Calmodulin Is Involved in Membrane Depolarization-Mediated Survival of Motoneurons by Phosphatidylinositol-3 Kinase- and MAPK-Independent Pathways

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In the present work, we find that the elevation of extracellular K⁺ concentration promotes the survival of chick spinal cord motoneurons in vitro deprived of any neurotrophic support. This treatment induces chronic depolarization of the neuronal plasma membrane, which activates L-type voltage-dependent Ca²⁺ channels, resulting in Ca²⁺ influx and elevation of the cytosolic free Ca²⁺ concentration. Pharmacological reduction of intracellular free Ca²⁺ or withdrawal of extracellular Ca²⁺ reversed the effects of depolarization on survival. The intracellular Ca²⁺ response to membrane depolarization developed as an initial peak followed by a sustained increase in intracellular Ca²⁺ concentration. The depolarizing treatment caused tyrosine phosphorylation of mitogen-activated protein kinase (MAPK) without involving tyrosine kinase receptor activation. The calmodulin antagonist W13 inhibited the survival-promoting effect induced by membrane depolarization but not the tyrosine phosphorylation of MAPK. Moreover, depolarization did not induce phosphatidylinositol-3 kinase (PI-3K) phosphorylation in our cells, and the PI-3K inhibitor wortmannin did not suppress the survival-promoting effect of K⁺ treatment. These results suggest that calmodulin is involved in calcium-mediated survival of motoneurons through the activation of PI-3K- and MAPK-independent pathways.

Key words: motoneuron; calmodulin; signal transduction; trophic factor; depolarization; apoptosis

During embryonic development of the vertebrate nervous system, approximately one half of all the neurons that are produced die as a result of a process known as natural or programmed cell death, which appears to be a strategy to adapt neuronal populations to their innervation target size and specificity (for review, see Oppenheim, 1991). It is now clear that specific target-derived neurotrophic factors play a decisive role in this (Barde, 1989; Oppenheim, 1989; Snider and Johnson, 1989). Motoneurons (MTNs) display the same behavior on trophic deprivation as other neuronal populations that respond to specific neurotrophic molecules (Comella et al., 1994). There is growing evidence that neuronal differentiation and survival may also be regulated by non-target-derived factors such as bioelectric activity (Franklin and Johnson, 1992; Franklin et al., 1995) or the cytoplasmic free Ca²⁺ concentration ([Ca²⁺]ᵢ) (Collins and Lile, 1989; Collins et al., 1991; Johnson et al., 1992; Larmet et al., 1992; Murrell and Tolkosky, 1993; Franklin et al., 1995).

Previous studies have demonstrated that an increase in the [Ca²⁺]ᵢ is able to activate Ras in PC12 cells (Rosen et al., 1994; for review, see Finkbeiner and Greenberg, 1996). Ras activation starts the sequential phosphorylation and activation of Raf, MEK, and MAPK protein kinases. This pathway provides a route through which Ca²⁺ influx could regulate cytoplasmic signaling and affect neuronal responses. The Ras pathway can also be activated by neurotrophins. In neurons, neurotrophins bind to specific tyrosine kinase receptors (Trks), resulting in tyrosine autophosphorylation of the receptor and tyrosine phosphorylation of Shc (for review, see Schlessinger, 1993; Segal and Greenberg, 1996).

PI-3K mediates another intracellular pathway that could be stimulated by increases on [Ca²⁺]ᵢ or neurotrophin treatment and has been shown to be required for NGF-mediated survival in PC12 cells (Yao and Cooper, 1995). Moreover, this pathway has been shown to be involved in growth factor-mediated survival of primary neurons (Dudek et al., 1997; D’Mello et al., 1997) or other types of cells (Vemuri and McMorris, 1996). However, it seems that activation of the PI-3 kinase pathway is not essential in the promotion of neuronal survival because of high potassium (Dudek et al., 1997; D’Mello et al., 1997), although there is not complete agreement on this (Miller et al., 1997).

In the present work, we analyze the effect of elevated extracellular K⁺ on the survival of primarily cultured MTNs from chick spinal cord and we explore some of the molecular mechanisms involved therein. We have explored the possibility that calmodulin may serve as a second messenger to this effect. To that end we have monitored the MAPK signaling pathway in the presence of calmodulin antagonists. We show that calmodulin modulates survival but not the activation of the MAPK pathway when MTNs are submitted to depolarization. We also show that block-
ade of the PI-3 kinase does not suppress the survival effects of K+ depolarization. Taken together, these results suggest that calmodulin is involved in the survival of depolarized MTNs and acts through mechanisms that are independent from both PI-3 kinase and MAPK.

MATERIALS AND METHODS

Cell isolation and culture. MTNs were purified from embryonic chicken according to Comella et al. (1994). Briefly, whole spinal cords were dissected out from 5.5-day-old Arbor Acres chick embryos (COPAGA, Lelystad, The Netherlands). MTNs were cultured in the presence of a saturating concentration of 18 mM glucose, 22.5 mM bicarbonate, 2.5 mM glutamine, and 20 U/ml penicillin plus 20 μg/ml streptomycin (L15) containing 10% heat-inactivated horse serum (Life Technologies, Gaithersburg, MD) (L15H). The single-cell solution was layered over 5 ml of L15 medium and 3.5% (wt/vol) BSA and spun at 100 × g for 5 min to remove cell debris. Cells were resuspended in GHEBS and layered over 4 ml of 28.75% (vol/vol) Nycodenz (N-2, 3-dihydroxypropylacetamido)-2, 4, 6-triiodo-9-[3-(3-dihydroxypropyl)aminol]-(2, 3-dihydroxypropyl)amido [Nycodenz, Norway] (supplied as an isotonic sterile solution of 1.15 g/ml density) in GHEBS and centrifuged at 400 × g for 10 min. The intermediate layer was collected and transferred to an appropriate amount of L15H, and cells were counted with a hemocytometer. For survival experiments, MTNs were plated in 96-well culture dishes (Corning, Corning, NY) precoated with poly-DL-ornithine (PORN) (30 μg/ml for 30 min) and laminin (2 μg/ml for 1 hr) (Life Technologies), and seeded at a density of 15,000 cells per well. For Western blot and immunoprecipitation experiments, 2–3 × 10^6 cells were plated in precoated 60 mm culture dishes (Corning). PC12 cells were grown on 75 cm² culture dishes (Corning) in DMEM (Sigma, St. Louis, MO) supplemented with 6% heat-inactivated fetal calf serum (Life Technologies) and 3% heat-inactivated horse serum (Life Technologies) containing 10 mM HEPES and 20 U/ml penicillin plus 20 μg/ml streptomycin. For Western blot and immunoprecipitation experiments, 5–6 × 10^6 PC12 cells were plated in 60 mm culture dishes (Corning) precoated with PORN.

All cultures were kept at 37°C in a saturating humidity atmosphere of 95% air, 5% CO₂.

Evaluation of neuronal survival and apoptosis. Unless indicated otherwise, MTNs were cultured in the presence of a saturating concentration (300 μg/ml) of muscle extract (MEX) for 48 hr (Comella et al., 1994). At this time, cells were washed with L15H and 50 μl of assay medium containing the appropriate amount of supplements or drugs. The number of cells was determined in the central area of every well using a 20× power objective on a phase-contrast inverted microscope. Only cells bearing neurites longer than two cell diameters were included in counts. This value represented our corrected 100% initial survival. Counts were performed every 24 hr in precisely the same microscopic field throughout the duration of the experiment, and survival was expressed as a percentage of neuronal counts with respect to the 100% initial value. Values shown are the mean ± SEM of these percentages for eight wells; each experiment was repeated at least three times. Where applicable, statistical analysis was performed with the nonparametric test for two independent samples: Mann–Whitney, Kruskal–Wallis test and one-way ANOVA and least-significant difference test.

To assess whether a given treatment induced an apoptotic cell death process, cultures were stained with the Hoechst 33258 dye. MTNs having grown in 35 mm culture wells for 48 hr in the presence of saturating concentrations of MEX were washed with L15H and were grown for an additional 15 min with NE, MEX, 30K, or W13 medium. At this time, media were removed, and cells were washed twice with PBS and fixed with 4% (wt/vol) paraformaldehyde (Fluka, Neu-Ulm, Germany) in PBS for 15 min. Thereafter, neurons were washed three times with PBS and stained for 30 min with 0.05 μg/ml Hoechst 33258 (Sigma). Cultures were then washed twice with PBS and mounted with glass coverslips using Fluoromount (Biomeda, Foster City, CA). Stained cells were observed and counted with a vertical microscope equipped with epifluorescence and UV filters.

Measurement of intracellular Ca²⁺. Neurons were loaded with fura-2 AM, and intracellular Ca²⁺ levels were measured microscopically in individual cell bodies. Cells were grown on PORN-laminin-coated glass coverslips and loaded with 2 μM fura-2 AM (Molecular Probes, Eugene, OR) for 1–2 hr. Cells were then washed in GHEBS–fura-1 AM solution (GHEBS supplemented with 2 mM CaCl₂ and 1.5 mM MgCl₂) and incubated for 30 min at 37°C to allow hydrolysis of ester. Ca²⁺ measurements were recorded on a Zeiss Axiovert 10 inverted microscope equipped with a Zeiss MPM microscope photometer. Light from a 75 W xenon lamp combined with interference filters of 340 ± 10 nm and 380 ± 10 nm in a wheel changer was deflected by a dichroic mirror at 425 nm into a 40× Plan-Neofluar objective, and fluorescence emission was collected through a 500–530 nm interference filter to a photomultiplier (Hamamatsu, R928). Excitation at 380 nm was attenuated by a corrective filter to compensate for higher attenuation of 340 nm light along the optical path. The output of the photomultiplier was fed to a specially written computer program (FFP, Zeiss). Random fields of neuronal cell bodies (see cell field) were examined in individual dishes to determine pretreatment calcium values. Media containing high K+ or drugs were quickly substituted for GHEBS, and calcium measurements were made at different times after medium replacement. Signal calibration as a function of intracellular Ca²⁺ was performed using standard Ca²⁺ solutions (Molecular Probes) and was converted to Ca²⁺ concentrations as described by Grynkiewicz et al. (1985). Data from different culture dishes receiving the same treatment were pooled. Values for Ca²⁺ are expressed as the mean concentrations ± SEM.

Tyrosine phosphorylation assay and immunodetection of p85 subunit of PI-3K. For immunodetection experiments, we determined that a minimum of 2–3 × 10⁶ MTNs were needed for each treatment (i.e., lane on SDS-PAGE gel). Alternatively an 80% confluence 60 mm culture dish of PC12 cells was used for each treatment. MTNs were plated on 60 mm tissue culture dishes and grown in the presence of 2 or 3 d before exposure to the agents was initiated. At appropriate times, MTNs or PC12 cells were rinsed three times with L15H or DMEM without serum, respectively, and were maintained for 3 hr in the presence of fresh medium containing the appropriate drug. After this time, PC12 cells or MTNs were incubated in medium containing NGF (200 ng/ml) or 30 mM KCl for 5 min at 37°C. At the end of the treatment, cultures were rinsed rapidly in ice-cold PBS and solubilized at 4°C in 0.4 ml of Tris/ NP-40 lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1% NP-40, 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, 2 mM benzamidine, and 20 μg/ml leupeptin). After a 15 min incubation on ice, the samples were rotated orbitally for 30 min at 4°C and spun in a microcentrifuge for 15 min at 4°C to remove nuclei and cellular debris. The amount of protein in lysates was quantified by the BIO-RAD DC Protein Assay (Bio-Rad, Munich, Germany).

To determine the level of tyrosine phosphorylation of MAPK, cell lysates were immunoprecipitated with specific antibodies. Supernatants were subjected to MAPK immunoprecipitation (MAPK-1P) overnight at 4°C with an anti-extracellular-regulated kinase 2 (ERK2) polyclonal antibody (Transduction Laboratories, Lexington, KY). Alternatively, cell lysates were immunoprecipitated with protein A-Sepharose beads, electro-phoresed in SDS-PAGE gels, and transferred onto polyvinylidene difluoride (PVDF) Immobilon-P transfer membrane filters (Millipore, Bedford, MA). Membranes were blocked with TBS-T20 (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20) containing 5% BSA for 1 hr at room temperature and blotted with the 4G10 anti-phosphotyrosine monoclonal (anti-P-Tyr) antibody for 1 hr at room temperature. Membranes were incubated with anti-mouse IgG antibody peroxidase conjugated for 1 hr at room temperature and developed with the enhanced chemiluminescence Western blotting system from Amersham (Arlington Heights, IL). Alternatively, tyrosine phosphorylation of MAPK was detected in cytoplasmic lysates. Thus 20 μg of cytoplasmic protein per well was separated by SDS-PAGE, transferred onto PVDF membranes, and blotted with the anti-P-Tyr antibody as described above. Filters from tyrosine phosphorylation assays were stripped with 100 mM β-mercaptoethanol, 2% SDS in 62.5 mM Tris-HCl, pH 6.7, for 30 min at 50°C, and processed for the immunodetection of ERK proteins with a mouse monoclonal anti-pan-ERK antibody (Transduction Laboratories).

Immunodetection of p85 subunit of PI-3K was performed in anti-P-Tyr immunoblotting. Staining was facilitated by treatment. To immunoprecipitate p85, protein extracts from MTNs depolarized with 30 K+ for 1 min or from PC12 cells stimulated with NGF for 1 min were subjected to immunoprecipitation overnight at 4°C with the anti-P-Tyr antibody. Immunocomplexes were precipitated with protein A-Sepharose coupled to rabbit.
anti-mouse polyclonal antibody (Sigma). Precipitates or alternatively 20 μg of protein extracts per well were electrophoresed and transferred as described above. Filters were blotted with an anti-p85 polyclonal antibody (UBI) as described by the provider.

Reagents. 1,2-bis 2-aminophenoxethane-N,N,N,N'-tetra-acetic acid (acetomethyl) ester (BAPTA/AM) was obtained from Molecular Probes. Bay K 8644 was from Calbiochem (La Jolla, CA). Nifedipine, amiloride, verapamil, wortmannin, N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide hydrochloride (W13), N-(4-aminobutyl)-2-naphthalenesulfonamide hydrochloride (W12), anti-mouse IgG antibody peroxidase-conjugated, and the other biochemicals were obtained from Sigma. NGF was prepared at the laboratory from mouse salivary glands, as described by Mobley et al. (1976). 4G10 anti-PTyr antibody was a generous gift of D. Martín-Zanca (Salamanca, Spain).

RESULTS

Elevated extracellular K⁺ promotes the survival of spinal cord motoneurons

MTNs were initially cultured in a saturating concentration of MEX for 2 d. Afterward, the culture medium was replaced and the different conditions were established. On readdition of a medium containing MEX, >90% of the MTNs remained alive after an additional 24 hr of culture (Fig. 1). However, when they were deprived of MEX and maintained in the basal medium, i.e., L15H, a significantly lower (p < 0.005) percentage of MTNs (~50%) survived (Fig. 2A).
to maintain MTN survival. In MEX-treated cultures the survival was 83 ± 3%, whereas in MEX-treated cultures to which 20 μM BAPTA/AM was added the observed survival was 82 ± 3%. However, BAPTA/AM completely prevented the effects of 30K on MTN survival. In cultures treated with 30K plus 20 μM BAPTA/AM (Fig. 3A), the survival percentage decreased significantly (44 ± 3%; p < 0.05) with respect to those treated with 30K alone (85 ± 4%). Thus, these results suggest that an increase in [Ca^{2+}]_{i} is required for high K\(^{+}\) medium to rescue MEX-deprived MTNs.

To ascertain whether the incremental increase in [Ca^{2+}]_{i} in high K^{+}-treated cells results from the influx of Ca^{2+} from the extracellular medium, we tested the effect of the withdrawal of extracellular Ca^{2+} using the extracellular calcium chelator EGTA. When 1.5 mM EGTA was added to 30K-treated cultures, the survival of MTNs decreased to 61 ± 3% (Fig. 3A). On the other hand, when the same concentration of EGTA was added to MEX-treated cultures, the survival percentage (80 ± 3%) was not significantly different from that observed in cells treated with MEX only (Fig. 3A). This finding indicates that the increase in [Ca^{2+}]_{i} is attributable to an influx of Ca^{2+} from the extracellular medium.

**Voltage-gated Ca^{2+} channel antagonists block the survival-promoting effect of high K^{+} medium**

To analyze which types of voltage-gated Ca^{2+} channels are involved in the rescuing effects of depolarization, we tested different Ca^{2+} channel antagonists. Amiloride, a T-type voltage-gated Ca^{2+} channel antagonist, did not affect the survival-promoting effect of 30K, suggesting that channels of this type were not involved (Fig. 4A). L-type Ca^{2+} channel antagonists, such as nifedipine and verapamil, prevented the ability of high K^{+} to maintain survival of MTNs. The effects of these drugs were dose dependent. The percentage of MTN survival in high K^{+} plus 1 μM nifedipine was 62 ± 3%; thus the values of survival in high K^{+} (98 ± 3%) were similar to those found in NE control cultures (61 ± 2%) (Fig. 4A). Toxicity of the drug was tested by adding 1 μM nifedipine to MEX-treated cultures. The percentages of survival in drug-treated and untreated cultures were not significantly different (data not shown). Likewise, the effects of verapamil, which blocks the L-type Ca^{2+} channels independently of depolarization (Striessing et al., 1986), were similar to those containing 30K, the survival percentage was ~60% in both cases. When MTNs were allowed to grow in the presence of MEX for 48 or 72 hr, 30K supported the survival of >90% of the cells. Thus, although MEX maintains MTN survival from the beginning of the culture period (12 hr), the ability of high K^{+} to do so is not fully developed until 48 hr in culture with MEX (Fig. 2B).
obtained with nifedipine, except for the fact that verapamil was less effective (Fig. 4).

To further investigate the role of L-type Ca\(^{2+}\) channels in mediating the depolarization survival effects, we used Bay K 8644. This L-type Ca\(^{2+}\) channel agonist is known to shift the voltage dependence of the Ca\(^{2+}\) channel opening to more negative potentials and to prolong the channel opening (Nowycky et al., 1985). As would be expected, Bay K did not have a direct effect on MTN survival in our cultures (data not shown), but it clearly potentiated the effect of high K\(^{+}\). At suboptimal concentrations of K\(^{+}\) (10 or 20 mM), MTN survival in Bay K-treated cultures was similar to that found in 30K-treated cultures (Fig. 3B). Taken together, these results imply that L-type Ca\(^{2+}\) channels are the mediators of the Ca\(^{2+}\) influx that promotes the survival-enhancing effect of high K\(^{+}\) medium in MTNs.

**Effects of high K\(^{+}\) medium on intracellular Ca\(^{2+}\) concentration**

Elevation of the extracellular K\(^{+}\) concentration to a level that promotes neuronal survival in different neuronal populations is associated with a sustained elevation of intracellular calcium obtained with nifedipine, except for the fact that verapamil was less effective (Fig. 4A).

To further investigate the role of L-type Ca\(^{2+}\) channels in mediating the depolarization survival effects, we used Bay K 8644. This L-type Ca\(^{2+}\) channel agonist is known to shift the voltage dependence of the Ca\(^{2+}\) channel opening to more negative potentials and to prolong the channel opening (Nowycky et al., 1985). As would be expected, Bay K did not have a direct effect on MTN survival in our cultures (data not shown), but it clearly potentiated the effect of high K\(^{+}\). At suboptimal concentrations of K\(^{+}\) (10 or 20 mM), MTN survival in Bay K-treated cultures was similar to that found in 30K-treated cultures (Fig. 3B). Taken together, these results imply that L-type Ca\(^{2+}\) channels are the mediators of the Ca\(^{2+}\) influx that promotes the survival-enhancing effect of high K\(^{+}\) medium in MTNs.

**Effects of high K\(^{+}\) medium on intracellular Ca\(^{2+}\) concentration**

Elevation of the extracellular K\(^{+}\) concentration to a level that promotes neuronal survival in different neuronal populations is associated with a sustained elevation of intracellular calcium

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**Figure 3.** Effect of BAPTA/AM, EGTA, and Bay K 8644 on the survival of depolarized MTNs. A, Percentages of MTN survival after treatment during 24 hr with no drugs (black bars), 20 \(\mu\)M BAPTA/AM (open bars), or 1.5 mM EGTA (cross-hatched bars) in MEX or 30K medium. B, Percentage of MTN survival after 24 hr in vitro in cultures supplemented with 10, 20, and 30 mM KCl, with (black bars) or without (open bars) 1 \(\mu\)M Bay K. Broken lines show survival of cells in sibling control cultures maintained in NE (A) or in MEX and NE (B) culture medium for the same culture period. Values are the mean ± SEM. Asterisks in A indicate values significantly different (\(p < 0.05\)) from control, 30K-treated cultures.

**Figure 4.** Effect of Ca\(^{2+}\) channel antagonists on MTN survival and on [Ca\(^{2+}\)]\(_i\). A, Percentages of MTN survival in cultures supplemented with 30K are plotted against different doses of the Ca\(^{2+}\) channel antagonists nifedipine (filled circles), verapamil (filled squares), and amiloride (filled triangles). Survival was evaluated 24 hr after treatment. Broken lines show survival of cells in sibling control cultures maintained in MEX or in NE culture medium for the same period of time. Survival values are plotted as the mean ± SEM. B, Effect of extracellular high K\(^{+}\) on [Ca\(^{2+}\)]\(_i\). KCl (30 mM) was added to the culture medium of MTNs at 0 min, and [Ca\(^{2+}\)]\(_i\) was measured 1, 5, and 15 min, and 24 and 48 hr after exposition. C, Effect of the addition of high potassium (*30K*) after 10 min in the presence of 10 \(\mu\)M nifedipine (filled circles) and 10 \(\mu\)M amiloride (filled squares). In B and C [Ca\(^{2+}\)]\(_i\) is plotted as the mean ± SEM, and the broken lines represent the average baseline calcium level in control medium.
(Collins et al., 1991; Koike and Tanaka, 1991). To study the intracellular Ca\textsuperscript{2+} response to membrane depolarization in MTNs, cells were loaded with fura-2 AM, and Ca\textsuperscript{2+} levels were measured microscopically in individual cell bodies. The basal [Ca\textsuperscript{2+}]i in MTNs cultured for 2 d in the presence of MEX was 36.3 ± 2.60 nm (mean of 25 cells from five different experiments) (Fig. 4B). When the neurons were exposed to 30K, [Ca\textsuperscript{2+}]i increased rapidly within the first minute to 297 ± 35 nm (mean of 25 cells from five different experiments). After 15 min the concentration decreased to 189 ± 23 nm (10 cells from two different experiments); after 24 hr in the continued presence of 30K this value was 165 ± 11 nm (33 cells from six different experiments), and after 48 hr of exposure of the MTNs to a 30K medium, [Ca\textsuperscript{2+}]i decreased to 82 ± 7.5 nm (20 cells from four different experiments). This value was still significantly higher than that found in the basal culture conditions. The addition of 1.5 nm EGTA or 20 μM BAPTA/AM to the 30K medium completely suppressed the intracellular calcium elevation observed at 1 and 15 min (data not shown). Therefore, we observed a good correspondence between neuronal survival and the variation of [Ca\textsuperscript{2+}]i caused by chronic depolarization.

The dihydropyridine L-type calcium channel antagonist nifedipine suppressed the elevation of intracellular calcium. When the extracellular K\textsuperscript{+} concentration was raised to 30 mM in cultures previously exposed to 10 μM nifedipine for 10 min, the [Ca\textsuperscript{2+}]i increased from 30 ± 5 nm to 60.5 ± 11 nm within 1 min, but decreased to 47 ± 7 nm within 30 min (Fig. 4C). The T-type Ca\textsuperscript{2+} channel antagonist amiloride did not affect the increase in the [Ca\textsuperscript{2+}]i after addition of 30 mM K\textsuperscript{+} to the culture medium (Fig. 4C). These results suggest that the observed increase in [Ca\textsuperscript{2+}]i after depolarization of the plasma membrane by high K\textsuperscript{+} is attributable to Ca\textsuperscript{2+} entry from the extracellular space through L-type Ca\textsuperscript{2+} channels.

**Role of calmodulin in depolarization-enhanced survival of MTNs**

A possible role for calmodulin in mediating the effect of depolarization on neuronal survival has been suggested previously (Gallo et al., 1987; Hack et al., 1993; Franklin et al., 1995). To determine whether calmodulin antagonists play a role in neuronal survival in depolarized MTNs, we tested W13, a specific calmodulin antagonist that binds to calmodulin and inhibits Ca\textsuperscript{2+}/calmodulin-regulated enzyme activities. As a control we used W12, a related compound that lacks chlorine in its molecule and is a much less effective calmodulin blocker than W13 (Hidaka et al., 1981; Hidaka and Tanaka, 1983). Application of 5 μg/ml of W13 to 30K-treated cultures (Fig. 5A) reversed the survival to the level found in NE-treated cultures. The same dose applied to MEX-supplemented neurons had no effect on survival. On the other hand, W12 (5 μg/ml) had no effect on neuronal survival in any of the culture conditions tested (Fig. 5A). To assess whether W13 suppresses the survival effects of depolarization, therefore inducing neuronal death, we have quantified the percentage of apoptotic MTNs after W13 treatment. Experiments were performed with the Hoechst 33258 dye, which binds specifically to the double-stranded DNA and emits at 490–500 nm when excited at 360 nm. Apoptotic cells display a highly condensed DNA that is normally fragmented in two or more chromatin aggregates. In MTN cultures grown in the presence of MEX or 30K the percentage of cells displaying this morphology was found to be 2.2% and 2.1%, respectively; however, after 15 hr of MEX deprivation, a substantial increase (4.8%) in the percentage of cells that displayed apoptotic nuclei was found. In 5 μg/ml W13–30K-treated cultures, this percentage was found to be similar (4.3%) to that found in MEX-deprived cultures.

These results suggest that calmodulin plays a role in mediating the survival effects of depolarization. It has been suggested, however, that some calmodulin antagonists (e.g., calmidazolium and W7) can interact with voltage-dependent calcium channels and reduce Ca\textsuperscript{2+} influx (Greenberg et al., 1987; Doroshenko et al., 1988; Franklin et al., 1995). To ascertain whether W12 and W13 might affect the sustained elevation of intracellular Ca\textsuperscript{2+} caused by depolarization in MTNs, cells were depolarized with 30K in the presence of the calmodulin antagonists, and [Ca\textsuperscript{2+}]i was measured. After 5 min in these conditions, intracellular Ca\textsuperscript{2+} increased to ~90 nm. This value was significantly lower (p < 0.05) than that observed in 30K-stimulated control cultures. After 24 hr of treatment, the intracellular calcium in 30K–W13- and 30K–W12-treated cells had increased to 98 ± 7 and 97 ± 13.5 nm, respectively. These values are similar to those found in 30K control cultures after the same period (98 ± 13 nm) (Fig. 5B). Taken together, these results confirm that W13 induces apoptotic...
Inhibitors of PI-3 kinase did not block depolarization-promoted survival of MTNs

Recent experiments reported by Miller et al. (1997) suggest a role of PI-3 kinase in neuronal survival induced by depolarization. We therefore investigated the possible involvement of PI-3 kinase in signaling pathways that promote cell survival in MTNs treated with high K⁺ medium. To this end we used wortmannin, a PI-3 kinase inhibitor. It has been demonstrated that at a wortmannin concentration of 100 nm, the activity of PI-3 kinase was inhibited almost completely (Yano et al., 1993; Kimura et al., 1994; Okada et al., 1994a,b). The addition of wortmannin to MTNs maintained in 30K did not have any effect on survival. The percentage of MTN survival in cultures treated with 100 nm wortmannin and 30K was 79.4 ± 4.2%. This value is similar to that observed in cultures treated with 30K alone (84.1 ± 4.7%). To confirm that PI-3K was not involved in the survival effect of depolarization, we studied the activation of this enzyme by the tyrosine phosphorylation of its p85 subunit. Thus, MTNs were depolarized for 1 min with 30 K⁺. Cytoplasmic lysates from these cells were subjected to immunoprecipitation with a specific anti-PTyr antibody (4G10) and submitted to Western blot analysis with an anti-p85 polyclonal antibody. This assay showed that depolarization failed to activate the PI-3K (Fig. 6A). The MTNs immunoprecipitates were compared with those of NGF-stimulated PC12 cells that were used as positive control of the experiment and that showed a clear band of tyrosine phosphorylated p85. The positive signal obtained with the anti-p85 antibody on western blots of MTNs cell lysates (Fig. 6B) discard the possibility that the lack of p85 signal in the MTNs immunoprecipitates of the Figure 6A was due to failure of the antibody to recognize the chicken p85 subunit of PI-3K. Taken together, these results suggest that activation of PI-3 kinase is not involved in the MTN survival promoted by membrane depolarization.

Depolarization induces MAPK tyrosine phosphorylation in MTNs

Our previous results show that the elevation of [Ca²⁺]ᵢ, promotes MTN survival in the absence of neurotrophic support. Many neurotrophic factors promote neuronal survival through the activation of the MAPK pathway, the state of activation of which can be monitored by assessing the level of tyrosine phosphorylation of ERK1 and ERK2, two members of the MAPK family (Thomas et al., 1992; Rosen et al., 1994; Rusansucu et al., 1995). To determine whether the membrane depolarization is also able to activate the MAPK pathway, we compared the level of MAPK tyrosine phosphorylation during our experimental condition, i.e., 30K. As a negative control, nonstimulated (N.S.) MTNs were used. Cytoplasmic lysates of the cells were extracted and submitted to Western blot analysis for detection of tyrosine-phosphorylated proteins with the anti-PTyr antibody 4G10. The results (Fig. 6C) showed a protein, with an apparent molecular weight of ~42 kDa (designated as ERK2), that increased its state of tyrosine phosphorylation after 5 min of depolarization. This tyrosine-phosphorylated protein was not detected in nonstimulated (N.S.) cells. ERK2 was immunoprecipitated from the same lysates with a specific anti-ERK2 antibody. When those immunoprecipitates were submitted to the same anti-phosphotyrosine analysis, a similar result was observed. Depolarization was able to specifically phosphorylate this protein on tyrosine residues (Fig. 6D).

Reblottings of the membranes that contained the cytoplasmic lysates or the immunoprecipitates with an anti-pan-ERK monoclonal antibody (Fig. 6C,D) confirmed the identity of the observed bands as the ERK2 kinase. Moreover, this result demonstrates that differences in band intensity between N.S. cells and treated cells were caused by the degree of phosphorylation and not differences in the protein content. We have also analyzed the state of phosphorylation of Trk in depolarized MTNs or PC12 cells by immunoprecipitating this protein with specific antibodies (pan-Trk antibodies) and submitting the immunoprecipitates to Western blot analysis using an anti-Ptyr antibody. We have never detected any autophosphorylative response to Trk in MTNs or PC12 cells stimulated with high K⁺ in the culture medium, although there was a clear phosphorylation of the MAPK in their corresponding cell lysates (data not shown).

Depolarization-induced MAPK activation was not blocked by calmodulin antagonists

The cell survival experiments showed that the calmodulin antagonist W13 is able to block the effect of membrane depolarization on MTN survival at concentrations of 5 μg/ml or lower. To test whether this calmodulin antagonist has any effect on MAPK...
Figure 7. Effect of calmodulin antagonists on depolarization-induced MAPK activation. A, MTN cultures were pretreated for 3 hr with 25 μg/ml of W12 (K12) or 25 μg/ml W13 (K13) or left untreated (N.S. and KC1). Then, cultures were stimulated for 5 min with high potassium (K12, K13, and KC1) or were left unstimulated (N.S.). Protein extracts were analyzed on Western blot with an anti-PTyr antibody (top panel). Membranes were stripped and reprobed with an anti-pan-ERK monoclonal antibody (bottom panel). B, MTN cultures were pretreated for 3 hr with 5 μg/ml W13 (K13) or left untreated (N.S. and KC1). Then, cultures were stimulated for 5 (5′) or 15 (15′) min with high potassium (KC1 and K13) or were left unstimulated (N.S.). Protein extracts were analyzed on Western blot with an anti-PTyr antibody (top panel). Membranes were stripped and reprobed with the anti-pan-ERK antibody (bottom panel). ERK2-labeled arrows indicate the position of ERK2 protein in A and B.

pathway activation in depolarized MTNs, tyrosine phosphorylation of ERK2 MAPK was monitored. In MTNs treated for 5 or 15 min with 30 mM K+, addition of 5 μg/ml W13 did not inhibit the tyrosine phosphorylation of ERK2 MAPK (Fig. 7B). The inhibition of ERK2 phosphorylation was observed only when the concentration of W13 was increased to 25 μg/ml (Fig. 7A). The inhibition was specific, because in the same experiment application of 25 μg/ml of the weaker calmodulin antagonist W12 did not have any effect on MAPK phosphorylation (Fig. 7A). These data suggest that calmodulin may play a primary role in the survival promoted by membrane depolarization without interrupting the signaling from membrane depolarization to the MAPK pathway.

DISCUSSION

In the present work, we have shown that elevated extracellular K+ promoted MTN survival in the absence of neurotrophic support. Increased extracellular K+ can prolong the survival of different types of nerve cells and can completely substitute the neurotrophic agents for in vitro survival of many populations of neurons (for review, see Franklin and Johnson, 1992). Our results show that 30 mM K+ added to the culture medium promoted the survival of MTNs deprived of MEX. It is well known that elevated extracellular K+ causes membrane depolarization on neurons. The consequence of membrane depolarization is the increase in $[\text{Ca}^{2+}]_i$, which appears to be attributable to Ca$^{2+}$ influx through dihydropyridine-sensitive L-type voltage-gated Ca$^{2+}$ channels. In accordance with these pharmacological studies, we have shown that depolarizing levels of K+ promote the survival of embryonic chick MTNs by activating L-type Ca$^{2+}$ channels. Our $[\text{Ca}^{2+}]_i$ measurements with fura-2 AM showed that membrane depolarization caused a sustained increment in the intracellular Ca$^{2+}$ level, which was prevented by the application of the L-type Ca$^{2+}$ channel antagonists. Therefore, it can be concluded that the depolarization-induced Ca$^{2+}$ increase was caused by Ca$^{2+}$ influx from the extracellular compartment.

MTN response to membrane depolarization differs depending on the stage of maturation of the cells in culture. Thus, when MTNs were treated with high K+ 12 or 24 hr after plating, this treatment did not promote as much MTN survival as MEX. However, when high K+ was applied after 2 or 3 d in culture with MEX, 30K supported a percentage of MTN survival similar to that of MEX. It is possible that although MTNs in ovo already show an elevated density of L-Ca$^{2+}$ current at the corresponding developmental age (Mc Cobb et al., 1989), MTNs in vitro need a longer time to express a sufficient quantity of L-type Ca$^{2+}$ channels to allow significant Ca$^{2+}$ currents in response to depolarization. Similar results have been reported by Franklin et al. (1995), who showed that superior cervical ganglion neurons did not survive in response to increases in $[\text{Ca}^{2+}]_i$ until the cells had been cultured for 3 d in the presence of NGF. However, intracellular Ca$^{2+}$ measurements on addition of 30 mM K+ to the MTN culture medium did not differ between neurons cultured for 1, 2, or 3 d in the presence of MEX (data not shown). These results raise the possibility that factors other than the increase in $[\text{Ca}^{2+}]_i$ are needed for neurons to develop a survival response to membrane depolarization that is able to prevent neuronal death after trophic deprivation. One possible explanation of this result would be that MTNs need to recover from the damage caused by dissociation procedure. It is also possible that the developmental age of the motoneurons is important because it could be related to the acquisition of some additional elements that allow the survival as a consequence of the intracellular calcium increases. This would be difficult to approach experimentally because purification of chicken motoneurons older than 6 embryonic days is very difficult technically and has never been reported.

One important issue examined in the present report is how chronic membrane depolarization is able to prevent MTN death after neurotrophic deprivation. A possible explanation would be that high K+ medium might induce the release from other cells of neurotrophic factors for this population of neurons; however, several results argue against this possibility. Our cultures are >95% pure in MTNs and do not contain glial cells (Comella et al., 1994). The small percentage of non-MTN cells corresponds to other types of neuronal cells. Therefore, the possibility that glial-derived neurotrophic factors such as GDNF, which have been reported to be neurotrophic for MTN (Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995), are released seems unlikely. Additionally, MTN survival attributable to high K+ did not significantly improve in high-versus low-density cultures (data not shown). Similar results have been reported by other groups that have described a relative insensitivity to changes in cell density of the survival response to high K+ (Collins and Lile, 1989; Franklin et al., 1995). However, a trophic factor might be produced in small quantities by depolarized motoneurons having local effects on the neighboring cells. According to this hypothesis, Ghosh et al. (1994) demonstrated recently that high K+ induces BDNF expression in cultured embryonic cortical neurons from rats. In our culture system,
BDNF has an obvious survival-promoting effect on 3-d-old MTN cultures but not on 1- and 2-d-old MTN cultures (J. X. Comella, unpublished results). It should be noted here that after 48 hr in culture MTNs showed a full rescuing response to depolarization. Moreover, we have never seen any autophosphorylative response of Trk in response to membrane depolarized in MTNs. Accordingly, in PC12 cells, the addition of high potassium to the culture medium is able to activate MAPK without inducing Trk autophosphorylation. Therefore, our results suggest strongly that depolarization promotes neuronal survival directly by affecting some process occurring within the cell rather than indirectly by autocrine BDNF release. However, we cannot rule out completely that part of the rescuing effect of 30K may depend on the synthesis and release of BDNF or other neurotrophic factors in cultures older than 48 hr.

Several reports using pharmacological antagonists of calmodulin (calmidazolium, W7, or trifluoperazine) have suggested that this molecule mediates the survival effects of high K\(^+\) (Gallo et al., 1987; Hack et al., 1993; Franklin et al., 1995). However, the major drawback of these inhibitors is that they also reduce the Ca\(^{2+}\) current induced by high K\(^+\), because they functionally block the voltage-dependent Ca\(^{2+}\) channels (Greenberg et al., 1987; Doroshenko et al., 1988; Franklin et al., 1995). Therefore, it cannot be determined whether the effects of these drugs were attributable to the blockade of calcium entry or an effect on an intracellular pathway related to neuronal survival. In the present work we have used the calmodulin inhibitors W12 and W13, which are related structurally. The only difference between these molecules is a chlorine residue present in W13 that renders this compound much more effective as a calmodulin inhibitor than W12. Therefore, W12 may be used as a control for the side effects of W13 on Ca\(^{2+}\) channels (Hidaka et al., 1981; Hidaka and Tanaka, 1983). W13 was able to block the survival response caused by membrane depolarization in our culture system, whereas W12 was ineffective. Data obtained by scoring apoptotic MTNs with a nuclear DNA staining support the view that W13 blocks the survival response, thus inducing apoptotic cell death. On the other hand, when Ca\(^{2+}\) measurements were performed in MTNs treated with the calmodulin inhibitors, both of them significantly and similarly reduced the initial elevation of [Ca\(^{2+}\)]\(_i\) after 30K addition, whereas no differences were observed between the long-term effects of W13 and W12 on [Ca\(^{2+}\)]\(_i\). Taken together, these results suggest that W13 inhibits the biological effect of membrane depolarization by directly blocking the calmodulin function rather than preventing calcium entry. At the same time, the differential effects of W12 and W13 on survival exclude a functional relevance of the initial [Ca\(^{2+}\)]\(_i\) peak, which was suppressed similarly by both drugs.

Recent experiments by several groups have shown that increases in [Ca\(^{2+}\)]\(_i\) activate the MAPK pathway (for review, see Finkbeiner and Greenberg, 1996; Segal and Greenberg, 1996). Our results showed that MAPK pathway is activated after membrane depolarization in MTNs. This activation is not involved in depolarization-induced neuronal survival, because calmodulin antagonists did not block the MAPK tyrosine phosphorylation at doses that are able to inhibit the neuronal survival.

It is known that in NGF-treated PC12 cells, the PI-3 kinase signaling pathway mediates neuronal survival (Yao and Cooper, 1995). In our culture system the presence of the PI-3 kinase inhibitor wortmannin did not suppress the neuronal survival promoted by elevated extracellular K\(^+\). Moreover, no phosphorylation of the p85 subunit of PI-3K was detected in response to depolarization. These results suggest that the PI-3 kinase pathway is not relevantly involved in the survival of chicken depolarized MTNs. At present we do not know whether PI-3K is relevant for MTN survival induced by other mechanisms, such as specific neurotrophic factors. Recent reports from other laboratories showed contradictory results about the role of the activation of this pathway in promoting cell survival of depolarized primary cultured neurons (D’Mello et al., 1997; Miller et al., 1997). Because a given neurotrophin can activate different signaling pathways that mediate neuronal survival in different neuronal populations (Borasio et al., 1993), we cannot rule out the possibility that MTN survival is mediated by unknown PI-3 kinase- and MAPK-independent pathways.

In conclusion, our results show that increases in [Ca\(^{2+}\)]\(_i\), after membrane depolarization are able to promote neuronal survival by PI-3K- and MAPK-independent pathways in chicken MTNs. The main intracellular mediator of this effect appears to be calmodulin, and therefore proteins regulated by calmodulin should be major targets of analysis when the study of the survival-promoting effects of intracellular calcium is approached.

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