Homeostatic Effects of Depolarization on Ca^{2+} Influx, Synaptic Signaling, and Survival

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Depolarization promotes neuronal survival through moderate increases in Ca^{2+} influx, but the effects of survival-promoting depolarization (vs conventional trophic support) on neuronal signaling are poorly characterized. We found that chronic, survival-promoting depolarization, but not conventional trophic support, selectively decreased the somatic Ca^{2+} current density in hippocampal and cerebellar granule neurons. Depolarization rearing depressed multiple classes of high-voltage activated Ca^{2+} current. Consistent with the idea that these changes also affected synaptic Ca^{2+} channels, chronic depolarization presynaptically depressed hippocampal neurotransmission. Six days of depolarization rearing completely abolished glutamate transmission but altered GABA transmission in a manner consistent with the alterations of Ca^{2+} current. The continued survival of depolarization-reared neurons was extremely sensitive to the re-establishment of basal culture conditions and was correlated with the effects on intracellular Ca^{2+} concentration. Thus, compared with cells reared on conventional trophic factors, depolarization evokes homeostatic changes in Ca^{2+} influx and signaling that render neurons vulnerable to cell death on activity reduction.

Key words: depolarization; HVA Ca^{2+} current; glutamate; GABA; synaptic depression; neuronal survival

Introduction

During development, correlated with the period of rapid synaptogenesis, excess neurons are removed by apoptosis, or physiological cell death. Classical studies have indicated that limiting quantities of peptide neurotrophic factors (NTFs) support neuronal survival during this period (Burek and Oppenheim, 1996). However, for many CNS neurons, endogenous NTFs remain undefined, and accumulating evidence strongly suggests that electrical excitation itself, perhaps from the activity of nascent synapses, can sustain neuronal survival (Gallo et al., 1987; Ikonomidou et al., 1999, 2000; Verhage et al., 2000). To some extent, the intracellular survival pathways activated by NTFs and electrical activity may overlap (Miller et al., 1997; Mennerick and Zorumski, 2000). However, one might expect phenotypic differences in the signaling properties of neurons sustained by electrical activity versus NTFs. For instance, activity-dependent homeostatic mechanisms might influence the membrane and signaling properties of a neuron supported by excess activity (Turrigiano et al., 1998). Alternatively, excess activity may increase electrical signaling (Bliss and Lomo, 1973), potentially enhancing the survival effects of activity.

Moderate Ca^{2+} influx and associated rises in intracellular Ca^{2+} levels participate in activity-dependent survival in many systems (for review, see Franklin and Johnson, 1994). Also, the depression of electrical activity with excess GABA_A receptor activation or ethanol exposure results in the persistent depression of

Ca^{2+} influx, changes beyond the acute actions of these drugs on membrane potential (Xu et al., 2000; Moulder et al., 2002). The depression of Ca^{2+} current may participate in the observed neuronal death (Xu et al., 2000; Moulder et al., 2002). Here we investigated the hypothesis that excess depolarization may result in opposite changes: an upregulation of Ca^{2+} channel function that could potentially participate in the protection afforded by depolarization. In contrast to our expectations, depolarization depressed Ca^{2+} current density in hippocampal neurons and cerebellar granule neurons, two cell types whose survival is sensitive to activity (D'Mello et al., 1993; Xu et al., 2000). Synaptic signaling was downregulated in a manner consistent with decreased Ca^{2+} influx into presynaptic terminals. Furthermore, the continued survival of depolarization-reared neurons was more sensitive to the re-establishment of basal culture conditions than was the survival of NTF-reared neurons. These results suggest that presynaptic homeostatic mechanisms shape the signaling properties of neurons whose survival is sustained by depolarization, at the expense of vulnerability to electrical inhibition.

Materials and Methods

Cell culture. Hippocampal cultures were prepared from postnatal rat pups according to established protocols (Mennerick et al., 1995). Briefly, tissue was digested with papain and mechanically dissociated before plating on a collagen-coated culture dish in Eagle's medium (Invitrogen, Gaithersburg, MD) supplemented with heat-inactivated horse serum (5%), fetal calf serum (5%), 17 mM glucose, 400 μM glutamine, 50 U/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin. For measurement of Ca^{2+} currents and cell death, mass cultures were plated at 2000 cells/ mm^2 . For synaptic studies, cells were plated at 100 cells/ mm^2 on microdots of collagen to facilitate the formation of autaptic connections. Neurons plated in microcultures exhibited a depolarization-induced decrease in Ca^{2+} current amplitude similar to that seen for mass cultures (data not shown). In synaptic experiments only solitary neurons were used, thus excluding polysynaptic contributions to measured responses.

Granule neuron culture techniques were adapted from those of Levi et

Received Oct. 3, 2002; revised Dec. 2, 2002; accepted Dec. 9, 2002.

This work was supported by National Institutes of Health (NIH) Grants AA12952 and NS40488, by the Klingenstein Fund (S.M.), and by Grants AA12951 and MH45493 and a gift from the Bantky Foundation (C.F.Z.). K.L.M. was supported by NIH Grant ST32DA07261. We thank Ann Benz for preparing the hippocampal cultures and members of our laboratory for advice and criticism.

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al. (1984) and have been described in detail previously by Miller and Johnson (1996). In brief, cerebella were dissected from rat pups at postnatal day 7, digested with trypsin, and mechanically dissociated before plating at a density of 2.3×10^5 cells/cm². Before plating, dishes were coated with 0.1 mg/ml poly-L-lysine. The plating medium for granule neurons consisted of Eagle's medium containing 10% fetal bovine serum, with KCl added to a final concentration of 25 mM, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

Additions to the hippocampal culture medium (35 mM total K⁺, 50 ng/ml IGF-I) were made at 4 d *in vitro* (DIV). Ca²⁺ currents were recorded at 9–11 DIV, and synaptic responses were recorded at 11–13 DIV. For cerebellar granule cells, medium changes were made at 5 DIV, and Ca²⁺ currents were recorded at 8 DIV. Chlorophenylthio-cAMP (CPT-cAMP), a membrane-permeant analog of cAMP, was used as the cAMP source.

Cell survival was quantified by an observer who was unaware of the hypotheses. Under phase-contrast optics, live neurons in 10 random microscopic fields (20 $\times$ objective) were counted in each dish. Numbers refer to the number of independent platings (litters) on which the experiments were performed.

Electrophysiology. For recording, the culture medium was exchanged for a saline solution containing (in mM): 138 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, and 0.025 D-APV, pH 7.25. For Ca²⁺ current recordings, Ba²⁺ was used as the charge carrier to increase the current size and to improve the passive properties of the cell; a 3 mM concentration was used for most experiments, but 15 mM was used for experiments examining the contributions of Ca²⁺ channel subtypes to total current (see Fig. 2). Also, 500 nM tetrodotoxin (TTX), 1 μ M 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[f]quinoxaline (NBQX), and 25 μ M bicuculline were included to block sodium currents and spontaneous synaptic currents. For the measurement of K⁺ currents, 50 μ M Cd²⁺ was also present in the extracellular solution to block Ca²⁺ current and Ca²⁺-activated K⁺ current. All Ba²⁺ currents were digitally leak-subtracted using a trace obtained in the presence of 50 μ M Cd²⁺. Whole-cell recordings were obtained with pipettes 3–5 M Ω for hippocampal neurons and 6–8 M Ω for granule cells. For Ca²⁺ current and Na⁺ current recordings, the whole-cell pipette contained (in mM): 140 cesium methanesulfonate, 4 NaCl, 0.5 CaCl₂, 5 EGTA, 10 HEPES, 0.5 GTP, and 2 ATP, pH 7.25. Cells were stimulated with 30–50 msec (Ca²⁺ currents) or 15 msec (Na⁺ currents) pulses to 0 mV from the holding potential of -70 mV. For potassium currents, the pipette solution contained 140 mM potassium gluconate in place of cesium methanesulfonate, and 300 msec pulses to $+20$ mV were used to elicit currents. Capacitance was estimated as described previously (Xu et al., 2000; Moulder et al., 2002).

For synaptic recordings, TTX, NBQX, and bicuculline were omitted from the extracellular solution, and whole-cell pipette solution contained potassium chloride in place of cesium methanesulfonate. Cells were stimulated with 1.5 msec pulses to 0 mV from -70 mV to evoke transmitter release (Mennerick et al., 1995). Paired pulses were delivered at an interval of 50 msec, and sweeps were collected at a rate of <0.05 Hz. All results are reported as means $\pm$ SEM. Paired and unpaired *t* tests were used to evaluate statistical significance. In all instances, cells were excluded from analysis if a leak current >300 pA was observed.

[Ca²⁺]_i imaging. Cells were loaded with membrane permeant fura-2 AM (5 μ M) for 45 min at 37°C. Cells were initially imaged in standard electrophysiology recording solution supplemented with the relevant trophic factor (30 mM K⁺ or 100 ng/ml IGF-I). After establishing baseline [Ca²⁺]_i, cells were washed and imaged in standard recording saline, without added trophic factor. Ratiometric imaging (excitation at 340 and 380 nm) was performed on a Nikon (Tokyo, Japan) inverted microscope equipped with a filter wheel and intensified CCD camera. *In vitro* calibrations were performed as described previously (Gryniewicz et al., 1985).

Results

Decrease in Ca²⁺ current density in depolarization-reared hippocampal neurons

In cultures reared in standard serum-containing medium, 20–80% of neurons were lost between 4 and 10 DIV, depending on

the plating ($47 \pm 15\%$ cell loss; $n = 5$ platings). Nearly all neurons present at 4 DIV were protected by 35 mM total extracellular K⁺, which pilot work suggested was optimal ($15 \pm 14\%$ cell loss between 4 and 10 DIV, $n = 5$ platings; $p < 0.05$ compared with untreated). The resting membrane potential of K⁺-reared neurons, measured at 11 DIV, was -20.6 ± 1.1 mV ($n = 15$), depolarized from a resting potential of -47.9 ± 1.5 mV ($n = 17$) in cells reared in unsupplemented culture medium.

Because the Ca²⁺ influx through high-voltage activated (HVA) Ca²⁺ channels under similar depolarizing conditions has been implicated in depolarization-induced survival (Franklin and Johnson, 1994; Moulder et al., 2002; but see Yu et al., 1997), we examined the persistent effects of chronic depolarization on HVA current. The electrical inhibition of neurons with GABAergic agents or ethanol produced decreases in Ca²⁺ influx beyond the acute actions of the drugs (Xu et al., 2000; Moulder et al., 2002). Therefore, we initially hypothesized that chronic depolarization might increase Ca²⁺ influx. Instead, we observed that Ca²⁺ current density, measured in recording saline with 4 mM K⁺, was smaller in K⁺-reared neurons than in untreated cells (Fig. 1A,B).

As a survival control, we reared neurons in 50–100 ng/ml IGF-I, a peptide factor that promotes hippocampal survival levels similar to those of KCl (see Fig. 5). In IGF-I-reared cultures, there was no difference in Ca²⁺ current density compared with untreated cells (Fig. 1B). Cell capacitance was not statistically different in KCl-reared cells compared with untreated or IGF-I-reared cells (22.6 ± 1.5 , 26.9 ± 3.0 , and 29.9 ± 5.1 pF; $n = 8$ –12 in each group; $p > 0.1$).

Other voltage-gated currents were not affected by depolarization rearing. Compared with control cultures, K⁺-reared neurons exhibited no significant difference in Na⁺ current density (Fig. 1C) (control, -165.6 ± 15.7 pA/pF; K⁺-reared, -191.6 ± 15.9 pA/pF; $n = 11$ in both groups; $p > 0.05$). Likewise, there was no difference in either peak or steady-state voltage-gated K⁺ current density (Fig. 1D) ($n = 12$ control and 12 K⁺-reared neurons; $p > 0.05$). Thus, the downregulation of Ca²⁺ current is a relatively selective effect of K⁺ depolarization.

We examined the current–voltage (*I*–*V*) curve for HVA currents reared under different conditions. The *I*–*V* relationship was similar across experimental conditions (Fig. 1E). This result suggests that the change in Ca²⁺ current density did not result from a change in the voltage dependence of activation or the reversal potential. Rather, the current decrease occurred at all potentials, possibly consistent with the loss of functional channels in the depolarization-reared cells.

Ca²⁺ current density decrease in depolarization-reared granule neurons

Because the survival of cerebellar granule neurons is also enhanced by depolarization rearing, we questioned whether the effect of depolarization on Ca²⁺ current extended to these cells. Unlike hippocampal neurons, basal medium supports the survival of almost no granule neurons; these cells are absolutely dependent on added trophic factors or depolarization for survival (D'Mello et al., 1993; Miller and Johnson, 1996). We reared granule neurons from the day of plating in 25 mM K⁺, which provides optimal protection (Gallo et al., 1987). At 5 DIV, cultures were continued in 25 mM K⁺ or switched to medium containing 5 mM K⁺ and either IGF-I or cAMP (D'Mello et al., 1993). IGF-I and cAMP produced survival that was 71.4 ± 5.3 and $78.5 \pm 2.1\%$, respectively, of K⁺-reared cultures, evaluated at 8 DIV.

Figure 1. Depolarization rearing specifically decreases Ca^{2+} current density in hippocampal neurons and in cerebellar granule neurons. *A*, Representative Ca^{2+} currents elicited by voltage steps from -70 to 0 mV in a hippocampal neuron reared in control medium and a neuron reared in medium containing 35 mM K^{+} . The capacitance of the control cell was 22.0 pF, and that of the K^{+} -treated cell was 22.3 pF. *B*, Summary of the Ca^{2+} current densities in control cultures (Ctrl), K^{+} -reared cultures, and cultures reared in 50 ng/ml IGF-I. * $p < 0.05$ compared with control. *C*, Representative Na^{+} currents elicited by 15 msec voltage steps from -70 to 0 mV in a hippocampal neuron reared in control medium and a neuron reared in medium containing 35 mM K^{+} . Na^{+} currents were leak-subtracted using 500 nM TTX. *D*, Representative K^{+} currents elicited by voltage steps from -70 to $+20$ mV in a hippocampal neuron reared in control medium and a neuron reared in medium containing 35 mM K^{+} . Potassium currents were obtained with a potassium-based pipette solution in the presence of extracellular 50 μM Cd^{2+} and 500 nM TTX. Currents were linearly leak-subtracted. *E*, Scaled current–voltage relationship for Ca^{2+} currents in hippocampal neurons reared in control medium or in medium supplemented with 35 mM K^{+} or 50 ng/ml IGF-I ($n = 9$ for each condition). Scaled currents (I/I_p) were normalized to the peak current at 0 mV. *F*, Summary of the Ca^{2+} current

Depolarization-reared granule neurons exhibited depression of Ca^{2+} current density qualitatively similar to that in hippocampal neurons (Fig. 1*F*). Cells raised in IGF-I plus 25 mM NaCl (as an osmotic and charge control for KCl) exhibited no decrease in Ca^{2+} current density (Fig. 1*F*). As expected from cell size differences, granule neuron capacitance was significantly smaller than hippocampal cell capacitance. However, depolarization-reared granule neurons exhibited larger capacitance (8.3 ± 0.3 pF; $n = 9$) than IGF-I-reared neurons (6.2 ± 0.4 pF; $n = 5$; $p < 0.001$) or cAMP-reared neurons (5.7 ± 0.7 pF; $n = 5$; $p < 0.001$). Thus, despite the larger cell size in K^{+} -reared granule neurons (compared with no detected difference in hippocampal neurons), the Ca^{2+} current density was still depressed.

K^{+} rearing mimics the innervation of granule neurons by glutamatergic synapses *in situ* (Balazs et al., 1988). Therefore, we examined the effect of 50 μM NMDA as a depolarizing agent. NMDA produced an intermediate level of survival ($60.7 \pm 4.1\%$ of that of 25 mM KCl; $n = 3$) and an intermediate level of reduction in Ca^{2+} current density, although not statistically different from the reduction observed with 25 mM K^{+} (Fig. 1*F*). These data strongly suggest that depolarization, rather than some other, unanticipated, result of K^{+} rearing, caused decreased Ca^{2+} current density.

To explore the Ca^{2+} channel subtype specificity of depolarization effects, we pharmacologically dissected components of HVA currents in granule neurons. The contributions of N, P, L, and Q/R subtypes to the total Ca^{2+} current were unchanged by K^{+} rearing versus IGF-I rearing (Fig. 2). In depolarization-reared cells the percentages of the total Ca^{2+} current attributable to each subtype were 11, 20, 15, and 54% for P, N, L, and Q/R type currents, respectively, and were similar to values reported previously in cerebellar granule cells (Randall and Tsien, 1995). These results suggest that depression occurred across HVA classes. This uniform effect of depolarization differs strikingly from the preferential role of L-type Ca^{2+} channels in the survival-promoting Ca^{2+} influx that initially accompanies K^{+} depolarization (Collins and Lile, 1989; Moulder et al., 2002). However, it is consistent with reports in other systems in which both inactivating and noninactivating (Franklin et al., 1992) and both low-voltage activated and HVA (Li et al., 1996) Ca^{2+} currents are depressed by chronic depolarization.

K^{+} rearing and hippocampal synaptic transmission

Because K^{+} depolarization appeared to affect multiple Ca^{2+} channel subtypes, including those responsible for driving transmitter release, we examined whether the persistent depression of somatic Ca^{2+} influx extended to hippocampal synaptic terminals (Figs. 3, 4). We examined recurrent (autaptic) synaptic signaling in solitary hippocampal microisland neurons reared in K^{+} or with no additions. The average IPSC size was depressed by $\sim 60\%$ in K^{+} -reared cells (Fig. 3*A,D*). In addition, there was less IPSC paired-pulse depression in depolarization-reared neurons (Fig. 3*A,E*), consistent with a depression of the baseline probability of vesicle release (p_r) (Thomson, 2000). Interestingly, EPSCs were completely eliminated by K^{+} rearing (Fig. 3*B,C*). This EPSC loss

density in cerebellar granule neuron cultures maintained in the presence of 25 mM K^{+} (K25+S), 100 ng/ml IGF-I, 800 μM CPT-cAMP, 50 μM NMDA, or 25 mM Na^{+} (plus 100 ng/ml IGF-I to promote survival). * $p < 0.05$ compared with 25 mM K^{+} . Inset, Ca^{2+} current from a granule neuron maintained in IGF-I (left) and a granule neuron maintained in 25 mM K^{+} (right). Calibration: 100 pA, 25 msec.

Figure 2. Multiple subtypes of HVA Ca^{2+} current are depressed by depolarization rearing. *A*, Representative Ca^{2+} currents elicited by steps from -70 to 0 mV in cerebellar granule neurons. Currents were recorded sequentially in the absence of any Ca^{2+} channel blockers (*Baseline*), in the presence of the P-type blocker ω -agatoxin IVA (ω -Aga IVA; 100 nM), in the presence of the N-type blocker ω -conotoxin GVIA (ω -CgTx GVIA; 1 μ M), and in the presence of the L-type blocker nifedipine (5 μ M). The *top* traces were obtained from a neuron maintained in 100 ng/ml IGF-I. The *bottom* traces were obtained from a neuron maintained in 25 mM K^{+} (K25+S). *B*, Summary of the percentage of the total current accounted for by each subtype in K^{+} - and IGF-I-treated neurons. The total current was determined using an application of 50 μ M Cd^{2+} ($n = 9$ for each condition).

was not attributable simply to the death of excitatory cells, because the percentage of cells with no autaptic response increased with the loss of EPSCs in depolarization-reared cultures (Fig. 3*B*). Also, there was no change in the percentage of neurons immunopositive for GABA (data not shown). Similar deficits in IPSCs and EPSCs were evident with 14 hr of treatment with elevated K^{+} (data not shown), suggesting that the changes in synaptic function did not require days of tonic depolarization.

Hypertonic solutions, which evoke the Ca^{2+} -independent release of transmitter from readily releasable vesicle pools (Rosenmund and Stevens, 1996), failed to elicit EPSCs from presumed glutamatergic cells in K^{+} -reared cultures (Fig. 3*C*). IPSCs were elicited normally by hypertonic solution in K^{+} -reared cultures (data not shown). The failure to elicit even hypertonicity-evoked EPSCs suggests that depolarization rearing depresses glutamate neurotransmission via mechanisms beyond the depression of Ca^{2+} current, the focus of the present work. Therefore, we characterized the less severe deficit in IPSC signaling as potentially relevant to Ca^{2+} -current depression.

If a decrease in synaptic-terminal Ca^{2+} current is responsible

Figure 3. Depolarization rearing depresses synaptic transmission. *A*, Representative autaptic IPSCs from solitary hippocampal neurons reared in control medium or in medium containing 35 mM K^{+} . Presynaptic stimulus transients have been blanked for clarity. *B*, Summary of the incidence of glutamatergic autapses (black columns), GABAergic autapses (white columns), and nonresponding cells (gray columns) in control and K^{+} -reared cultures. Glutamatergic transmission was abolished by K^{+} rearing, whereas the percentage of nonresponding cells increased proportionally. *C*, The absence of glutamate transmission was associated with no detectable Ca^{2+} -independent transmission. Hypertonic sucrose (500 mM) was used to elicit the release of readily releasable vesicles, causing an NBQX (1 μ M)-sensitive current from a control culture (*left*). The same hyperosmotic challenge produced no response in a K^{+} -reared cell (*right*) that also exhibited no action potential-evoked autaptic response (data not shown). No sucrose response was elicited in any of the 13 nonresponding K^{+} -reared neurons examined. All glutamate-releasing cells in control cultures exhibited sucrose-evoked release ($n = 6$). *D*, Summary of the effect of K^{+} rearing on initial peak amplitude; $*p < 0.05$. *E*, Summary of the effect of K^{+} rearing on paired-pulse modulation (PPM); $*p < 0.001$. For both *D* and *E*, $n > 20$ for each condition.

for the synaptic deficit in depolarization-reared neurons, depressed IPSCs might be at least partially rescued by increasing Ca^{2+} with elevated extracellular Ca^{2+} . To test this, IPSCs were recorded in the presence of 0.5 – 10 mM Ca^{2+} from potassium-

Figure 4. GABA synaptic depression is rescued by increasing $[Ca^{2+}]_o$. *A*, Concentration-response curves for the initial peak amplitude of IPSCs recorded in hippocampal neurons maintained in control medium ($\circ$) or in the presence of 35 mM K^+ ($\bullet$). The solid lines represent fits to the plotted data with the Hill equation. For control cultures, the EC_{50} value is 0.90, with a Hill coefficient of 2.31 ($n = 8$). For K^+ -treated cultures, the EC_{50} value is 2.88, with a Hill coefficient of 1.46 ($n = 10$). *B*, Summary of the initial peak amplitudes of IPSCs recorded in the presence of 2 or 10 mM extracellular Ca^{2+} in both control and K^+ -treated cultures; $p < 0.01$. *C*, Summary of the degree of paired-pulse modulation (PPM) in currents recorded in the presence of 2 or 10 mM extracellular Ca^{2+} in both control and K^+ -treated cultures; $p < 0.01$.

reared and control neurons. Consistent with this hypothesis, the Ca^{2+} concentration response for IPSCs was significantly shifted to the right in depolarization-reared cells, with no significant difference in maximum response size (Fig. 4*A,B*). The Hill coefficient for Ca^{2+} was slightly lower than that observed for autaptic EPSCs (Reid et al., 1998) but was within the range of cooperativity observed in other preparations (Wu et al., 1998). There was a nonsignificant trend toward a decreased Hill coefficient in K^+ -reared cells (Fig. 4*A*), possibly suggesting a nonuniform depression of Ca^{2+} influx across synaptic terminals (Reid et al., 1998). Importantly, we also overcame the deficit in paired-pulse depression by increasing Ca^{2+} levels in the extracellular recording solution of depolarization-reared cells (Fig. 4*C*). These data support the idea that in addition to effects on somatic Ca^{2+} influx, depolarization rearing depresses Ca^{2+} -dependent neurotransmission at synaptic terminals.

Depolarization rearing leaves neurons vulnerable to re-establishment of baseline culture conditions

If Ca^{2+} influx through L-type and perhaps other Ca^{2+} channels is important in the survival of neurons supported by depolarization, then depression of HVA Ca^{2+} currents might leave depolarization-reared neurons susceptible to the re-establishment of basal culture conditions (hyperpolarized membrane potential and no added trophic factors). We tested this possibility by rearing hippocampal neurons from 4 to 9 DIV in 35 mM K^+ , in 50 ng/ml IGF-I, or with no additions. At 9 DIV, we counted surviving cells. We then switched cultures to conditioned media from untreated sibling cultures. We recounted cells in each condition 24 hr after the switch back to basal conditions and compared survival at the two time points. Depolarization-reared cells were significantly more susceptible to the re-establishment of basal culture conditions than cells reared either in unsupplemented media or in the presence of IGF-I (Fig. 5*A,B*). The effect did not result from osmotic differences, because replacing KCl with NaCl at 9 DIV did not alter the results ($n = 4$ experiments) (data not shown).

We have shown previously that neuronal death induced by agents that depress electrical activity is associated with depressed $[Ca^{2+}]_i$ (Xu et al., 2000; Moulder et al., 2002). To determine whether $[Ca^{2+}]_i$ depression is associated with K^+ withdrawal, we examined basal $[Ca^{2+}]_i$ levels using ratiometric fura-2 imaging in IGF-I- and K^+ -reared cells before and after K^+ withdrawal. We found that basal $[Ca^{2+}]_i$ was elevated in K^+ -reared cells while they were maintained in elevated K^+ (Fig. 5*C*). However, on withdrawal of K^+ , levels dropped below those of control (IGF-reared) cultures (Fig. 5*C*). These results are consistent with the idea that the depression of Ca^{2+} current, and the resulting depression of $[Ca^{2+}]_i$, participates in the enhanced cell death observed after K^+ withdrawal.

Discussion

Membrane depolarization is essential to neuronal function, apparently including the regulation of neuronal survival during development. Because electrical activity and conventional NTFs likely produce different effects on the long-term signaling properties of neurons, we have begun to explore explicitly the effects of depolarization rearing on neuronal signaling. We find evidence for homeostatic changes in response to tonic depolarization that have implications for both synaptic function and survival.

We examined the effect of depolarization rearing on Ca^{2+} influx for two reasons. First, moderate increases in Ca^{2+} influx are implicated in the improved survival of neurons with depolarization (Franklin and Johnson, 1994). Second, persistent down-

Figure 5. Depolarization rearing leaves neurons vulnerable to the re-establishment of baseline culture conditions. *A*, Effect of K⁺ depolarization or IGF-I addition on neuronal survival at 9 DIV. Survival is plotted as follows: (experimental counts)/(average control counts) – 1, such that positive values represent increased survival compared with control. *B*, Effect of switching cultures to basal medium conditions at 9 DIV, with survival measured at 10 DIV. Survival is plotted as follows: (counts at 10 DIV)/(counts at 9 DIV) – 1, such that negative values represent decreased survival compared with 9 DIV; **p* < 0.001. For both *A* and *B*, *n* = 9. *C*, Changes in [Ca²⁺]_i consistent with increased vulnerability. Ca²⁺ levels, reported as fluorescence ratios at 340 and 380 nm excitation (340/380), were imaged in cultures at 10 DIV, while cells were still in either IGF-I- or K⁺-containing medium (*Baseline*). The cells were imaged again 10 min after the exchange of medium to standard recording solution containing no trophic support (*Switch*). Note that the K⁺-reared cells initially contained higher Ca²⁺ levels than IGF-I-reared cells, but levels fell to below those of IGF-I controls after trophic withdrawal (**p* < 0.01; K⁺ vs IGF-I; *n* = 37 IGF-I cells and 48 K⁺ cells from three independent platings). Calibrated Ca²⁺ concentrations for the conditions were 78 ± 6 nM (baseline IGF), 102 ± 3 nM (baseline K⁺), 93 ± 10 nM (switch IGF), and 61 ± 3 nM (switch K⁺). *Ctrl*, Control.

regulation of Ca²⁺ current is implicated in inactivity-induced neuronal death (Xu et al., 2000; Moulder et al., 2002). In contrast to our expectations, we found that depolarization rearing produced robust downregulation of Ca²⁺ influx, a homeostatic response to depolarization that actually rendered the supported neurons more vulnerable to subsequent inhibition than neurons reared on peptide trophic factors. In other cell types the depression of Ca²⁺ current with tonic depolarization has been observed in some (Franklin et al., 1992; Murrell and Tolkovsky, 1993; Li et

al., 1996) but not all (Garcia et al., 1994; Toescu, 1998) studies. Furthermore, a single study that examined depolarization and peptide growth factors in the same system found similar depression of Ca²⁺ current with both forms of trophic support (Stewart et al., 1995). This clearly differs from our results, which suggest that depolarization and peptide growth factors produce very different outcomes in the signaling properties of neurons.

In vivo, depolarization may interact with peptide trophic factors to support young neurons (Schmidt and Kater, 1993). In our work, 25–35 mM total K⁺ was used to depolarize neurons, in the absence of added trophic peptides, and provide maximal survival benefit. *In situ* it seems likely that more moderate depolarization and limiting quantities of trophic factors may act together to promote optimal survival. Accordingly, the effects on Ca²⁺ influx and signaling observed in our model systems are likely to be more extreme than might be observed *in situ*. Nevertheless, our results highlight the possibility that during development the nervous system may produce neurons with varied signaling properties by altering the proportion of depolarization used to support the cells.

How K⁺ depolarization supports survival downstream of increased [Ca²⁺]_i remains unclear, as do the intracellular pathways responsible for the signaling changes observed in the present study. The biochemical cascades responsible for depolarization-induced survival may be parallel to or interact with those activated by NTFs. Some studies have linked depolarization with an increase in NTF sensitivity or production (Ghosh et al., 1994; Meyer-Franke et al., 1995). Other studies have linked depolarization to the activation of intracellular pathways activated by NTFs, such as the phosphatidylinositol 3-kinase pathway (Miller et al., 1997) and potential downstream targets such as proapoptotic Bcl-2 family members, and Forkhead, a transcription factor (for review, see Datta et al., 1999). If similar pathways are indeed responsible for survival mediated by depolarization and survival mediated by NTFs, our results suggest that depolarization must trigger additional parallel changes, because NTF support did not cause the same signaling changes caused by depolarization.

We found two major changes in depolarization-reared neurons predicted by the observed downregulation of Ca²⁺ influx. First, we found Ca²⁺-sensitive depression of synaptic currents in hippocampal cultures. As the most parsimonious explanation, we favor the interpretation that increased [Ca²⁺]_o rescued the synaptic deficit in hippocampal IPSCs by compensating for depressed Ca²⁺ current in the presynaptic terminal. However, we cannot exclude the possibility that depolarization rearing had more direct effects on the secretory machinery, perhaps on the Ca²⁺ sensor itself. Although the effects of prolonged disuse (inactivity) on neurotransmission have been examined previously (Sharpless, 1975; O'Brien et al., 1998; Turrigiano et al., 1998; Bacci et al., 2001; Luthi et al., 2001; Murthy et al., 2001), few reports have examined the effect of depolarization on neurotransmission. One recent study found decreased postsynaptic responsiveness (Leslie et al., 2001); another reported presynaptic depression of transmission (Shi et al., 2001).

Our results also suggest severe changes in glutamate neurotransmission beyond effects that can be accounted for by changes in Ca²⁺ current. Work is underway to characterize the mechanisms of this phenomenon and to determine whether this deficit reflects a developmental arrest of glutamate synapse formation or a more dynamic plasticity mechanism operating on glutamate synapses. The severe depression of glutamate neurotransmission that we observed appears to represent yet another homeostatic regulatory mechanism in response to tonic depolarization.

A second major effect of depolarization rearing was a change in the susceptibility of neurons to a return to basal culture conditions. This vulnerability could be important physiologically. Inhibition tends to develop later than excitation in many brain areas. Thus, inhibition could actually participate in sculpting the final numbers of neurons in the developing brain. Neurons reared primarily on depolarization may find themselves particularly vulnerable to apoptosis triggered by the maturation of inhibition during development.

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