cells (Yaari et al., 1987) and sen-

sory neurons (Gottmann et al., 1991). We were interested in additional modes of regulation that could reflect new synthesis of Ca channels and may result in changes of I_{Ca} enduring several days. To obtain reliable controls, full differentiated hippocampal cells were chosen for the present study. Since glutamate has been associated with important processes like excitatory synaptic transmission (Mayer and Westbrook, 1987), the hippocampal cells were stimulated repetitively with glutamate during several days. Periodical stimulation was preferred instead of permanent exposure to glutamate because of possible toxic effects of this neurotransmitter during prolonged periods of time (Randall et al., 1992). Membrane depolarization was also induced periodically because sustained depolarization resulted in downregulation of I_{Ca} (Franklin et al., 1992). Here, we report upregulation in I_{Ca} of hippocampal cells following daily-induced Ca_i transients.

Materials and Methods

Cell cultures. Hippocampal cell cultures were prepared from rat embryos (18–19 d) by conventional techniques (Banker and Cowan, 1977; Segal, 1983). Briefly, the tissue from six to eight dissected hippocampi was mechanically dissociated. Cells were plated at a density of 10^5 cells per dish in basal medium (Eagle, GIBCO, Eggenstein, Germany) enriched with 2 mM glutamine, 10% glucose, and 10% horse serum. Glia proliferation was suppressed by adding β -cytosine arabinoside (10^{-8} M) on the fourth day of culture for 24 hr. Dishes were incubated at 37°C during 2–3 weeks. Only well-differentiated pyramidal-like or stellate-like neurons were employed for measurements.

Glutamate and KCl treatment. The experimental protocol consisted in a single daily exposure of the cells to 10 μM glutamate or 45 mM KCl for 1 hr. Glutamate or KCl was applied directly into the medium and the cells were incubated at 37°C for 1 hr. Afterward, exhaustive washout was performed in order to restore the basal culture conditions. The same washout procedure was applied to the control cells in order to discard effects introduced by solution exchange. The glutamate exposures were performed from day 12 to 16 of culture, and these experimental days were termed from day 0 (d0) to day 5 (d5), respectively.

Electrical measurements. Whole-cell recordings (Hamill et al., 1981) were made from the somatic region of the neurons with low-resistance (2–3 M Ω) microelectrodes using a EPC-7 patch-clamp amplifier (List Medical, Darmstadt, Germany). All measurements were made 24 hr after each glutamate or KCl session at room temperature (20–22°C). I_{Ca} was isolated from other voltage-dependent membrane currents by ionic substitution and addition of sodium and potassium channel blockers to the external and internal solutions, respectively. Bath solution contained (mM) 120 NaCl, 10 CaCl_2 , 2 MgCl_2 , 10 HEPES, and 3 μM TTX, pH 7.4 (NaOH). Pipette solution contained (mM) 130 CsCl, 4 MgCl_2 , 10 EGTA, 10 HEPES, and 3 Na_2ATP , pH 7.4 (CsOH). Cell capacitance was calculated from the integral of the capacity transient elicited by a pulse of 10 mV from -120 mV. For each individual cell, the peak value of the maximal I_{Ca} was determined from the current–voltage relationship. Peak I_{Ca} was divided by the membrane capacitance value and the result is showed as I_{Ca} density. Signals were digitized on line at 7–12 kHz and analyzed off line. Leakage and residual capacitative currents were minimized by subtracting appropriately scaled current responses

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Correspondence should be addressed to Dr. Adolfo Cavalié, Max-Planck-Institut für Psychiatrie, Abt. Neurophysiologie, Am Klopferspitz 18a, D-82152 Martinsried, Germany.

^a Permanent address: Department of Physiology, Fac. de Medicina, UNAM, Apdo. Post. 70250, CP 04510, Mexico, D.F.

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Figure 1. Comparison of I_{Ca} in a control and in a glutamate-treated cell. Membrane current records were made 24 hr after the second daily exposure to 10 μ M glutamate for 1 hr. **A**, Superimposed records of LVA I_{Ca} in control (Con) and glutamate (Glu) conditions at the potentials indicated. No major changes can be appreciated. **B**, Superimposed records of HVA I_{Ca} of the same cells at the potentials indicated. A strong enhancement of HVA I_{Ca} amplitude occurred in the glutamate-treated cell. **C**, Current-voltage relationship of the same cells under control (open symbols) and glutamate (solid symbols) conditions. Holding potential, hp = -100 mV. Membrane capacitance, C_m ; control cell, 25.2 pF; glutamate cell, 25.5 pF.

from -120 mV of holding potential. Membrane potential measurements were made with microelectrodes filled with 3 mM KCl giving resistance of 50–80 M Ω . Membrane potential measurements were performed in a basal culture medium-like solution containing (mM) 142.19 NaCl, 5.36 KCl, 1.8 CaCl₂, 0.81 MgSO₄, 1.02 NaH₂PO₄, 5.05 glucose, 25 HEPES, pH 7.4 (NaOH).

Ca²⁺ imaging. The Ca transients were measured with the Ca indicator Fura-2. The ratio method, $R_{360/380}$ (Grynkiewicz et al., 1985), was used with fluorescence excitation at 360 and 380 and emission at 510 nm. Cells were loaded with 2.5 μ M Fura-2/AM in 0.001% pluronic acid (Calbiochem, Bad Soden, Germany) in basal culture medium at 37°C during 30–40 min. After exhaustive washout, optical measurements were performed in a culture medium-like solution (see above). Fura-2 calibration was performed either *in vivo* (1–2 μ M ionomycin) or *in vitro* (samples of solution in capillary glasses) with equivalent results (Williams et al., 1985; Tsien and Pozzan, 1989). The Ca_i concentration, [Ca²⁺]_i, was calculated using equations equivalent to those of Grynkiewicz et al. (1985), and the calibration parameters R_{min} , R_{max} and K were determined by fitting to the data the equation

$$R = (R_{max}[Ca^{2+}]_i + R_{min}K)/([Ca^{2+}]_i + K),$$

where $R_{min} = 0.46$ and $R_{max} = 7.84$ were the limiting fluorescence intensity ratios for low and high [Ca²⁺]_i, respectively, and $K = 3.72 \times 10^{-6}$ M was proportional to the binding constant of Fura-2, K_d . The latter was calculated from $K = K_d/\beta$, where $\beta = 15.75$ was the ratio of the fluorescence intensity coefficient at 380 nm in the virtual absence of Ca to that in the presence of an excess amount of Ca, S_{280}/S_{380} . Hence, $K_d = 2.36 \times 10^{-7}$ M.

Statistical methods. Data values that appear in text and figures are given as mean $\pm$ standard error of the mean (SEM). Statistical comparisons were made as independent variables, using the Student's *t* test. Values were considered statistically significant if $p < 0.05$.

Results

Long-term effects of glutamate

Whole-cell I_{Ca} was elicited by step depolarizations from membrane holding potentials (hp) of -100 or -40 mV in an extracellular solution containing 10 mM Ca²⁺. Typical records from hp = -100 mV showed clearly the LVA and HVA I_{Ca} components (Fig. 1, *A* and *B*, respectively). In good agreement with previous observations in cultured hippocampal neurons (Yaari et al., 1987), LVA I_{Ca} was activated at relatively low membrane potentials (-50 to -40 mV), and it was fully inactivated at hp

= -40 mV. HVA I_{Ca} was activated at relatively high membrane potentials (-30 to -20 mV). It was considerably larger than LVA I_{Ca} and could be activated from both hp, -100 and -40 mV. Both LVA I_{Ca} and HVA I_{Ca} activation thresholds remained unchanged after glutamate treatment. Surprisingly, HVA I_{Ca} increased dramatically after two glutamate sessions (Fig. 1*B,C*) while LVA I_{Ca} remained unchanged (Fig. 1*A,C*). Peak of maximal HVA I_{Ca} , usually found at $+10$ mV, was 1220 ± 52 pA ($n = 45$) for control and 1858 ± 108 pA ($n = 47$) for glutamate-treated cells from d2 to d5 (see Materials and Methods). However, no significant change was found from d0 to d1 (control, 1181 ± 52 pA, $n = 24$; glutamate, 1262 ± 49 pA, $n = 26$), indicating that at least two exposures to glutamate are required to enhance I_{Ca} . Maximal LVA I_{Ca} did not change between d2 and d5 under glutamate treatment (124 ± 6 pA, $n = 45$, vs 121 ± 8 pA, $n = 42$), indicating that HVA I_{Ca} is specifically affected. The enhancement of I_{Ca} after glutamate treatment could be the consequence of an increase in specific conductance or an increase in membrane surface. Therefore, cell membrane capacitance (C_m), as an indicator of the size of the cells, was measured between d2 and d5 in the same group of cells where HVA I_{Ca} enhancement was observed. No significant difference was found after glutamate treatment (24.4 ± 1.1 pF, $n = 43$, vs 26.7 ± 1.3 pF, $n = 41$).

Besides I_{Ca} amplitudes, voltage dependency, inactivation, and deactivation of HVA I_{Ca} were studied in a group of cells taken from d2 to d5 ($n = 20$). The activation, as judged by the time to peak, sped up with increasing membrane potential. Consequently, the time to peak decreased and reached at $+10$ mV the same value in control (4.67 ± 0.21 msec) as in glutamate-treated cells (4.54 ± 0.21 msec). Inactivation and deactivation of HVA I_{Ca} were adequately described by the equation $I(t) = Ae^{-t/\tau_1} + Be^{-t/\tau_2} + C$, which was employed to estimate the time constants (τ_1 and τ_2) at $+10$ mV of membrane potential, where maximal I_{Ca} was usually detected. The current decay was similar in the control ($\tau_1 = 15.3 \pm 1.1$ msec, $\tau_2 = 76.1 \pm 4.5$ msec) and in the glutamate-treated cells ($\tau_1 = 14.8 \pm 1.3$ msec, $\tau_2 = 73.5 \pm 4.1$ msec). Similarly, tail I_{Ca} did not reveal any significant

A

B

Figure 2. Cell morphology under glutamate treatment: photomicrographs comparing the typical aspect of a control (*A*) and a glutamate-treated (*B*) cell at the fourth day of treatment. No obvious differences can be appreciated. The somatic membrane surface and the proximal dendritic radius of 38 control and 37 glutamate-treated cells are shown in *C* and *D*, respectively. Measurements were done between experimental days 2 and 5 at 320 $\times$ magnification. No differences were found. Values are given as mean $\pm$ SEM.

change in the deactivation (control: $\tau_1 = 0.43 \pm 0.07$ msec, $\tau_2 = 3.23 \pm 0.28$ msec; glutamate: $\tau_1 = 0.42 \pm 0.05$ msec, $\tau_2 = 3.38 \pm 0.25$ msec).

Cell morphology

After 2 weeks in culture, most of the cells became morphologically well differentiated with two or three main processes and abundant arborizations. As illustrated in Figure 2, *A* and *B*, no obvious morphological changes could be observed between control and glutamate-treated cells. Supporting this suggestion,

measurements of the somatic membrane surface and the radius of proximal dendrites between d2 and d5 revealed no statistical difference in these parameters (Fig. 2*C,D*).

Intracellular calcium transients

Intracellular calcium plays an important role in cellular functions as a second messenger, and it is well known that glutamate induces Ca^{2+} elevations in hippocampus (Kudo and Ogura, 1986; Mayer and Westbrook, 1987; Murphy and Miller, 1988; Furuya et al., 1989). Therefore, the amplitude and time course of the

Figure 3. Ca_i transients in pyramidal-like cells during exposure to glutamate or KCl. Glutamate or KCl was added into the bath at the time indicated (arrows) and cells were continuously exposed for up to 60 min. *A*, Exposure to $10 \mu\text{M}$ glutamate. A strong Ca_i transient is produced during the first 1–2 min after glutamate application, showing complete recovery in about 10 min. *B*, KCl at 45 mM was added to obtain a final concentration of 50 mM . No complete recovery can be seen up to 20 min. Symbols represent mean $\pm$ SEM. Peak Ca_i : glutamate, $n = 15$; KCl, $n = 12$. Ca_i signal value at 20 min: glutamate, $n = 8$; KCl, $n = 6$. Significant difference was found at 20 min. Insets show typical examples of the changes observed in the membrane potential during the exposure to glutamate (*A*) or KCl (*B*).

Ca_i during the exposure to $10 \mu\text{M}$ glutamate were examined (Fig. 3*A*). Basal $[\text{Ca}^{2+}]_i$ varied from 50 to 200 nM. A strong Ca_i transient with a peak of $1.27 \pm 0.11 \mu\text{M}$ was observed during the first 1–2 min after glutamate application and substantial recovery in about 10 min. At 20 min, Ca_i was $0.18 \pm 0.07 \mu\text{M}$. In five cells, Ca_i was recorded for up to 60 min and no additional changes were observed. Because glutamate also induces mem-

brane depolarization, the membrane potential was measured during exposure to $10 \mu\text{M}$ glutamate (Fig. 3*A*, inset). In average, the depolarization induced by glutamate was $-12.4 \pm 1.35 \text{ mV}$ ($n = 6$). Similar to glutamate effects, the peak of the Ca_i transient induced by 45 mM KCl was $1.14 \pm 0.11 \mu\text{M}$ (Fig. 3*B*). However, only partial recovery could be observed within 20 min ($0.55 \pm 0.14 \mu\text{M}$). This residual Ca_i was statistically higher than in glutamate and remained evenly elevated in four cells followed up to 60 min. As illustrated in the Figure 3*B* inset, the depolarization recorded during KCl exposure was $-7.66 \pm 1.31 \text{ mV}$ ($n = 4$). Differences in glutamate-induced depolarization compared to that by KCl can be noticed, not only in amplitude but also in temporal course. Depolarization by glutamate developed slower and tended to recover even though no substantial recovery could be founded. Amplitude of depolarization by KCl, although only subtly different, was significantly greater than that by glutamate. Moreover, both glutamate ($10 \mu\text{M}$) and KCl (45 mM) raise the Ca_i to values just above $1 \mu\text{M}$. However, the fact that Ca_i was restored to basal levels during the exposure to glutamate but not during KCl-induced membrane depolarization suggests different actions not only on the induction of Ca_i transients but also on the Ca_i buffering mechanisms.

Development of I_{Ca} under glutamate treatment

If Ca_i transients account for the changes observed in I_{Ca} (Fig. 1*B*), it can be inferred from Figure 3*A* that only a few minutes of exposure to glutamate would be sufficient to induce such response. This is based on the observation that, during glutamate exposure, Ca_i returned to its basal level within 10 min and no additional changes occurred thereafter. However, the glutamate exposures were standardized to 1 hr per day. Since vigorous Ca_i elevations were also observed upon KCl-induced depolarization (Fig. 3*B*), the long-term effects of glutamate and elevated KCl on I_{Ca} were compared. In control cells, the membrane current elicited by depolarizing steps from -100 mV to $+10 \text{ mV}$ increased slightly in parallel with membrane capacitance (Fig. 4*A*, left). By contrast, a strong increment in the amplitude of I_{Ca} was observed between d1 and d2 during the treatment with glutamate (Fig. 4*A*, middle), which correlated well with a similar response of I_{Ca} induced by the treatment with KCl (Fig. 4*A*, right). In order to bring out the specificity of the effect on HVA I_{Ca} , the current densities obtained from hp of -100 and -40 mV were examined. Interestingly, one single exposure to glutamate or KCl did not change I_{Ca} (Fig. 4*B,C*). However, a strong increment of I_{Ca} density was observed after 2 d of glutamate or KCl treatment at both holding potentials in the absence of significant changes in C_m . We found a significant response to glutamate from d2 to d5 at hp = -100 mV ($77.6 \pm 7.2 \text{ pA/pF}$, $n = 15$, vs $42.9 \pm 3.3 \text{ pA/pF}$, $n = 14$) and at hp = -40 mV ($71.4 \pm 6.5 \text{ pA/pF}$ vs $43.1 \pm 2.9 \text{ pA/pF}$) in the same cells. C_m remained unchanged during the same period ($28.7 \pm 1.7 \text{ pF}$ vs $29.5 \pm 3.2 \text{ pF}$). The addition of glycine ($0.5 \mu\text{M}$; data not shown) did not change this result (at hp = -100 mV : $82.8 \pm 5.9 \text{ pA/pF}$, $n = 12$, vs $47.1 \pm 2.8 \text{ pA/pF}$, $n = 15$; at hp = -40 mV : $83.1 \pm 4.6 \text{ pA/pF}$ vs $44.8 \pm 3.1 \text{ pA/pF}$; C_m : $20.2 \pm 0.8 \text{ pF}$ vs $23.3 \pm 2.1 \text{ pF}$). This observation suggested that glycine was already present in the culture medium. On the other hand, indicating the importance of Ca_i increase in the long-term enhancement of I_{Ca} , a response similar to that for glutamate was found for KCl (at hp = -100 mV : $75.4 \pm 4.9 \text{ pA/pF}$, $n = 27$, vs $43.3 \pm 2.5 \text{ pA/pF}$, $n = 22$; at hp = -40 mV :

Figure 4. Development of I_{Ca} during glutamate treatment and periodical membrane depolarization. Cells were exposed to 10 μ M glutamate or 45 mM KCl every day for 1 hr. Glutamate or KCl sessions started at day 12 in culture, termed experimental day zero (d0). All I_{Ca} measurements were made 24 hr after each exposure to glutamate or KCl. **A**, Typical records of I_{Ca} from control cells (left) and from cells treated with glutamate (middle), or KCl (right). The membrane capacitance (C_m) and the day of treatment are indicated close to each record. I_{Ca} was elicited by voltage pulses to +10 mV from a holding potential of -100 mV. Notice the small increment in the amplitude of I_{Ca} in parallel to the value of C_m in the left panel, and the strong increment of I_{Ca} at d2 in the middle and right panels. **B**, Development of I_{Ca} density in a population of cells in successive days of glutamate treatment. Mean I_{Ca} density: open symbols, control conditions; solid symbols, glutamate treatment. Triangles, $hp = -100$ mV; squares, $hp = -40$ mV. Notice the strong increment after 2 d of glutamate treatment at both holding potentials. Significant changes were found between d2 and d5. No major changes in the membrane capacitance (circles) can be appreciated. **C**, Development of I_{Ca} during KCl treatment. **D**, Responsiveness of cultured hippocampal cells to the treatment with glutamate or KCl. Bars represent I_{Ca} density of a number of cells in control (C) and in glutamate (Glu) or KCl treatment. I_{Ca} density for control cells ($n = 79$) was obtained between d0 and d5; that for glutamate ($n = 33$) and KCl ($n = 29$), between d2 and d5. Cells with I_{Ca} densities greater than the control mean value plus two standard deviations were considered as positive (+). Negative (-) cells had I_{Ca} densities comparable to control cells. For glutamate, positive cells were 18 of 33, and for KCl, 16 of 29. Values are given as mean $\pm$ SEM.

69.1 ± 4.9 pA/pF vs 39.3 ± 2.2 pA/pF; C_m : 24.4 ± 1.7 pF vs 29.2 ± 1.6 pF).

Because the present experiments were conducted in cultures from the whole hippocampus, a heterogeneous population of cells could be expected. This was supported by the observation that at least two morphological types, pyramidal-like and stellate-like cells, could be easily distinguished. Functionally, a variability between individual cells in the glutamate response and in I_{Ca} amplitude was observed as well. We considered that a cell had a positive response to glutamate or KCl, if its I_{Ca} density value was greater than the mean value of control I_{Ca} densities (43.34 pA/pF, $n = 79$) plus two standard deviations (21.42 pA/pF). Using this criterion, cells tested at $hp = -100$ mV were taken from the preceding results and classified as positive or negative (Fig. 4D). For glutamate, 54.6% (18 of 33 cells) were positive responses presenting an increment of 111.3% in I_{Ca} density compared to control cells. For KCl, 55.2% (16 of 29 cells) presented a similar increment (107.6%). From these re-

sults, it is concluded that the HVA I_{Ca} density enhanced twofold in about 50% of the treated cells (see an example in Fig. 1B,C).

Effects of cycloheximide and AP5

The Ca^{2+} conductance (g_{Ca}) was calculated from I_{Ca} measured at $hp = -100$ mV and assuming $[Ca^{2+}]_i \approx 10^{-8}$ M. Maximal I_{Ca} was divided by the driving force for Ca^{2+} at the appropriate membrane potential. As illustrated in Figure 5, g_{Ca} in positive cells under glutamate or KCl treatment was greater than in control and in negative cells. On the basis that I_{Ca} enhancement appeared clearly after 2 d of glutamate treatment (Fig. 4B), a mechanism mediated by protein synthesis was suggested. To test this hypothesis, the cells were incubated with 2 μ M cycloheximide continuously during the glutamate treatment. In preliminary experiments, higher concentrations of cycloheximide produced excessive cell damage. Clearly, g_{Ca} , calculated from current records obtained between d2 and d3, showed that cycloheximide prevented the glutamate effects (Fig. 5). This result

Figure 5. Membrane Ca^{2+} conductance of hippocampal cells exposed periodically to glutamate and depolarizing concentrations of extracellular potassium: blockade of glutamate and KCl responses by cycloheximide and AP5. Control (Con), glutamate (Glu+ and Glu-), and KCl (KCl+ and KCl-) responses were obtained from Figure 4D. Cycloheximide ($2\text{ }\mu\text{M}$) was added from d0, while AP5 ($30\text{ }\mu\text{M}$) was applied 10 min before glutamate ($10\text{ }\mu\text{M}$) and KCl (45 mM) every day. Ca^{2+} conductance was calculated from maximal I_{Ca} elicited by voltage-clamp steps from $h_p = -100\text{ mV}$. I_{Ca} was recorded between d2 and d3 for cycloheximide (Cyx, $n = 9$; Cyx + Glu, $n = 9$) and between d2 and d4 for AP5 (AP5, $n = 14$; AP5 + Glu, $n = 9$; AP5 + KCl, $n = 17$). No positive responses to glutamate and KCl were found in the presence of cycloheximide or AP5.

confirms the suggestion that protein synthesis supports the g_{Ca} enhancement induced by glutamate.

L-Glutamate interacts with at least four classes of membrane receptors in neurons, grossly classified as NMDA and non-NMDA receptors (MacDermott et al., 1986; Mayer and Westbrook, 1987; Murphy and Miller, 1988; Furuya et al., 1989). As an interesting possibility, we explored the involvement of the NMDA receptor in the long-term enhancement of g_{Ca} induced by glutamate. The competitive antagonists 2-amino-5-phosphonopentanoic acid (AP5) and 2-amino-7-phosphonoheptanoic acid (AP7) have been the key compounds in identifying NMDA receptor action in a variety of CNS regions (Mayer and Westbrook, 1987). Accordingly, AP5 ($30\text{ }\mu\text{M}$) clearly prevented the enhancement of g_{Ca} in hippocampal cells treated periodically with glutamate or elevated KCl (Fig. 5). Because $30\text{ }\mu\text{M}$ AP5 was sufficient to prevent the long-term enhancement of g_{Ca} , higher doses were not employed. However, measurements of Ca_i during glutamate application in presence of AP5 revealed a significant but only partial decrease in the peak of the transient

(Fig. 6). Moreover, only partial block of the Ca_i transient was observed even when applying higher concentrations of AP5 (250 and $500\text{ }\mu\text{M}$; not shown). AP5 was also able to induce Ca_i elevation by itself, in agreement with previous observations (Kudo and Ogura, 1986).

Discussion

Dissociated cultures provide a high degree of experimental accessibility, including the relative ease whereby voltage-clamp recording can be performed. We found that repetitive exposure of hippocampal cultured cells to glutamate specifically enhanced HVA I_{Ca} . Moreover, this effect was blocked by cycloheximide and mimicked by periodic membrane depolarization.

Specificity of HVA I_{Ca} enhancement

Undoubtedly, the most robust and widely accepted break point among the various Ca channel types identified in neurons is that between the LVA and the HVA channels (Tsien et al., 1988; Hess, 1990; Swandulla et al., 1991). The present study shows that only HVA I_{Ca} is affected by glutamate treatment, and not LVA I_{Ca} . The increase in HVA I_{Ca} could be the consequence of enhanced specific conductance or enlarged membrane surface. However, on the basis that changes neither in size of the cells nor in membrane capacitance were found, a specific enhancement in HVA Ca conductance is proposed. Our results not only point out distinct regulation of the two major Ca channel types, but also strengthen the view that different channel types correspond to separate molecular entities.

Glutamate stimulation versus membrane depolarization

L-Glutamate induces Ca_i elevations by acting directly on NMDA and non-NMDA receptors (Kudo and Ogura, 1986; Murphy and Miller, 1988; Furuya et al., 1989) and, indirectly, on voltage-gated Ca channels (Kudo and Ogura, 1986; MacDermott et al., 1986; Murphy et al., 1987; Murphy and Miller, 1988). Our results show that periodic Ca_i elevations by glutamate induce an enhancement of I_{Ca} in a time frame of days. Moreover, we observed that KCl induced vigorous Ca_i elevations by a membrane depolarization comparable to those induced by glutamate. Under normal circumstances, such rise in Ca_i might function

Figure 6. Ca_i transients during application of glutamate ($10\text{ }\mu\text{M}$) in the presence of AP5 ($30\text{ }\mu\text{M}$). Symbols are mean $\pm$ SEM. Peak transient, $n = 11$; at 20 min, $n = 7$. AP5 produced a partial but significant blockade of the Ca_i transient induced by glutamate (see Fig. 3A).

as a common link between cooperative mechanisms that converge in the appropriate signal and trigger adaptive changes in neuronal functions. Initially, we designed experiments with elevated KCl to simulate electrical activity in cultured hippocampal cells. The results show that periodic membrane depolarizations with elevated potassium mimic the effect of glutamate treatment on I_{Ca} . Yet, AP5, a NMDA receptor antagonist, prevents the long-lasting effects of glutamate and membrane depolarizations on I_{Ca} . Thus, elevated KCl may induce the release of neurotransmitters, which in turn participate in the enhancement of I_{Ca} generated under periodical membrane depolarization. Although, Ca_i increase may underlie the action of membrane depolarization and glutamate, a detailed dissection of Ca_i transients is required to establish their relative importance in the induction of the long-term enhancement of HVA I_{Ca} . Predominance of a specific glutamate-activated mechanism leading to increase in Ca_i , would depend on cell type (Cline and Tsien, 1991).

It has recently been reported I_{Ca} decreases progressively in myenteric neurons exposed to depolarizing concentrations of KCl for several days (Franklin et al., 1992). However, neurons *in vivo* hardly experience sustained depolarizations as prolonged as those caused by elevated potassium in cultured cells. A short rise of glutamate induced runup of I_{Ca} in tens of minutes and overlapped the exposure time (Mironov and Lux, 1992). The present study shows that HVA I_{Ca} density can be upregulated in hippocampal cells by periodical glutamate stimulation or membrane depolarization. This effect required at least 2 d of treatment and lasted for up to 5 d. In our hands, chronic exposures to glutamate or KCl downregulated I_{Ca} .

Block of I_{Ca} enhancement by cycloheximide

The significant delay and the long duration of the I_{Ca} enhancement are in line with previous reports on changes in neuronal functions that depend on protein synthesis (Staton and Sarvey, 1984; Goelet et al., 1986). Accordingly, glutamate response was significantly reduced by cycloheximide, suggesting that a neo-synthesis of Ca channels is involved in the evolution of I_{Ca} under glutamate treatment. The lack of modifications in kinetic and voltage dependence characteristics of the increased I_{Ca} rules out possible changes in the gating behavior of Ca channels. As previously suggested for Na^+ channels in other types of neurons (Brismar and Gilly, 1987; Tribut et al., 1991), this evolution of I_{Ca} could be due to an enhancement in the density of functional Ca channels in the membrane of hippocampal cells. Alternatively, increased unitary channel conductance may underlie the I_{Ca} enhancement, though there is no precedent for increases of unitary channel conductance when kinetics and current-voltage relationships remain unchanged. Nevertheless, single-channel measurements would be desirable to test possible changes at the level of unitary conductance.

Possible involvement of the NMDA receptor

NMDA and non-NMDA receptors coexist in hippocampal cells (Mayer and Westbrook, 1987). Among various alternatives, we explored the possibility whether NMDA receptors could account for the long-lasting glutamate effect observed on I_{Ca} . Consistently, we did not find this enhancement in the presence of AP5, a selective antagonist of NMDA receptors. However, the partial effect of AP5 on the Ca_i transient induced by glutamate limits the interpretation of this result, even when the presence of AP5-sensitive sites in hippocampus has been confirmed by

several groups in cultures (Harada et al., 1992), in slice preparations (Collingridge and Singer, 1990), and *in vivo* (Morris et al., 1986). Moreover, it is well documented that block of these sites with AP5 does not detectably affect synaptic transmission but prevents the induction of hippocampal long-term potentiation (LTP) following brief high-frequency stimulation (Bliss and Lomo, 1973; Collingridge et al., 1983; Harris et al., 1984). It might be that Ca entry through NMDA receptor channels plays a critical role under glutamate stimulation (Lynch et al., 1983; Malenka et al., 1988). Under physiological conditions, such Ca entry may occur through NMDA-receptor channels, activated during repetitive stimulation of the afferent pathway (Mayer and Westbrook, 1987). Experimentally, exposures to elevated potassium could indirectly emulate effects of this kind. Accordingly, AP5 inhibits the enhancement of HVA I_{Ca} in cells under treatment with elevated KCl. This observation reinforces the proposal that KCl-induced depolarizations may release neurotransmitters, which trigger the functional expression of HVA calcium channels in hippocampal cells maintained in primary cultures. Whatever the mechanism turns out to be, the experiments with AP5 show evidence indicating NMDA receptors are involved in the long-term modulation of I_{Ca} .

Possible physiological significance of I_{Ca} enhancement

Regulation of neuronal Ca channels is important because entry of Ca through voltage-gated channels is a major mechanism by which changes in membrane potential can influence cellular processes. It was initially proposed that either ω -conotoxin (Miller, 1987) or dihydropyridine-sensitive HVA I_{Ca} (Perney et al., 1986; Rane et al., 1987) could mediate synaptic transmission. However, Regan et al. (1991) have recently shown that in hippocampal cells, as in other mammalian neurons, much of the HVA I_{Ca} is resistant to both ω -conotoxin and dihydropyridine blockers. In our hands, at least, 60% of HVA I_{Ca} seems to be resistant to dihydropyridine antagonists and ω -conotoxin. Recently, it has been reported that an ω -conotoxin-resistant Ca channel, which is blocked by ω -Aga-IVA, seems to be associated with glutamate release (Turner et al., 1992). In the present study, we preferred to use the most widely accepted break point between the various classes of calcium channels. Hence, we simply separated HVA from LVA I_{Ca} , despite the fact that currents were recorded from cell bodies and not from presynaptic terminals as desirable. Accordingly, we found a consistent HVA I_{Ca} enhancement under experimental conditions that roughly emulate those of increased neuronal activity. Notoriously, the I_{Ca} enhancement we observed was produced in a time frame of days. We suggest that this enhancement may correspond to a long-lasting process depending on protein synthesis, though no solid reasons can be argued in favor of models of neuronal plasticity such as LTP (Bliss and Lomo, 1973) or kindling (Godard et al., 1969). Coincidentally, a long-term enhancement of HVA I_{Ca} induced by kindling has been recently observed in hippocampal neurons as well (Vreugdenhil and Wadman, 1992). Hence, it is tempting to speculate that the Ca influx resulting from an increased density of Ca channels could modulate calcium-dependent factors leading to changes in neuronal excitability. Experimental evidence supports the idea that Ca is an important intracellular regulator of neuronal excitability in the hippocampus (Kennedy, 1989).

In addition to being a determinant of the electrophysiological characteristics of many cells, voltage-gated Ca influx can trigger cellular events such as activation of enzymes (Kaczmarek and

Levitan, 1987), even leading to genomic responses (Morgan and Curran, 1986). Interestingly, *c-fos* and *c-jun*, as prototypes of immediate-early genes, trigger a long-term enhancement of I_{Ca} when they are cotransfected in PC12 cells (A. Cavalié, B. Berninger, D. E. García, D. Lindholm, and H. D. Lux, unpublished observations). We suggest that the reported form of Ca channel regulation could cause neurons to adapt to altered levels of electrical activity and may contribute to changes in synaptic strength occurring after periods of increased electrical activity.

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