BDNF-Induced Potentiation of Spontaneous Twitching in Innervated Myocytes Requires Calcium Release From Intracellular Stores

ROBIN J. KLEIMAN,1 NING TIAN,2 DAVID KRIZAJ,1 THOMAS N. HWANG,1 DAVID R. COPENHAGEN,1,2 AND LOUIS F. REICHARDT1,3
1Department of Physiology, 2Department of Ophthalmology, and 3Howard Hughes Medical Institute, University of California, San Francisco, California 94143-0723

Received 8 December 1999; accepted in final form 5 April 2000

Kleiman, Robin J., Ning Tian, David Krizaj, Thomas N. Hwang, David R. Copenhagen, and Louis F. Reichardt. BDNF-induced potentiation of spontaneous twitching in innervated myocytes requires calcium release from intracellular stores. J Neurophysiol 84: 472–483, 2000. Brain-derived neurotrophic factor (BDNF) can potentiate synaptic release at newly developed frog neuromuscular junctions. Although this potentiation depends on extracellular Ca2+ and reflects changes in acetylcholine release, little is known about the intracellular transduction or calcium signaling pathways. We have developed a video assay for neurotrophin-induced potentiation of myocyte twitching as a measure of potentiation of synaptic activity. We use this assay to show that BDNF-induced synaptic potentiation is not blocked by cadmium, indicating that Ca2+- influx through voltage-gated Ca2+- channels is not required. TrkB autophosphorylation is not blocked in Ca2+-free conditions, indicating that TrkB activity is not Ca2+- dependent. Additionally, an inhibitor of phospholipase C interferes with BDNF-induced potentiation. These results suggest that activation of the TrkB receptor activates phospholipase C to initiate intracellular Ca2+ release from stores which subsequently potentiates transmitter release.

INTRODUCTION

Neurotrophins play pivotal roles in acute and long-term changes in synaptic plasticity. Acute potentiation of synaptic strength by neurotrophins is accomplished by increasing neurotransmitter release (Kang and Schuman 1995; Li et al. 1998; Lohof et al. 1993; Sala et al. 1998) and by modulation of neurotransmitter receptor sensitivity and ion channel conductance (Holm et al. 1997; Levine et al. 1995, 1998). Neurotrophins and their receptors can be up-regulated in response to activity (Merlio et al. 1993; Schmidt-Kastner et al. 1996; Shieh et al. 1998; Tao et al. 1998), and neurotrophin release can be enhanced in response to depolarization (Blöchl and Thoenen 1995; Xie et al. 1997). Experimental increases in neurotrophins produce long-lasting changes in neuronal function (Cabelli et al. 1995; Cohen-Cory and Fraser 1995; McAllister et al. 1995, 1997). Reductions in neurotrophin levels produce deficits in long-term potentiation (Patterson et al. 1996) and other forms of activity-dependent synaptic plasticity (Cabelli et al. 1997).

Bath application of brain-derived neurotrophic factor (BDNF) acutely potentiates neurotransmitter release in Xenopus motor neuron-myocyte co-cultures (Lohof et al. 1993). Potentiation does not require protein synthesis and occurs without an intact cell body but requires extracellular Ca2+ (Stoop and Poo 1995, 1996). It is not known whether the need for extracellular Ca2+ stems from a requirement for a Ca2+- influx, a Ca2+-sensitive activation of the TrkB receptor, or some other Ca2+-sensitive process on the extracellular surface of the cell. Although BDNF has been shown to produce a rise in [Ca2+]i, (Stoop and Poo 1996), the source for the rise in [Ca2+]i, triggered by BDNF has not been elucidated.

Little is known about which TrkB-linked intracellular signaling pathways are required for acute BDNF-induced synaptic potentiation. Among the signal transduction pathways known to be activated by Trk receptors are those leading to activation of MAP kinase, PI3 kinase and phospholipase Cγ (PLCγ) (reviewed in Kaplan and Miller 1997). Activation of PLCγ is one attractive candidate to mediate synaptic potentiation because its activation would result in intracellular Ca2+ release via the second messenger IP3 (Obermeier et al. 1993). Changes in cytoplasmic Ca2+ concentrations can regulate a wide variety of cellular processes, including neurotransmitter release (reviewed in Matthews 1996) and transcriptional activity (reviewed in Gallin and Greenberg 1995). To investigate the source of the Ca2+ required for BDNF-induced potentiation and whether there is an essential link between TrkB receptor activation and the PLCγ pathway, we developed a video assay for synaptic activity. We demonstrate that, although extracellular Ca2+ is required to produce BDNF-induced potentiation, a Ca2+ influx through voltage-gated Ca2+ channels is not required. We show that an inhibitor of PLC prevents BDNF-induced synaptic potentiation, suggesting an essential role for the TrkB-induced activation of PLC and subsequent release of Ca2+ from intracellular stores.

METHODS

Reagents

Recombinant human BDNF was generously provided by Amgen (Thousand Oaks, CA).

Tissue culture

Oocyte-positive female Xenopus (NASCo) were injected with 1,000 units of human chorionic gonadotropin (Sigma, St. Louis, MO; The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.)

Address for reprint requests: L. F. Reichardt, Howard Hughes Medical Institute, University of California, San Francisco, CA 94143-0723 (E-mail: lfr@cgl.ucsf.edu).
pared by dissolving 25 mg gramicidin into 500
interference of gramicidin with seal formation. The stock solution of
then back-filled with pipette solution containing gramicidin to avoid
were washed in five changes of sterile 0.1 times Ringer solution [1
the vitelline membrane was removed with fine forceps, the embryos
pared essentially as described previously (Tabti and Poo 1991). After
testes, freshly harvested from euthanized male
Xenopus
pipette solution. The final concentration of gramicidin in the pipette
conductance was monitored every 5–10 min. Recordings began after
borosilicate glass (type 7502, Garner Glass Company, Claremont,
recording pipettes using, Nomarski DIC optics. We recorded from
water immersion objective lens was used for visualizing the cells and
scope (Zeiss Axioskop, Carl Zeiss, Oberkochen, Germany). A
Xenopus
spinal motor neurons and myocytes were pre-
pared essentially as described previously (Tabti and Poo 1991). After
stimulation membrane was removed with fine forceps, the embryos
were washed in five changes of sterile 0.1 times Ringer solution [1
times Ringer (in mM): 115 NaCl, 2.6 KCl, 2 CaCl2, and 10 HEPES,
and 100 M HEPE, pH 7.6]. The embryos were then transferred to Ca2+
and Mg2+-free Ringer [CMF Ringer (in mM): 115 NaCl, 2.6 KCl, 10 HEPES, and 0.4
EDTA] where the neural tube and associated myotomal tissue was
dissected from the dorsal surface of the embryo. The epithelial layer
was removed and the tissue partially dissociated after 20–30 min in
CMF Ringer. The dissected tissue was drawn up into a finely drawn
Pasture pipette and plated onto autoclaved 22 mm \times 22 mm glass
coverslips (Gold Seal No. 1 3306), which were submerged in frog
medium [1 part Ringer solution to 1 part L-15 (GIBCO) with 1% fetal
bovine serum]. Cultures were plated at a density of three embryos per
35-mm dish. Cultures were left undisturbed for at least 30 min before
moving them into a Tupperware container lined with wet paper towel.
Cultures were grown at least 20–24 h at room temperature before use
in experiments.

Excitatory postsynaptic potential (EPSP) recordings

The culture dish was mounted on the stage of an upright micro-
scope (Zeiss Axioskop, Carl Zeiss, Oberkothen, Germany). A \times 40
water immersion objective lens was used for visualizing the cells and
recording pipettes using, Nomarski DIC optics. We recorded from
visually identified twitching myocytes. Patch pipettes were made of
borosilicate glass (type 7502, Garner Glass Company, Claremont,
CA) on a Brown-Flaming horizontal puller (model P-80/PC, Sutter
instruments, CA). The pipette-to-bath resistance ranged from 3 to 5
MΩ. The electrodes were tip-filled with a small volume (300–500 μm
in length) of gramicidin-free pipette solution (in mM: 1 NaCl, 150
KCl, 2 CaCl2, 1 MgCl2, and 10 mM Na-HEPES, pH 7.3) and were
then back-filled with pipette solution containing gramicidin to avoid
interference of gramicidin with seal formation. The stock solution of
gramicidin [gramicidin D (Dubos), Sigma, St. Louis, MO] was
prepared by dissolving 25 mg gramicidin into 500 μl dimethylsulfoxide
(DMSO). This solution was aliquoted and stored frozen. Each day
before an experiment, 2 μl of stock solution was added to 1 ml of
pipette solution. The final concentration of gramicidin in the pipette
was 100 μg/ml (0.2% DMSO). During experiments, cells were held
at –70 mV once the GΩ seal was formed. Access resistance and cell
conductance was monitored every 5–10 min. Recordings began after
the access resistance reached a stable plateau (≤50 MΩ), that gener-
al took 20–30 min. Electrode capacitance was compensated by
80–90% after the GΩ seal. Cell capacitance was not compensated.
Membrane potentials were recorded with an Axopatch 1D amplifier
(Axon Instruments, Burlington, CA) in current-clamp mode without
injection of current, and data were collected using a Macintosh-based
interface (ITC-16 Mac computer interface, Instrutech, Great Neck,
NY) run by HEKA software (Pulse + PulseFit, HEKA Elektronik
GmbH, Germany). The signals were filtered at 1 kHz. The movement
of recorded myocytes was imaged simultaneously.

Twitching recordings

Neuron–myocyte cultures were imaged on either an inverted Nikon
microscope or an Axioptix microscope with a \times 40 objective. A
charge-coupled device (CCD; Hamamatsu C2400) camera was used
to transmit the images to a VCR, which recorded cell movement
in real time. Videotapes were played back into a PC-compatible com-
puter (Dell Optiplex GXMT5166) and were analyzed using a program
called “TWITCH,” which utilizes a frame-grabber to measure
changes in pixel intensities to detect cell movement. The digitized
data were downloaded into the software program Igor Pro (Wave-
metrics, Portland, OR). TWITCH was used to monitor up to nine
separate regions of the screen simultaneously. A macro was written
for Igor Pro that counted the twitching events and plotted frequency
histograms of the data. The threshold for detecting a twitch was set at
twice the magnitude of the mean to average peak amplitude of the
noise.

To determine the fold change in frequency of muscle cell move-
ment in response to BDNF, we plotted frequency histograms of each
experiment using 5-min binwidths. The average frequency per 5 min
was determined during the control recording period by dividing the
total number of twitching events during the control period by
the number of 5-min intervals recorded. The peak frequency following
BDNF application was determined from frequency histograms of each
experiment using a 5-min binwidth. The fold change in frequency
was determined by comparing the average frequency per 5 min before
BDNF application to the peak frequency after BDNF application.
The fold change in frequency was calculated by (PF – ACF)/ACF, where
PF is the peak frequency after treatment and ACF is the average
control frequency.

BDNF and other drugs were introduced into the cultures as 2 times
solutions in frog medium. Over several minutes, half the volume of
the dish (2 ml) was slowly drawn out of the dish using a sterile 6-ml
syringe with a 18-gauge needle and attached tubing and was replaced
slowly with the BDNF and/or drug containing medium. Medium
change alone sometimes produced up to a 3.5-fold increase in the
frequency of twitching. Therefore increases in the frequency of <3.5-
fold were not considered to represent potentiation. Some recordings
were done in Ca2+-free medium (in mM: 115 NaCl, 2 MgCl2, 2.6
KCl, and 3 EGTA, 0.1% BSA and 10 mM Na-HEPES, pH 7.3).

Immunoprecipitations

PC12 cell lines that stably express the rat TrkB receptor were
grown on collagen (Vitrogen 100, diluted 1:100 in PBS; Collagen,
Fremont, CA) coated 100-mm tissue culture plates (Fisher Scientific,
Houston, CA, No. 08772E) (Ip et al. 1993). Cells were washed three
free, Mg 2+
FBS
) containing 0.18 mg/ml so-
3
PC12 cell lines that stably express the rat TrkB receptor were
grown on collagen (Vitrogen 100, diluted 1:100 in PBS; Collagen,
Fremont, CA) coated 100-mm tissue culture plates (Fisher Scientific,
Houston, CA, No. 08772E) (Ip et al. 1993). Cells were washed three
times with Dulbecco’s phosphate-buffered saline (PBS) or with Ca2+-
free, Mg2+-free Dulbecco’s PBS plus 2 mM MgCl2 and 3 mM EGTA.
Cells were incubated in PBS (with or without Ca2+) for 10 min before
adding 100 ng/ml recombinant human BDNF. After incubation of
the cells in BDNF for 10 min, cells were rinsed with PBS containing
0.018 mg/ml sodium orthovanadate, scraped, and homogenized in 500
μl of rapid immunoprecipitation assay (RIPA) buffer [50 mM NaCl,
50 mM NaF, 5 mM EDTA, 10 mM Tris, pH 7.5, 1% Triton plus 2
μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, and 100 μg/ml
phenylmethylsulfonyl fluoride (PMSF)] containing 0.18 mg/ml sodium
orthovanadate per plate. Supernatants of 10,000 g of extract with 3
l of affinity purified anti-TrkB antibody (Huang et al. 1999) and protein A coupled sepharose beads
Upstate Biotechnology, Lake Placid, NY) or
or

Upstate Biotechnology, Lake Placid, NY) or
473
BDNF-INDUCED POTENTIATION REQUIRES INTRACELLULAR Ca2+
anti-rat TrkB. Alkaline phosphatase-conjugated secondary antibodies (anti-mouse and anti-rabbit, respectively) were used with ECF substrate (Amersham-Pharmacia, Piscataway, NJ) to visualize and enable quantitation of signals on a Fuji Multiimager FLA 2000.

**Fura imaging**

Cells were loaded with (7–10 μM) Fura-2 AM (Molecular Probes, Eugene, OR) in frog culture medium for 10 min and rinsed for an additional 20 min before imaging in a laminar flow perfusion stage (Warner Instruments, Hamden, CT) on an inverted microscope equipped with a cooled CCD camera (PXL, Photometrics, Tucson, AZ) using a ×40 objective. \([\text{Ca}^{2+}]_i\), was estimated by determining the 340:380 ratio using standard techniques (Grynkiewicz et al. 1985). Background subtracted ratios were acquired every 5 s. Metafluor software (Universal Imaging, West Chester, PA) was used to control the image acquisition. \(\text{Ca}^{2+}\) levels were calibrated in motor neurons using the equation

\[
[\text{Ca}^{2+}]_i = K_d(R - R_{\text{min}})/(R_{\text{max}} - R)S_{380}/S_{340},
\]

in which \(R\) is the measured ratio, \(S_{380}/S_{340}\), are the extremes of the \(\text{Ca}^{2+}\)-bound and \(\text{Ca}^{2+}\)-free Fura-2 AM at 380 and 340 nm, respectively, and \(K_d\) is the Fura-2 dissociation constant for \(\text{Ca}^{2+}\) (224 nM). Since we did not directly determine \(K_d\), calibrated values should be considered estimates. \(R_{\text{min}}, R_{\text{max}}, S_{380}, S_{340}\) were determined by the addition of 10 μM ionomycin to the \(\text{Ca}^{2+}\)-free (0 μM \(\text{Ca}^{2+}\)-EGTA) perfusate and to Imaging Buffer with \(\text{Ca}^{2+}\) (4 mM \(\text{Ca}^{2+}\)), respectively.

**RESULTS**

**BDNF increases the frequency of spontaneous twitching in innervated myocytes**

BDNF increases the frequency of spontaneous synaptic events recorded under voltage clamp from innervated myocytes (Lohof et al. 1993; Stoop and Poo 1995, 1996; unpublished observations). Potentiation of transmitter release begins within minutes and peaks approximately 15 min after BDNF application, as evidenced by an increase in the frequency of spontaneous synaptic events (Lohof et al. 1993). Because there is no change in the amplitude of these spontaneous events, this is strong evidence that the effects of BDNF are largely on the presynaptic motor neuron. However, there is evidence that TrkB receptors on the muscle cell may also mediate additional effects postsynaptically on acetylcholine receptor function (Wang and Poo 1997).

*Xenopus* myocytes contacted in culture by motor neurons often contract spontaneously (Fig. 1). Figure 1, A and B, shows images of five myocytes as marked in Fig. 1 C. All five myocytes twitched spontaneously in this preparation. A and B show the morphology of nerve terminals (indicated by arrows). The overlay of profiles before (green) and during (red) spontaneous twitching event is shown in Fig. 1C. Open arrows indicate areas of movement. Note that only muscle cell 1 twitched in this example.
BDNF-induced potentiation requires intracellular Ca\(^{2+}\)

FIG. 2. Simultaneous recording of spontaneous twitching activity and excitatory postsynaptic potentials (EPSPs) using current-clamp recordings. A, top trace: EPSPs recorded under current clamp. Bottom trace: spontaneous twitching activity detected with Twitch video assay during the same period of time. B: the same data shown in the boxed area of A. Note that every twitching event corresponds to an EPSP. Some EPSPs do not have a corresponding twitching event, as indicated by the arrowheads. EPSPs that do not have a corresponding twitching event tend to have relatively smaller amplitudes.

of myocyte 1 captured at rest and during twitching, respectively. Figure 1C outlines the edges of the cells and reveals changes in the boundaries of myocyte 1. Unlike cardiac myocytes, cultured myocytes do not contract in the absence of contact with neurons (Peng et al. 1991; unpublished observations). Spontaneous twitching is readily observed in the presence of the sodium channel blocker TTX, suggesting that action potentials are not required to produce this activity (Fig. 3, C and D). Moreover, consistent with this conclusion, we rarely observed synchronous twitching from multiple muscle cells contacted by a single motor neuron (Fig. 1). To validate that spontaneous twitching activity resulted from presynaptically driven EPSPs, we recorded spontaneous synaptic events under current clamp and videotaped the cells simultaneously. Figure 2 illustrates that every recorded-twitching movement detected by image analysis (see METHODS) corresponded to a recorded EPSP. These dual recordings revealed that not every EPSP resulted in a twitching event. In particular, small EPSPs often failed to elicit a twitching event, while larger ones consistently produced movement. Figure 2B is an enlarged version of the data outlined in the box drawn in Fig. 2A, and the arrows denote the positions of small amplitude EPSPs that did not result in detectable twitching events. These data suggest that the frequency of spontaneous twitching events represents the frequency of a subset of EPSPs that are large enough to elicit a twitching response. Thus our image analysis computer program justifiably provides a simple assay for a synaptic activity in cultured motor neuron–myocyte pairs.

Figure 3A shows an example of a BDNF-induced increase in the frequency of spontaneous twitching of an innervated myocyte. The upward deflections show the timing of twitching events. Following 30 min in control medium, 100 ng/ml BDNF was added to the medium. An increase in the frequency of twitching was evident approximately 20 min after BDNF application and peaked after approximately 30 min. Although the timing of twitches can be determined with an accuracy of \(<100\) ms, the amplitude of these deflections is not a good indicator of the strength of contraction because the amplitude of the events varied with the placement of the detection window. Since this approach is useful for examining changes in relative frequency, but not changes in amplitude, twitching frequency histograms were used as our measure of synaptic activity. Figure 3B shows an example of the frequency histograms, which are plotted using a 5-min binwidth. The average frequency per 5 min during the control recording period was 18.6 twitches. The peak frequency per 5 min, after BDNF application was 127 twitches. This represented a 5.8-fold increase in the frequency of twitching.

We considered the twitching frequency to be potentiated by BDNF when the peak twitching frequency following the application of BDNF was more than 3.5-fold that of the control frequency. The rationale for this criterion was based on the finding that occasionally just switching media elicited an increase. The average increase for exchanging the media alone was 0.65-fold, and the largest increase we saw from exchanging the media alone was 3.5-fold. Twenty-one of 31 myocytes (68%) observed to twitching in control solution responded to BDNF with a more than 3.5-fold increase in frequency (Fig. 3C). Including all cells, the average increase in the frequency of twitching was approximately 15-fold (Fig. 3D) and peaked approximately 25 min after the BDNF application, although individual cells varied considerably with respect to the magnitude and timing of maximal potentiation. The delay between BDNF application and potentiation seemed to vary within a 5- or 10-min range from cell to cell and may vary somewhat from one batch of BDNF to another, although we did not examine this systematically.

Addition of TTX (1 μM) significantly decreased the number of cells that exhibited a potentiated twitching response in response to BDNF (11 of 28 cells; Fig. 3C). The cells that exhibited BDNF-induced potentiation in the presence of TTX did so with a similar time course to BDNF alone (not shown). Including all cells, the average increase in frequency was lower (3.3-fold in TTX plus BDNF vs. 14.9-fold in BDNF alone).

These results demonstrate that the potentiating actions of BDNF on synaptic activity are reflected in the frequency of myocyte twitching. BDNF treatment produced an increase in the frequency of twitching that paralleled the time course and magnitude of the increase in the frequency of recorded spontaneous synaptic activity as well as the percent of BDNF-responsive cells (Lohof et al. 1993; unpublished data). Therefore we used the twitching assay to investigate the signaling pathways involved in BDNF-induced potentiation.

Removal of extracellular Ca\(^{2+}\) severely retards the ability of BDNF to produce potentiated twitching

Previous studies have demonstrated a requirement for extracellular calcium to produce BDNF-induced potentiation of spontaneous synaptic currents (Stoop and Poo 1996). We were able to observe a similar requirement for extracellular calcium to produce BDNF-induced potentiation of twitching using our video assay. Removing Ca\(^{2+}\) from the medium decreased the frequency of spontaneous twitching (Fig. 4, A and B), but the cells twitched at a stable rate for at least 1 h in the absence of Ca\(^{2+}\) (not shown).
The rate of twitching was observed for 10 min in the absence of Ca\(^{2+}\) before the addition of BDNF. In most cases no BDNF-induced potentiation was seen in the absence of extracellular Ca\(^{2+}\) (16 of 18 cells; Fig. 4C). The average fold increase in the absence of Ca\(^{2+}\) was only 1-fold as compared with a increase of 15-fold in the presence of Ca\(^{2+}\) (Fig. 4D).

These results confirm that extracellular Ca\(^{2+}\) plays an important role in producing BDNF-induced potentiation of twitching activity. There are several Ca\(^{2+}\)-dependent processes that could be affected by the removal of extracellular Ca\(^{2+}\). One possibility is that influx of extracellular Ca\(^{2+}\) is required. Alternatively, extracellular Ca\(^{2+}\) is required for some step in TrkB activation, such as ligand binding or dimerization. An alternative possibility is that in the absence of extracellular calcium, internal stores of calcium are depleted by leakage, and calcium entry via store-operated calcium channels cannot replace what is lost. Thus if calcium release from internal stores is required for potentiation, partial depletion of these stores could prevent potentiated transmitter release. To evaluate these putative mechanisms we designed the following experiments.

**Voltage-gated Ca\(^{2+}\) channel blocker cadmium does not interfere with BDNF-induced potentiation of twitching**

Neurotransmitter release is known to be triggered by Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels. The divalent cation Cd\(^{2+}\) blocks Ca\(^{2+}\) entry through all types of voltage-gated Ca\(^{2+}\) channels found in this preparation (Barish 1991; reviewed in Scott et al. 1991). To evaluate the potential role of Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels in BDNF-evoked potentiation, we examined the effects of BDNF in the presence of Cd\(^{2+}\). Figure 5, A and B, shows a typical response to BDNF in the presence of Cd\(^{2+}\) ions. Addition of 100 μM Cd\(^{2+}\) to the cultures had no effect on the ability of BDNF to produce potentiation or on the time course of the potentiation observed. The example shown in Fig. 5, A and B, demonstrates that an increase in the frequency of twitching was evident between 5 and 10 min after BDNF application and peaked between 30 and 35 min after BDNF application. The average frequency in the presence of Cd\(^{2+}\) for this cell was 6 twitches per 5 min, while the peak frequency after BDNF application increased to 80 twitches per 5 min. This represented a 12.3-fold increase in the frequency of twitching. Most of the cells examined (71%) responded to BDNF with a >3.5-fold increase in the frequency. The peak frequency occurred approximately 25 min after the bath application of BDNF. C: summary of potentiation data. No cells exhibited potentiation above the criterion when the media was exchanged with fresh medium. Potentiation is defined as an increase in the frequency that is >3.5-fold of control. Sixty-eight percent of cells exhibited potentiation when 100 ng/ml BDNF was added to the bath. Thirty-nine percent of cells exhibited potentiation when 1 μM TTX and 100 ng/ml BDNF were present in the bath. D: magnitude of potentiation response. On average, the addition of BDNF resulted in a 14.9-fold increase in the frequency. This was a significant increase compared with exchanging the media with fresh medium (P < 0.01). In the presence of TTX the addition of BDNF resulted, on average, in a 3.3-fold increase in the frequency. This was significantly more than changing media alone (0.65-fold average; P < 0.01), but was significantly less than adding BDNF alone (P < 0.05).
no significant inhibitory effect, it seemed possible that the TrkB receptor is sensitive to Ca\(^{2+}\) and unable to signal in its absence (Zhou et al. 1997). To evaluate this possibility, tyrosine phosphorylation of the TrkB receptor was quantified in response to BDNF in the presence and absence of Ca\(^{2+}\), using a PC12 cell line expressing TrkB receptors. Figure 6 shows a western blot with an antibody to phosphotyrosine of protein immunoprecipitated with antibodies directed against TrkB, except for lane 1, which did not include any antibody in the precipitation step. In response to 100 ng/ml BDNF, tyrosine phosphorylation of TrkB was observed in the presence and absence of extracellular Ca\(^{2+}\) (lanes 3 and 5 of Fig. 6), demonstrating that extracellular Ca\(^{2+}\) is not essential for ligand-dependent activation of TrkB.

A shows the same blot in B after it was stripped and blotted with antibodies to TrkB. C shows graphically that quantitative estimates of the amount of tyrosine phosphorylation of TrkB in response to BDNF were the same in the presence and absence of extracellular Ca\(^{2+}\).

**Inhibitor of PLC prevents BDNF-induced potentiation of twitching**

There does not seem to be a requirement for extracellular calcium to influx via voltage-gated calcium channels or to enable the Trk receptor to signal. One of the most parsimonious explanations for the inability of BDNF to produce a potentiated twitching response in the absence of extracellular calcium is a secondary depletion of intracellular stores of calcium that are required either for the potentiation of transmitter release or for the expression of the potentiated twitching by the muscle cell. Next, we investigated the possibility that TrkB activation regulates synaptic transmission via actions on an intracellular Ca\(^{2+}\) signaling pathway.

Neurotrophin receptors are capable of activating PLC\(\gamma\), which hydrolyses PIP\(_2\) to generate DAG and IP3. IP3 in turn, interacts with IP3 receptors present on endoplasmic reticulum (ER) membrane to release Ca\(^{2+}\) into the cytoplasm. An IP3-induced rise in the cytoplasmic concentration of Ca\(^{2+}\) could play an important role in producing the BDNF-induced potentiation of synaptic activity. To evaluate the potential contribution of PLC activity to the production of BDNF-induced potentiation, we examined the effects of a specific inhibitor of PLC (Smith et al. 1990). Treatment of cells with 5 \(\mu\)M of the PLC inhibitor U73122 (Calbiochem, San Diego, CA) prevented potentiation of the twitching response by BDNF in 10 of 10 cells examined (Fig. 7, A, B, D, and E). The average increase in response to BDNF was 0.65-fold in the presence of U73122 as compared with 14.9-fold in BDNF alone (Fig. 7E). To test whether the myocytes’ ability to twitch had been impaired by the U73122 treatment, 300 \(\mu\)M ATP was added to
U73122-treated myocytes. A significant increase in the frequency of twitching was observable in response to ATP treatment in 7 of 10 cells examined (Fig. 7C). This was consistent with previous reports that ATP potentiates the frequency of spontaneous synaptic currents in innervated *Xenopus* myocytes (Fu and Poo 1991) and suggests that the postsynaptic cell is capable of expressing a potentiated response in the presence of this PLC inhibitor. The severe disruption of BDNF-induced potentiation of the twitching response by U73122 suggests that activation of PLC is essential for potentiation.

**BDNF induces a rise in intracellular Ca$^{2+}$ concentrations that can be blocked by inhibitors of intracellular stores**

To examine the potential role of PLC-dependent Ca$^{2+}$ release from intracellular stores using a second approach, Fura-2 imaging was used to monitor BDNF-induced changes in intracellular Ca$^{2+}$ concentrations in the presence and absence of drugs that deplete intracellular stores. First, the effect of BDNF on intracellular Ca$^{2+}$ concentrations was determined. Every neuron examined (7 of 7) exhibited an increase in intracellular Ca$^{2+}$ with a time course that approximates that of the increase in spontaneous twitching. Before application of BDNF, the mean resting concentration of intracellular Ca$^{2+}$ was 71 ± 18 nM (mean ± SE, n = 7). This increased to 258 ± 40 nM (n = 7) between 20 and 30 min following the BDNF application. In some cases, BDNF application was followed by very large transient increases in intracellular Ca$^{2+}$ concentration superimposed on the already elevated intracellular Ca$^{2+}$ concentration (Fig. 8A). Myocytes did not exhibit any rise in [Ca$^{2+}$]$_i$ in response to BDNF treatment.

Our twitching data suggest that since a Ca$^{2+}$ influx is not required and inhibitors of PLC prevent the potentiation, the increase in intracellular Ca$^{2+}$ that we observe in response to BDNF application is likely to be due to release from intracellular stores. To test this possibility further, cultures were preincubated for 3 h with a mixture of thapsigargin (2 µM) and cyclopiazonic acid (CPA; 5 µM) to deplete the intracellular stores of Ca$^{2+}$. Pretreatment of neurons with these drugs prevented any significant increase in the cytoplasmic calcium concentration in response to 100 ng/ml BDNF application (n = 4). The basal cytoplasmic concentration of Ca$^{2+}$ in these cells was 53 ± 24 nM. Following BDNF application the basal Ca$^{2+}$ concentration was 69 ± 17 nM (n = 3; Fig. 8C). These results argue strongly for an essential BDNF-induced rise in intracellular Ca$^{2+}$ from intracellular stores.
BDNF-induced potentiation requires intracellular Ca\(^{2+}\)

Our results with the PLC inhibitor suggest that this rise in \([Ca^{2+}]_i\) is mediated by a PLC-mediated pathway.

**DISCUSSION**

**BDNF-induced potentiation is mediated by a PLC-sensitive pathway**

Our studies suggest that PLC-regulated Ca\(^{2+}\) stores play a critical role in the induction of BDNF-induced potentiation.

Figure 9 shows a schematic diagram of our proposed BDNF-evoked signaling pathway. We confirm previous observations that extracellular Ca\(^{2+}\) is required for BDNF-induced potentiation (Stoop and Poo 1996). Our data show, however, that the voltage-gated Ca\(^{2+}\) channel blocker Cd\(^{2+}\) had no effect on the BDNF-induced potentiation, suggesting very strongly that Ca\(^{2+}\) influx, at least through voltage-gated Ca\(^{2+}\) channels, is not required to produce potentiation. This is very similar to what has been described for BDNF-induced potentiation in cultured hippocampal neurons (Li et al. 1998). The somewhat paradoxical requirement for extracellular Ca\(^{2+}\), but not for Ca\(^{2+}\) influx can be explained either by a Ca\(^{2+}\) requirement for the Trk receptor to signal, or by a requirement for a secondary calcium influx, perhaps through store-operated channels to replenish intracellular calcium stores. We examined the former possibility by looking at BDNF-induced tyrosine phosphorylation of the TrkB receptor in the presence and absence of extracellular Ca\(^{2+}\) in a stable TrkB expressing PC12 cell line. These experiments demonstrate that, in PC12 cells, BDNF does not require extracellular Ca\(^{2+}\) to induce tyrosine phosphorylation of the TrkB receptor. This suggests that, although the Trk receptor signal transduction cascade is initiated in the absence of extracellular Ca\(^{2+}\), there must be an extracellular Ca\(^{2+}\) requirement downstream of receptor activation. This would be possible if removal of extracellular Ca\(^{2+}\) results in a secondary depletion of intracellular Ca\(^{2+}\) stores in the neuron and/or myocyte. To replenish intracellular Ca\(^{2+}\) stores, an influx of Ca\(^{2+}\) through store-operated Ca\(^{2+}\) channels (capacitative Ca\(^{2+}\) channels) may be required. These plasma-membrane Ca\(^{2+}\) channels are activated in response to decreases in Ca\(^{2+}\) concentrations in ER, such as those that occur when IP3 receptor–activated Ca\(^{2+}\) stores are released into the cytoplasm (reviewed in Barritt 1999). Although we have not addressed this issue directly, a similar influx has been demonstrated in other neurons (Li et al. 1999; Simpson et al. 1995).

TRPC (TRP-Ca) channels are vertebrate homologues of the *Drosophila* transient receptor potential (TRP) channels, which represent the best candidates for mediating a store-operated conductance. These channels mediate store-operated and/or PLC-dependent cation conductance in nonexcitable cells. The role of the TRPC-mediated conductance in neurons has not been fully explored; however, most family members are expressed in mammalian brain, and TRPC3, TRPC4, and TRPC5 are highly expressed in brain. A recent study by Li and colleagues has described a role for TRPC3, which co-localized and co-immunoprecipitated with TrkB, in mediating a PLC-dependent cation influx in response to BDNF in mammalian neurons (Li et al. 1999). Thus one possibility is that activation of a PLC-dependent cation influx via the TRPC3 channel is needed to produce the BDNF-induced potentiation. Alternatively, it is possible that the extended period in Ca\(^{2+}\)-free medium reduced the ability of the store-operated channels to replenish intracellular calcium stores. Under these conditions, activation of PLC\(\gamma\)-dependent pathway may not have been able to release sufficient Ca\(^{2+}\) from the intracellular stores to produce potentiation. While our experiments in the absence of extracellular Ca\(^{2+}\) cannot distinguish between an effect on the presynaptic cell and the postsynaptic cell, our Fura-2 data argue for some presynaptic locus of action.

A requirement for Ca\(^{2+}\) release from neuronal internal stores is supported by Fura-2 experiments demonstrating that BDNF...
treatment of cultured motor neurons results in a rise in the intracellular concentration of Ca\(^{2+}\) (Stoop and Poo 1996; unpublished observations). The release of Ca\(^{2+}\) from internal stores is likely to be at least partly mediated by TrkB activation of PLC because we are able to demonstrate that an inhibitor of PLC function prevents the BDNF-induced potentiation of twitching. Activated TrkB is autophosphorylated at tyrosine residues 670, 674, 675, and 785 and can phosphorylate and activate PLC\(\gamma\) (Guiton et al. 1994; Middlemas et al. 1994). The TrkB phosphotyrosine residue 785 can complex with phosphorylated PLC\(\gamma\) via interaction with its SH2 domain (Middlemas et al. 1994). Activated PLC\(\gamma\) stimulates the generation of IP3, which opens the IP3 receptor-gated channels in the ER. The resultant rise in the cytoplasmic concentration of Ca\(^{2+}\) is likely to be critical for the potentiation of the release of neurotransmitter (Guo et al. 1996; Li et al. 1998; Tse et al. 1997). Our observation that both the rise in intracellular calcium and the increased frequency of twitching often takes between 10 and 20 min to reach its peak suggests that the signal transduction cascade involved in producing these effects may have additional components. Similarly, Tanaka and colleagues (1997) have reported that BDNF treatment attenuates inhibitory postsynaptic potentials (IPSPs) in cultured rat hippocampal neurons in a U73122-dependent fashion, and this effect takes approximately 10–15 min to reach its peak. Other factors that would certainly influence the time course of this response in our system include the rate of association for human BDNF with the \(Xenopus\) TrkB receptor at 25°C. Trk receptors appear to associate with their ligands with unusually slow kinetics (Mahadeo et al. 1994).

**BDNF-induced potentiation of twitching mimics BDNF-induced potentiation of spontaneous synaptic currents**

The video assay used in this study has several attractive features. It is noninvasive and convenient and permits monitoring of cells over extended time. In addition, multiple cells can be monitored in a field of view simultaneously. The TWITCH software can monitor up to nine different locations within the field at the same time, allowing collection of data on...
more cells, more quickly. However, this video assay will not differentiate between presynaptic and postsynaptic effects since it only collects meaningful information on relative changes in the frequency of spontaneous activity.

The time course of the twitching response was similar but somewhat slower than that reported in frequency of spontaneous synaptic currents recorded (Lohof et al. 1993). Subtle changes in the experimental preparation may account for these differences, as our unpublished voltage-clamp data of myocytes is, on average, somewhat slower than that of Lohof et al. (1993).

**BDNF-induced potentiation of twitching is enhanced by action potentials**

We were able to detect BDNF-induced potentiation in the presence of TTX, suggesting that sodium action potentials are not required for this effect. However, we did not observe potentiation in as many cells when TTX was present. This suggests that action potentials, while not required, do enhance the likelihood that potentiation will develop. Recent studies demonstrate that presynaptic depolarization paired with concentrations of BDNF that are normally not sufficient to produce BDNF-induced potentiation (10 ng/ml) will allow induction of BDNF-induced potentiation (Boulanger and Poo 1999). Together with our data, this suggests that presynaptic depolarization greatly increases the likelihood that BDNF can produce potentiation. In cultured rat motor neurons, depolarization and

**FIG. 8.** Fura-2 imaging of changes in intracellular \(\text{Ca}^{2+}\) concentrations in response to BDNF. A and B: 2 examples of changes in intracellular \(\text{Ca}^{2+}\) concentration in response to BDNF application. The duration of the BDNF treatment is indicated in red. C: BDNF-induced increases were inhibited when cells were treated with store-depleting drugs for 3 h prior to BDNF. Top trace: control cell responding to BDNF. Bottom trace: cell pretreated with thapsigargin and cyclopiazonic acid.

**FIG. 9.** Role of calcium in BDNF-induced potentiation of synaptic activity. Binding of BDNF to the TrkB receptor initiates a calcium-insensitive activation receptor autophosphorylation. Phosphorylated TrkB recruits activated PLC to TrkB and promotes production of IP3. Blockade of this step with U73122 prevents BDNF-induced potentiation. IP3 binding to receptors on intracellular stores initiates a release of \(\text{Ca}^{2+}\), and an increase in \([\text{Ca}^{2+}]_i\). The rise in \([\text{Ca}^{2+}]_i\) leads to potentiated neurotransmitter release. Depletion of the intracellular stores may trigger a \(\text{Ca}^{2+}\) influx needed to replenish the intracellular stores via a store-operated mechanism. In the absence of extracellular \(\text{Ca}^{2+}\), BDNF-induced potentiation is blocked.
treatments that elevate cAMP levels produce a marked increase in the amount of TrkB receptor expressed on the cell surface within 30 min (Meyer-Franke et al. 1998), suggesting that surface expression of the Trk B receptor exhibits rapid and dynamic regulation. It is possible that under normal circumstances, spontaneous action potentials may provide sufficient presynaptic depolarization for BDNF to induce potentiation by promoting the insertion of TrkB receptors into the plasma membrane. Blocking action potentials for 30 min may decrease the spontaneous amount of presynaptic activity enough to reduce the amount of TrkB receptor expressed on the surface of the neurons, and decrease the likelihood of producing potentiation. We did not observe BDNF-induced potentiation from every cell even in the absence of TTX, which may reflect the existence of a subpopulation of cells lacking sufficient surface TrkB receptor (or any at all) to produce potentiation.

The role of BDNF in regulating synaptic function has emerged as a central theme in understanding activity-induced plasticity. TrkB receptors and BDNF are also required for trophic support of many neurons throughout the life of the organism. Understanding how TrkB receptor activation can orchestrate such complex cascades to result in each of its requisite functions are complicated questions, but some common themes are beginning to emerge. The pattern of Ca\(^{2+}\) transients induced by BDNF could play a significant role in mediating both acute and long-term changes in structure or function. The surface distribution of the TrkB receptors in combination with the subcellular localization of Ca\(^{2+}\) stores determines the range of patterns of Ca\(^{2+}\) transients that BDNF can produce in a particular neuron. Both surface expression of Trk receptors and neurotrophin release can be regulated in response to depolarization. Thus the activity-dependent regulation of both of these processes could produce associative properties of neurotrophin-induced plasticity. Key issues to address will be how the surface expression of TrkB receptor is regulated by activity and how this alters the dynamics of Ca\(^{2+}\) release from internal stores.

L. F. Reichardt is an investigator at the Howard Hughes Medical Institute. R. J. Kleiman was the recipient of an American Heart Association fellowship. We are grateful to P. Wang for help with data analysis. This work was supported by National Institutes of Health grants to D. R. Copenhagen and L. F. Reichardt.

REFERENCES


CABELLI RJ, HOHN A, and SHATZ CJ. Inhibition of ocular dominance column formation by infusion of NT-4/5 or BDNF. Science 267: 1662–1666, 1995.


