Temporal Characteristics of Activation, Deactivation, and Restimulation of Signal Transduction following Depolarization in the Pheochromocytoma Cell Line PC12

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This study focuses on the transient and dynamic activation of intracellular signal transduction following different protocols of depolarization. During chronic depolarization, phosphorylation of extracellular signal-regulated kinases (ERKs) was observed to peak and subsequently fall to low levels within 10 min of depolarization. Short periods of depolarization, from 1 to 5 min in duration, also led to phosphorylation of ERK, and the rate of ERK dephosphorylation was not affected by the duration of depolarization. Phosphorylation of the cyclic AMP response element binding protein (CREB) also peaked as a result of chronic depolarization but decreased to intermediate levels that were maintained for more than 1 h. Pulsatile depolarization was explored as a means to circumvent the deactivation of intracellular signaling activity during chronic depolarization.

Both ERK and CREB were rephosphorylated by a second period of depolarization that followed a recovery period of 10 min or more. The effects of the durations of depolarization and interpulse recovery on reactivation of ERK and CREB were characterized. Measurements of free cytoplasmic Ca^{2+} confirmed the transient rise in the intracellular calcium concentration ([Ca^{2+}]_i) during chronic depolarization and the pulsatile increase in [Ca^{2+}]_i that can be achieved with short periods of depolarization. This study characterizes the dynamic activities of signal transduction following depolarization. Electrical stimulation of neurons induces many cellular changes that unfold over time, and the influx of Ca^{2+} ions that mediate these events is transient. This study suggests that pulsatile activity may be a means of maintaining signaling activity over long periods of time.

Electrical simulation of neurons in culture activates a series of cellular processes that unfold over time, starting within seconds of stimulation and continuing several hours to days later. Membrane depolarization immediately activates, by way of voltage-gated calcium channels, various intracellular signaling pathways. These pathways include members of the mitogen-activated protein (MAP) kinase family, the protein kinase A (PKA) cascade, the calcium-calmodulin kinase (CaMK) cascade, and the PKC cascade (reviewed in reference 14). The combined action of these intracellular signaling pathways leads to activation of various transcription factors within 30 s to 1 min of depolarization. The best characterized of these Ca^{2+}-sensitive transcription factors is the cyclic AMP response element binding protein, CREB (25; reviewed in reference 10). CREB is activated by several transduction cascades and thus acts as an integrator of signaling activity (25).

The Ca^{2+}-activated transcription factors subsequently bind to regulatory regions and initiate transcription of various immediate-early genes (IEGs). Since no new protein synthesis is required, transcription of IEGs begins within minutes of electrical stimulation (2, 25). Some of these IEGs, such as c-fos, jun, NGFI-A (zif268), and NGFI-B (nur77), are transcription factors that proceed to activate other genes (2, 9). Others, such as arg3.1/arc and brain-derived neurotrophic factor, are involved in synaptic plasticity and neuronal survival, respectively (11, 26). As a result of the expression of the IEGs, delayed-response genes begin to be expressed. Examples include the vgf gene, which starts to be expressed 2 h after the start of depolarization and peaks after 6 h; and the gene for tyrosine hydroxylase, which is expressed after 6 to 24 h of stimulation (4, 23). Thus, a wide array of events begins immediately after stimulation and progresses over the course of many hours.

Stimulation of cells with various growth factors also leads to a complex cascade of events that ultimately lead to phenotypic changes. In some cases, multiple signaling cascades that convey messages independently of each other are activated. For example, it has been shown that neurotrophins transmit their prosurvival signals to the nucleus by way of the Akt/PKB signaling cascade, while proliferation and differentiation instructions are mediated via the Ras/mitogen-activated protein kinase pathways (5, 7). In other cases, multiple signaling cascades that act to either antagonize or amplify each other’s activities via cross talk are activated (27). In a third paradigm of signal transduction, the temporal pattern of activity of a single cascade has been shown to confer different messages to the cell. For example, there is considerable evidence supporting the hypothesis that sustained activation of Ras/MAP kinase leads to growth arrest and differentiation of PC12 cells, while transient activation of this same pathway gives a moderately proliferative stimulus to the cells (20; reviewed in reference 17). The former pattern of activation results from nerve growth factor (NGF) treatment, and the latter pattern results from epidermal growth factor (EGF) treatment of PC12 cells.

Taking the experimental observations of intracellular signal-
ing together, it is clear that the immediate effects of many extracellular stimuli, including depolarization, are dynamic and transient and that the precise timing of this evolution is an important, instructive influence. Motivated by these observations in the present study, we thus characterized in detail the temporal signature of intracellular signaling activity in response to membrane depolarization. Focusing on the neuron-like cell line PC12, we characterized the time courses of ERK and CREB activation and deactivation following chronic and short-term depolarization. Furthermore, we wanted to determine whether cascade activity could be maintained over long periods of time using repetitive stimulation. We thus characterized the activities of ERK and CREB in response to a second depolarizing event that occurred after a variable recovery period. The significance of pulsatile stimulation is discussed in the context of stimulus-specific responses of cells.

MATERIALS AND METHODS

**Cell culture.** Rat pheochromocytoma PC12 cells (American Type Culture Collection) were cultured in complete Dulbecco’s modified Eagle’s medium (cDMEM), which consists of DMEM supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 100 U of penicillin per ml, and 100 U of streptomycin per ml (all components from Gibco BRL). Cells were maintained in a humidified atmosphere of 5% CO₂ and 37°C. Cells were plated at a density of 10⁵ cells/cm² on poly- l-ornithine (PLO)-coated tissue culture plates (Sigma). Cells were fed cDMEM every 5 days and passed every 10 days with 0.05% trypsin-EDTA to detach adherent cells (Gibco BRL). Cells were not used in experiments after 20 passages.

**Stimulation for assessing protein phosphorylation.** For stimulation experiments where the phosphorylation level was determined by Western blotting, cells were seeded 24 to 48 h before experiments on 35-mm-diameter PLO-coated dishes at a density of 10⁶ cells/cm². Prior to stimulation, cells were maintained in serum-free DMEM for 3 h to remove any artifacts of protein phosphorylation due to serum factors. Cells were depolarized by adding an equal volume of high-potassium (HiK) solution (135 mM KCl, 30 mM D-glucose, 1 mM MgCl₂, 2 mM CaCl₂, 40 mM NaHCO₃, 1 mM Na₂HPO₄; all components cell culture grade from Sigma) to the cells in DMEM to give a final KCl concentration of 70 mM. In experiments where cells were returned to a polarized state to measure the dephosphorylation rate in the absence of elevated intracellular calcium concentration ([Ca²⁺]ₗ), the HiK solution was removed by washing the cells with PBS. The cells were then centrifuged at 8,000 × g for 5 min, and the cell lysate was kept on ice and sonicated to shear the DNA. The lysates were thawed and boiled once more for Western blots. Cell lysates were separated on 7.5% precast Tris-glycine acrylamide gels (Bio-Rad) by electrophoresis. For analysis of CREB phosphorylation, 5 µg of total protein was used in each lane. For analysis of phosphorylated ERK (pERK), 7 µg of total protein was used per lane. Proteins were transferred from the acrylamide gels to Immobilon-P polyvinylidene difluoride membranes (Millipore) at 300 mA for 70 min in transfer buffer (20% methanol, 25 mM Tris, 192 mM glycine; Millipore). Membranes were blocked for 4 h at room temperature in PBS containing 5% nonfat milk and 0.05% Tween 20. Membranes were then exposed overnight to primary antibody in fresh blocking solution at 4°C. Free primary antibody was removed with two successive 10-min washes in blocking solution, followed by two successive 15-min washes in PBS containing 0.05% Tween 20. Horseradish peroxidase (HRP)-conjugated secondary antibody was then added in fresh blocking solution. Excess secondary antibody was removed by the same wash protocol used for removal of primary antibody. Membranes were then rinsed several times in PBS, and the presence of immobilized HRP was detected on Hyperfilm ECL film (Amersham) using enhanced chemiluminescence (ECL; Amersham Pharmacon). Images were digitized using a transparency scanner at 600 dots per inch. The optical density (OD) of each lane was calculated using Scion Image 4.02 software, with the GelPlot macro installed. In most cases, the OD counts of the lanes of interest were divided by the OD count from the positive control. In this manner, OD counts for the same experimental conditions could be pooled from different Western blots, and semiquantitative comparisons between different experimental conditions could be made. All reported values are averages of four separate measurements. In the case of Fig. 4C, the OD counts from cells stimulated twice were reported relative to the signal from cells stimulated once.

The following antibodies were used. To detect phosphorylated CREB (pCREB), polyclonal rabbit immunoglobulin G (IgG) against rat CREB in which Ser-133 was phosphorylated (catalog no. 06-519; Upstate Biotechnology) was used at a concentration of 1:3,000. To detect the whole CREB molecule, polyclonal rabbit IgG (catalog no. 9192; New England Biolabs) was used at a concentration of 1:2,000. pERK was detected with mouse monoclonal IgG raised against the phosphorylated tyrosine/phosphorylated threonine peptide analog of p42ERK and p44ERK (catalog no. 05-481; Upstate Biotechnology) at a concentration of 1:2,000. HRP-conjugated secondary antibodies against mouse and rabbit IgG were purchased from Santa Cruz Biotechnology and used at a dilution of 1:4,000.

**Calcium imaging.** Relative changes in calcium concentration during depolarization and repolarization of PC12 cells were monitored with a single-wavelength fluorescent dye, Fluo-4 (Molecular Probes). The quantum yield of Fluo-4 increases approximately 10-fold when the concentration of free calcium ([Ca²⁺]ₗ) is increased from 50 nM to 1 µM, which is the range over which the cytoplasmic concentration of calcium changes during depolarization (13, 19).

Several days prior to imaging, cells were seeded on number zero glass coverslips (Carolina Sciences) that had been pretreated with PLO and mouse laminin (Gibco BRL). The cells were maintained at 37°C and 5% CO₂ in cDMEM until the day of the experiment, at which time the coverslips were transferred to a new dish containing a mixture of 4 µM Fluo-4AM and Pluronic (Molecular Probes) in Tyrode’s solution (125 mM NaCl, 5 mM KCl, 30 mM glucose, 25 mM HEPES, 2 mM CaCl₂, 1 mM MgCl₂ [pH 7.4]; 300 mOsm). Cells were loaded with dye for 40 min at 37°C, after which they were washed three times with Tyrode’s solution, and then left for 10 min in Tyrode’s solution at room temperature before imaging. The coverslip containing Fluo-4-loaded PC12 cells was transferred to a microscope stage (Warner Instruments) containing an imaging chamber that allowed continuous flow of solution. In all cases, the chamber initially contained room temperature Tyrode’s solution.

Imaging was performed with an epifluorescence microscope (Zeiss) equipped with a charge-coupled device (CCD) camera for image collection and a mercury arc lamp for fluoro excitation. Prior to imaging the cells, the gain of the CCD camera was set to use a solution of 1 µM Fluo-4 pentapotassium salt (F4-K5) (Molecular Probes) in Tyrode’s solution containing 0.1% Pluronic acid. This calibration procedure ensured a large response of the camera over the range of [Ca²⁺] variations during the experiment. The exposure of the cells to the exci-
tation beam was limited to 100 ms per time point using a computer-controlled shutter assembly to minimize photobleaching. The total number of images collected during a single time series did not exceed 100 images in any of the experiments.

Fluorescence changes in individual cells during each depolarization and washing procedure were collected and plotted after correcting for the effects of bleaching. From calibration experiments, it was found that the change in fluorescence, \( F - F_0 \), where \( F \) is fluorescence and \( F_0 \) is the baseline fluorescence of the cells in Tyrode’s solution prior to the start of the experiment, was linearly proportional to the logarithm of the ratio of free calcium ions, \( \log([Ca^{2+}]/[Ca^{2+}]) \), where \( [Ca^{2+}] \) is the baseline \( [Ca^{2+}] \) prior to the start of the experiment over the range of \( Ca^{2+} \) fluctuations in these experiments. However, the ratio of proportionality depended strongly on the concentration of Fluo4 used, and the uptake of the Fluo4 was heterogeneous in the cell population. Consequently, we could not accurately calculate the \( [Ca^{2+}]/[Ca^{2+}])_0 \) ratio, and the data were reported as \( F - F_0 \).

RESULTS

Phosphorylation of ERK and CREB during chronic depolarization. Previous studies have shown that chronic depolarization of PC12 cells elicits both immediate and delayed responses from activation of intracellular signaling molecules and transcription of IEGs to expression of the neuronal differentiation-specific delayed-response gene vgf (10, 21, 23). Therefore, first we wanted to determine the length of time the ERKs and CREB remained phosphorylated during chronic depolarization.

PC12 cells were depolarized with 70 mM KCl and the phosphorylation of p42ERK, p44ERK, and CREB were monitored over the course of 2 h by using Western blots. As shown in Fig. 1A, the level of phosphorylation of the pERKs peaked within the first 5 min of depolarization and then decreased to a low and stable level that was maintained for up to 2 h of depolarization. In addition, the peak level of pERK after membrane depolarization was lower than the peak level after stimulation with EGF by a factor of approximately 3 to 10, in agreement with previously reported results (22, 29). Similarly, the phosphorylation of CREB at serine 133 (pCREB) peaked within the first 2 to 5 min of depolarization (Fig. 1B). However, in contrast to the response of ERK, pCREB levels decreased by only a factor of 2 and were maintained at this intermediate level for up to 60 min of depolarization. Also, the peak level of pCREB after depolarization was lower (by a factor of 2) than that after forskolin treatment and approximately an order of magnitude greater than after treatment with EGF or NGF, in agreement with previous results (12). OD measurements of pERK and pCREB versus time are plotted in Fig. 1C.

We next wanted to confirm that activation of the signal transduction cascades was due to the influx of calcium ions. Cells were treated with nifedipine, a selective inhibitor of L-type voltage gated calcium channels, prior to depolarization. As shown in Fig. 1D, nifedipine effectively eliminated induction of pCREB, in agreement with previous reports (21). Interestingly, pCREB was reduced, though not completely eliminated, when cells were pretreated with the CaM antagonist W-13. This latter observation indicates that one or more CaM-independent pathways are partly responsible for activation of CREB. These latter pathways are also independent of the MAP kinase family, since both we and others have observed that W-13 completely eliminates the activation of ERK following membrane depolarization (8; A. H. Nashat and R. Langer, unpublished observations).

Effects of short periods of depolarization on activation and deactivation of ERK. Since chronic depolarization led to only transient activity of ERK, we wanted to determine whether a short depolarizing event was sufficient to activate the signal transduction cascades. Cells were depolarized for either 1 or 5 min and then immediately placed in physiological (repolarizing) solution. The levels of pERK were measured at various times after the end of the depolarizing pulse. As shown in Fig. 2A, the levels of pERK after 1- and 5-min periods of depolarization were approximately the same. To obtain an estimate of the dephosphorylation rate, we plotted OD measurements of

FIG. 1. ERK and CREB activation during chronic depolarization. (A) Western blot of pERK during chronic depolarization for various durations from 2 to 60 min (m) with 70 mM KCl. For controls, pERK was stimulated with 30 ng of EGF per ml for 10 min (as a positive control) and with Tyrode’s solution (Tyr) for 5 min (as a negative control). (B) Western blot of pCREB protein during chronic depolarization for various durations from 2 to 120 min (m) with 70 mM KCl. For controls, pCREB was stimulated with 10 \( \mu \)M forskolin (Fk) for 10 min (as a positive control) and with Tyrode’s solution (Tyr) for 5 min (as a negative control). (C) OD counts from Western blots versus duration of depolarization. Phosphorylation values are relative to the value for the positive control (PC) for each Western blot (n = 4 for each measurement). (D) Western blot of pCREB protein after treatment with inhibitors. Cells depolarized for 3 min with 70 mM KCl (-) and cells pretreated with 5 \( \mu \)M nifedipine (Nif) and 70 \( \mu \)M W-13 prior to depolarization are shown. For controls, pCREB was stimulated with 10 \( \mu \)M forskolin (Fk) for 10 min (as a positive control) and with Tyrode’s solution (Tyr) for 5 min (as a negative control). The position of the 46-kDa molecular mass marker is indicated to the right of the blots.
pERK as a function of time after repolarization. From the plot of Fig. 2B, we observed that, at the level of sensitivity and time scale of our measurements, the rate of dephosphorylation (estimated as the inverse of the time to full dephosphorylation from the peak level) is unaffected by the duration of depolarization. Furthermore, the estimated dephosphorylation rates from Fig. 2B are equivalent to the rate of dephosphorylation from the peak level during chronic depolarization (estimated from Fig. 1C).

**Effect of recovery period on the reactivation of ERK with two pulses of depolarization.** Since a short period of depolarization induced the same rise to peak and decay of pERK as did chronic depolarization, we wanted to test whether the ERKs could be reactivated by short periods of depolarization that were separated by a recovery period in the polarized state. As a first step, we varied the length of the recovery period and determined the extent of reactivation. As shown in Fig. 3A, for a stimulus duration of 5 min and recovery periods from 30 to 100 min in length, pERK increased after as little as a 10-min recovery period, although reactivation was qualitatively lower than a single stimulation or a second stimulation after 30 or 60 min of recovery. In contrast, CREB phosphorylation was unaffected by shortening the duration of the recovery period; at all three time points, pCREB levels were 80% as high after the second pulse as after the first (Fig. 4B; OD plotted in Fig. 4C). Thus, both ERK and CREB were reactivated in a pulsatile fashion at interpulse intervals of 10 min or more.

**Effect of duration of depolarization on the reactivation of MAP kinases with two pulses of depolarization.** To further characterize the relationship between depolarization and recovery, we varied the duration of depolarization in two succes-
sive pulses while keeping the recovery period constant. Cells were depolarized by two pulses of KCl that lasted 1, 2, or 5 min each, with a fixed recovery period of 30 min in all cases. The phosphorylation of ERK and CREB was measured at the end of the second depolarization period. As shown in Fig. 5A, pERK was highest following a 2-min pulse duration; ERK activity was qualitatively lower after 1- and 5-min depolarization pulses than after 2-min pulse. In contrast, pCREB levels were equally high after two 1- or 2-min pulses and lower with a 5-min pulse (Fig. 5B). Thus, while there are qualitative differences, pulses from 1 to 5 min in duration are all effective at reactivating ERK and CREB following 30-min recovery periods.

Free Ca\(^{2+}\) concentrations during short periods of depolarization. To better characterize the use of novel depolarization protocols to activate intracellular signaling cascades, we monitored changes in cytoplasmic free Ca\(^{2+}\) during various depolarization protocols using a single-wavelength Ca\(^{2+}\) dye. The fluorescence from individual cells was imaged, and changes in fluorescence intensity were plotted as a function of time. As shown in Fig. 6A, in all cells studied, \([\text{Ca}^{2+}]_i\) peaked within the first 10 min of chronic depolarization and then decreased to low levels that were maintained for at least 90 min. In contrast, when cells were exposed to depolarizing 70 mM KCl for 5 min and then switched back to repolarizing Tyrode’s solution, \([\text{Ca}^{2+}]_i\) remained elevated throughout the initial depolarizing pulse and then fell to the baseline (initial) level within 60 s of repolarization (Fig. 6B). When 70 mM KCl was reintroduced after a 30-min recovery period, \([\text{Ca}^{2+}]_i\) increased a second time in all cells that were studied, although not to the same level as for the first depolarizing pulse. Similarly, a 1-min exposure to 70 mM KCl led to a sharp increase in \([\text{Ca}^{2+}]_i\), and a subsequent decrease to the baseline level upon repolarization (Fig. 6C). However, a second exposure to depolarizing KCl, after a 30-min recovery period, led to an increase in \([\text{Ca}^{2+}]_i\), almost equivalent to the first peak (Fig. 6D). Thus, comparing panels B and D of Fig. 6, PC12 cells responded more effectively to multiple depolarizing stimuli when the duration of depolarization was shorter.

**DISCUSSION**

Transient activity of ERK and CREB. These findings show that in PC12 cells, the activation of MAP kinase family member ERK is transient in response to chronic membrane depolarization. These results are consistent with previous observations in PC12 cells (22) and in vivo (24). Furthermore, studies of another calcium-activated messenger, CaM kinase I, in PC12 cells, also showed a peak in activity within the first 10 min of depolarization and a subsequent drop to intermediate levels that were maintained for up to 60 min (1).

These results showed that, in contrast to the full deactivation of ERK, phosphorylation of CREB peaked and then was maintained at intermediate levels for up to 60 min. In a previous study, chronic depolarization of PC12 cells led to elevated levels of pCREB after 5, 20, and 45 min, but not after 1, 2, or 4 h (25), in agreement with our results. Other studies also...
highlight the transient activation of CREB following depolarization of PC12 cells (3, 22, 28). However, the transient activation of CREB might be cell type specific, as a recent study in primary sensory neurons found that CREB phosphorylation did not decrease at all after more than 2 h of depolarization in cultures of primary sensory neurons (4).

Since several messenger pathways can activate CREB (16), pathways other than MAP kinase may remain maximally or partially active and thus maintain intermediate CREB activity during prolonged depolarization. Two observations from the present study support this hypothesis. First, depolarization induced CREB phosphorylation much more efficiently than ERK phosphorylation, as can be seen by comparing the levels of pCREB and pERK following depolarization to the levels following EGF stimulation. Since ERK lies upstream of CREB, a large portion of the CREB activation during depolarization must arise from one of the other Ca²⁺-sensitive pathways. Second, the CaM inhibitor, W-13, only partially inhibited CREB phosphorylation. However, it has been shown that pretreatment with W-13 completely eliminates activation of Erk1 and Erk2 (8; unpublished observations). Taken together, these results indicate that depolarization activates signaling pathways distinct from MAP kinase and that at least some of these pathways are CaM independent.

Along these lines, the signaling pathways PKA and CaM kinase have both been shown to induce CREB phosphorylation and to be activated by calcium entry into the cell (15). In a recent study of hippocampal neurons, the MAP kinase path-

![FIG. 6. Free cytoplasmic Ca²⁺ changes during depolarization protocols. (A) Fluorescence change versus time during chronic depolarization for 90 min. Each data point was the average of six cells. (B) Fluorescence change versus time during a 5-min pulse of depolarizing KCl, followed by a 30-min recovery period in Tyrode’s solution, and then a second 5-min pulse of depolarizing KCl. Each data point was the average of five cells. (C) Fluorescence versus time during a single 1-min pulse of depolarizing KCl. Each data point was the average of six cells. (D) Fluorescence versus time when the first pulse of panel C was followed by a 30-min recovery in Tyrode’s solution and then a second 1-min pulse of depolarizing KCl. Each data point was the average of six cells.](http://mcb.asm.org/)

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way was shown to contribute to the later stages of CREB phosphorylation (approximately 60 min after a 3-min pulse of KCl), while the CaM kinase pathway was the dominant contributor to phosphorylation of CREB in the first 10 min following depolarization (28). In the present study, we have found that CREB activity mirrors ERK activity in some cases (during the early minutes of depolarization and during pulsatile re-stimulation), but not in others (during the later stages of chronic depolarization).

Interestingly, the activity of CREB may also be under the influence of several dephosphorylating factors. In a recent study, it was found that electrical stimulation with 0.20-ms pulses of depolarization delivered continuously at 5 Hz, rather than chronic depolarization, maintained CREB phosphorylation for only 1 h (4). The researchers suggested that several phosphatases respond differentially to the patterns of stimulation and thus give diverse responses. Similarly, it was observed in hippocampal neurons that calcineurin, a negative regulator of CREB activity, was modulated by different patterns of stimulation (3). When the duration of stimulation was increased from 18 to 180 s, CREB activity was prolonged as a result of the inactivation of calcineurin at longer stimulation times.

In the present study, dephosphorylation of the MAP kinases was found to be unaffected by the duration of depolarization. In all three depolarization protocols (chronic or 1- or 5-min pulses), the rates at which pERK decreased from the peak level were equivalent within the accuracy of our measurements. Since the levels of [Ca\(^{2+}\)] were different during the dephosphorylation time period in all three protocols (comparing panels in Fig. 6), it does not appear that the rate of dephosphorylation is strongly affected by the instantaneous level of [Ca\(^{2+}\)], or by the transmembrane voltage. However, it is possible that phosphatase activity is a function of peak [Ca\(^{2+}\)], which was the same in all three protocols we studied, and that this change in activity is maintained throughout dephosphorylation.

**Pulsatile activation of the MAP kinases and CREB.** While [Ca\(^{2+}\)], and ERK phosphorylation decrease during chronic depolarization, the present study shows that if duration of stimulation is short and is followed by a sufficiently long recovery period in a polarized state, both ERK and CREB can be successfully reactivated as soon as 10 min after the first depolarization event. Although the [Ca\(^{2+}\)] measurements showed some effects of buffering with 5-min pulses separated by a 30-min interval, there was no statistically significant effect on ERK or CREB phosphorylation. Therefore, patterns of stimulation where electrical activity occurs repetitively for short periods of time might be a mechanism to maintain CREB activity over long periods of time.

In the present study, measurements of Ca\(^{2+}\)-induced fluorescence showed that [Ca\(^{2+}\)] increased only transiently in response to chronic depolarization but could be raised in a pulsatile fashion if depolarization occurred for only a short period of time. Similarly, in a study of the effect of the A2A adenosine receptor, Park and colleagues showed that [Ca\(^{2+}\)] could be raised by two successive 30-s periods of chronic depolarization if the cells were allowed to rest for 10 min between pulses (19). Combining our results with the results of Park and coworkers, it is clear that [Ca\(^{2+}\)], can be reliably increased by multiple, short periods of depolarization (1 min or less) if sufficient (10 min or more) time is allowed between stimuli for recuperation. Furthermore, taken together with the measurements of phosphorylation, the calcium-imaging measurements suggest that the parameters that affect successful reactivation of signal transduction are the durations of depolarization and interpulse recovery.

The transient activities of ERK and CREB can lead to complex mechanisms of gene regulation by a given cellular stimulus. For example, VGF is a protein that is secreted from vesicles of PC12 cells following NGF-induced differentiation of these cells. Expression of vgf is Ras and MAP kinase dependent (6). Interestingly, the vgf promoter requires both activated CREB transcription factor and transcription factor NGFI-A. The gene encoding NGFI-A is an IEG that is expressed as a result of CREB activation. Thus, it would be predicted that expression of the vgf gene will require CREB activity for a length of time that is sufficient for transcription and translation of NGFI-A. Consistent with the above model for vgf transcription, NGF induces robust expression of vgf, while EGF does not, presumably due to the transient induction of CREB activity by EGF. Depolarization also induces expression of vgf, beginning 2 h after depolarization, peaking after 6 h, and still maintained 12 h after the onset of depolarization (23). Thus, there must be sufficient CREB activity, even at the intermediate levels which exist after 60 min or more of chronic depolarization, to drive transcription of this gene.

In the present study, we have observed that pulsatile depolarization can induce well-defined, maximal increases in pCREB. It will be interesting to determine whether short pulses of depolarization can be timed in such a way that the first pulse induces expression of NGFI-A and the second pulse coincides with the presence of NGFI-A protein in the nucleus. In theory, two well-timed pulses should induce stronger vgf expression than chronic depolarization, given that pCREB levels will be higher when needed for induction of the second gene (shown schematically in Fig. 7). If the model proposed by D’Arcangelo and coworkers (6) for the regulation of vgf represents a general paradigm for gene regulation, then it is possible that a number of genes will respond to specific patterns of electrical stimulation.

![FIG. 7. Schematic of gene regulation requiring sustained or pulsatile activation of a Ca\(^{2+}\)-activated transcription factor. The first Ca\(^{2+}\) transient rise activates a transcription factor, such as CREB, that regulates expression of a second transcription factor, NGFI-A (P1). P1 acts at the promoter of a second protein, VGF (P2), whose expression induces delayed, phenotypic cellular changes. However, expression of P2 requires both functional P1 protein and Ca\(^{2+}\)-activated transcription factor. Thus, delayed cellular changes occur only as a result of a second Ca\(^{2+}\) transient rise.](http://mcn.asm.org/)
Conclusions. Electrical stimulation of neurons, as a result of Ca\(^{2+}\) entry into the cytoplasm, induces many cellular changes that occur from minutes to hours or even days after stimulation. Many of the downstream effects of Ca\(^{2+}\) influx have been uncovered in previous research. In the present study, we focus specifically on the transient nature of activation of intracellular signal transduction following depolarization. During chronic depolarization, phosphorylation of the MAP kinase ERK was transient, peaking and falling to low levels within 10 min of depolarization. Short periods of depolarization, from 1 to 5 min in duration, also successfully phosphorylated ERK. In addition, the rate of deactivation of ERK was not affected by the duration of depolarization. Phosphorylation of the transcription factor CREB also peaked as a result of chronic depolarization but decreased to intermediate levels that were maintained for more than 1 h. Phosphorylation of both ERK and CREB was reinduced by a second period of depolarization after a recovery period of 10 min or more. The effects of the durations of depolarization and interpulse recovery on reactivation of ERK and CREB were characterized. Measurements of free cytoplasmic Ca\(^{2+}\) confirmed the transient rise in [Ca\(^{2+}\)]\(_i\) during chronic depolarization and the pulsatile increase in [Ca\(^{2+}\)]\(_i\) that can be achieved with short periods of depolarization. The present study suggests that pulsatile activity may be a means of maintaining signaling activity over long periods of time.

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