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Strong Calcium Entry Activates Mitochondrial Superoxide Generation, Upregulating Kinase Signaling in Hippocampal Neurons

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Large increases in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) activate several kinases that are important for neuronal plasticity, including Ca^{2+}/calmodulin-dependent kinase II (CaMKII), protein kinase A (PKA), and protein kinase C (PKC). Because it is also known, mainly in non-neuronal systems, that superoxide radicals (O_2^-) activate these (and other) kinases and because O_2^- generation by mitochondria is in part $[Ca^{2+}]_i$ dependent, we examined in hippocampal neurons the relationship between Ca^{2+} entry, O_2^- production, and kinase activity. We found that, after large stimulus-induced $[Ca^{2+}]_i$ increases, O_2^- selectively produced by mitochondria near plasmalemmal sites of Ca^{2+} entry acts as a modulator to upregulate the two kinases, namely, CaMKII and PKA, whose activities are directly or indirectly phosphorylation dependent. The common mechanism involves O_2^- inhibition of inactivating protein phosphatases. Conversely, because small $[Ca^{2+}]_i$ increases do not promote mitochondrial respiration and O_2^- generation, weak stimuli favor enhanced phosphatase activity, which therefore leads to suppressed kinase activity. Enhanced O_2^- production also promoted PKC activity but by a phosphatase-independent pathway. These results suggest that Ca^{2+}-dependent upregulation of mitochondrial O_2^- production may be a general mechanism for linking Ca^{2+} entry to enhanced kinase activity and therefore to synaptic plasticity. This mechanism also represents yet another way that mitochondria, acting as calcium sensors, can play a role in neuronal signal transduction.

Key words: **superoxide; reactive oxygen species; mitochondria; calcium; calcium signaling; calcium/calmodulin-dependent protein kinase II; CaMKII; protein kinase A; PKA; protein kinase C; PKC; protein phosphatases; hippocampus**

Introduction

Several Ca^{2+}-sensitive protein kinases that control the phosphorylation status of signaling molecules are known regulators of neuronal plasticity (Tokuda and Hatase, 1998; Soderling and Derkach, 2000). For example, large increases in cytosolic free calcium ($[Ca^{2+}]_i$) preferentially trigger one or more Ca^{2+}-dependent kinase cascades that lead to long-term potentiation (LTP), an activity-dependent strengthening of synaptic efficacy (Hardingham and Bading, 1999; Soderling and Derkach, 2000; Hardingham and Bading, 2003). Several classes of Ca^{2+}-sensitive kinases are known to be important in this role, including Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) (De Koninck and Schulman, 1988; Lisman et al., 2002), c-AMP-dependent protein kinase A (PKA) (Impey et al., 1996, 1998), and Ca^{2+}/phospholipid-dependent protein kinase (PKC) (Oancea and Meyer, 1998).

During physiological stimulation, transient elevations in the concentration of reactive oxygen species (ROS) affect signaling pathways in a variety of neuronal and non-neuronal cell types (Klann and Thiels, 1999; Hancock et al., 2001; Dröge, 2002). In non-neuronal cells, elevated but sublethal levels of ROS increase the activity of regulatory kinases, including PKC and PKA, whereas such elevations decrease the activity of protein phosphatases 2A (PP2A) and 2B (PP2B) (Whisler et al., 1995; Wang et al., 1996; Winder and Sweatt, 2001). In hippocampal neurons, stimulation increases the levels of Ca^{2+} (Hardingham et al., 2001) and superoxide (O_2^-) (Bito et al., 1996) in the vicinity of the plasma membrane. Increased production of the latter, an important member of the ROS family, has been shown to suppress protein phosphatase activity and thereby prolong the lifetime of phosphorylated cAMP response element-binding protein (pCREB) (Bito et al., 1996, 1997; Hongpaisan et al., 2003).

There are numerous pathways for the production of intracellular O_2^-. Among these, O_2^- generated by mitochondrial respiration is generally thought not to be involved in cell signaling (Hancock et al., 2001; Dröge, 2002). However, mitochondria do accumulate and release Ca^{2+} during and after physiological stimulation (Friel, 2000; Nicholls and Budd, 2000; Rizzuto et al., 2000; Brocard et al., 2001; Hongpaisan et al., 2001), and, with stimuli strong enough to generate high-amplitude $[Ca^{2+}]_i$ transients and activate robust mitochondrial Ca^{2+} uptake, mitochondria appear to be a major source of O_2^- in hippocampal neurons (Bindokas et al., 1996; Sengpiel et al., 1998; Hongpaisan et al.,

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2003), as well as in certain other neuronal and non-neuronal cells (Nicholls and Budd, 2000; Werner and Werb, 2002). This generally underappreciated aspect of mitochondrial function is potentially significant, as suggested by our recent finding that Ca^{2+} uptake-induced mitochondrial O_2^- production underlies the stimulus-dependent stabilization of pCREB mentioned above (Hongpaisan et al., 2003).

The present study examines in living hippocampal neurons the relationship between stimulus-induced $[Ca^{2+}]_i$ elevations, mitochondrial Ca^{2+} uptake, O_2^- production, and activation of three kinases (CaMKII, PKA, and PKC) that are important for synaptic plasticity and gene expression. The results indicate that mitochondrial O_2^- plays a key role in neuronal Ca^{2+} signaling, significantly modulating the link between Ca^{2+} entry and plasticity.

Materials and Methods

Primary cell culture. Primary cultures of hippocampal neurons were prepared by plating papain-dissociated hippocampal cells from embryonic day 21 Sprague Dawley rat embryos onto a previously prepared confluent feeder layer of hippocampal glial cells, as described by Lu et al. (1998). The cultures were maintained in a 37°C incubator with 10% CO_2 and fed twice weekly with half changes of Eagle's minimum essential medium (MEM) containing Earle's salts, 6 gm/l glucose, and 3.7 gm/l sodium bicarbonate and supplemented with 5% (v/v) heat-inactivated horse serum (HyClone, Logan, UT), 1% (v/v) fetal bovine serum, 2 mM Glutamax 1, 136 mM uridine, 54 mM 2-deoxy-5-fluoro-uridine (FUDR), and N3 supplement. Glial cells were prepared from papain-dissociated embryonic rat hippocampi and plated onto glass coverslips coated with a purified collagen substrate (Vitrogen 100; Cohesion, Carlsbad, CA) and poly-L-lysine (Sigma-Aldrich, St. Louis, MO) and subsequently UV sterilized. Glial cells were maintained in MEM containing 2 mM Glutamax 1 and 10% (v/v) fetal bovine serum and were later supplemented with 136 μM uridine and 54 μM FUDR to arrest cell division when the culture reached confluence, usually ~1 week before neurons were plated onto the glial feeder layer. Except as noted, cell culture reagents were from Invitrogen (Grand Island, NY). Reagents for N3 supplement, FUDR, and uridine were from Sigma-Aldrich.

In a few experiments, hippocampal neurons and glial cells were grown together as mixed cultures. Hippocampi of 19-d-old Sprague Dawley embryos were disaggregated by gentle trituration using a Pasteur pipette without enzyme digestion and plated onto glass coverslips. The plating medium was MEM containing 10% heat-inactivated horse serum, 5% fetal calf serum, 2 mM glutamine, 0.6% glucose, and 15 μg/ml gentamycin. Cells were incubated at 37°C with 8% CO_2. When glial cells were almost confluent, uridine and FUDR were added to prevent glial overgrowth. The cultures were fed thereafter one or two times per week with Eagle's MEM containing 10% horse serum and 1% fetal bovine serum with 8% CO_2.

All experiments were performed on 2- to 3-week-old cultures. No differences were observed between the two types of cell culture. Therefore, data collected from both preparations under the same experimental conditions were pooled.

Transfection. Cultured neurons 6–12 d *in vitro* were transfected (4–6 hr) with 0.5 μg of green fluorescent protein (GFP) plasmid (Gene Therapy Systems, San Diego, CA) and 1.5 μg of manganese superoxide dismutase (Mn-SOD) cDNA (pRK5 Mn-SOD plasmid; kindly provided by Genentech, South San Francisco, CA) by means of Effectene transfection reagent (Qiagen, Valencia, CA). Subsequently, cells were returned to culture medium in the absence of plasmid and cultured for at least 6 additional days. Overexpression of Mn-SOD protein was quantified by immunohistochemistry using an Mn-SOD-specific rabbit antibody (1:200; Upstate Biotechnology, Lake Placid, NY). On average, Mn-SOD immunoreactivity was elevated ~45% in GFP-positive neurons ($n = 60$) compared with GFP-negative cells.

Field stimulation. Before stimulation, cultured neurons were incubated overnight in 1 μM tetrodotoxin (TTX) in cultured medium to block spontaneous activity. Neurons were activated by field stimulation, using 1 msec constant voltage pulses at 15 V, applied with a biphasic stimulus isolator (BAK Electronics, Mt. Airy, MD), in Tyrode's solution containing the following (in mM): 129 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 30 glucose, and 25 HEPES, pH 7.3 (osmolarity, 313 ± 2 mOsm; at 25°C). Viability tests (0.2% Trypan blue staining) revealed no long-term damage 24 hr after stimulation. Survival fractions were 97.6 ± 1.1% (control), 97.2 ± 1.2% (single 100 Hz/18 sec pulse), and 97.0 ± 1.1% (three trains of 100 Hz/18 sec). For cells overexpressing Mn-SOD, experiments were repeated using isotonic 90 mM K^+ as a depolarizing stimulus.

When appropriate, cells were preincubated as follows: blockers of mitochondrial respiration were present for 3–5 min before stimulation; drugs affecting O_2^- concentration were present for 30 min before experiments, except NAc, which was present for ~15 hr; and protein phosphatase blockers were present for 45 min before experiments. All drugs were also present during and after stimulation. During poststimulation periods, cultures were incubated in Tyrode's solution in the presence of TTX (25°C). For loading Ca^{2+} chelators, cells were incubated at 25°C in Tyrode's solution containing either 1 mM BAPTA-AM plus 0.05% (w/v) pluronic acid for 45 min or 3 mM EGTA-AM without pluronic acid for 25 min.

Immunocytochemistry. Cultured neurons were fixed in 4% paraformaldehyde, 4 mM EGTA, and 4% (w/v) sucrose at 25°C for 20 min and stored at 4°C; PBS was used in all fixation and immunocytochemical procedures. For immunostaining, cells were washed and permeabilized with 0.5% Triton X-100, followed by incubation with 15% horse serum at 25°C for 1 hr to suppress nonspecific binding. Cells were then incubated with primary antibodies against autophosphorylated CaMKII (Affinity Bioreagents, Golden, CO), against PKCα phosphorylated at serine 657 (Upstate Biotechnology), or against the catalytic domain of PKA (BD Biosciences, San Jose, CA). Incubations were performed in PBS containing 0.5% Triton X-100 and 15% horse serum at 25°C for 3 hr, followed by 4°C overnight. Alexa-conjugated antibodies (Molecular Probes, Eugene, OR) were used as secondary antibodies (25°C, 2 hr). To inhibit fluorescence quenching, cover glasses were mounted with Vectashield H-1200 (Vector Laboratories, Burlingame, CA) containing 4′,6-diamidino-2-phenylindole to counterstain nuclei. Immunostaining was visualized using a Zeiss (Thornwood, NY) 410 or 510 confocal scanning microscope fitted with a 63×, 1.4 numerical aperture oil immersion objective. Random fields were imaged and stored for subsequent measurements of fluorescent intensity using NIH Image software. Parameters analyzed and taken as measures of enhanced kinase activity were as follows: (1) elevated immunofluorescence, integrated over the cytoplasm, from an antibody specific for autophosphorylated CaMKII; (2) elevated immunofluorescence from peripheral, subplasma membrane cytoplasm that reflects translocation of phospho-PKCα to the plasma membrane; and (3) the ratio of nuclear/cytosolic immunofluorescence that tracks nuclear import of PKA catalytic subunits. For each experimental condition, data were obtained from four to six cultures, six random regions in each culture, and two to six cells in each region. All data as presented were normalized to paired controls that were treated identically but without stimulation to eliminate the possible effects of vehicle, culture-to-culture variability, and other potential artifacts. Population measurements were expressed as mean ± SEM. Statistical significance was assessed using Student's *t* test.

Calcium and superoxide measurements. For $[Ca^{2+}]_i$ measurements, cultured cells were loaded with 5 μM fluo-3 AM (Molecular Probes) in Tyrode's solution at 25°C for 30 min. Cells were then washed and mounted in a modified perfusion imaging chamber (PDMI-2; Harvard Apparatus, Holliston, MA). Simultaneously with fluo-3 measurements, superoxide was estimated by the oxidation of hydroethidine to the fluorescent ethidium cation. Hydroethidine (2 μg/ml; Molecular Probes) plus 0.5 μM TTX was present in the Tyrode's perfusate throughout the entire experiment; there was no preincubation period. Both fluo-3 and ethidium fluorescence were recorded on a confocal laser scanning microscope with a 63×, 1.4 numerical aperture oil immersion objective. Optics used to measure ethidium fluorescence were 488 nm excitation and >510 nm emission. Images were averaged by line scanning (8:1) and recorded at 256 × 256 pixels. Fluorescent intensity was analyzed by

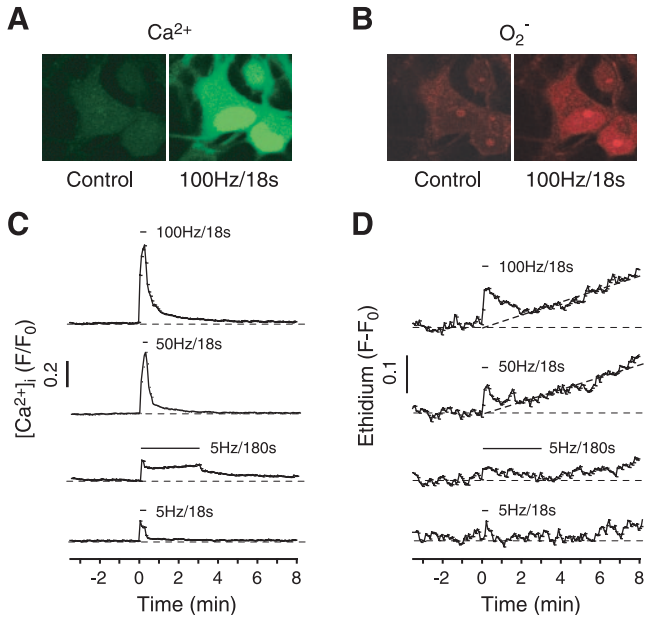

Figure 1. High-frequency field stimulation elicits parallel elevations of cytosolic calcium and superoxide radicals. *A*, *B*, In cultured rat CA1/CA3 hippocampal neurons, electrically evoked $[Ca^{2+}]_i$ elevations were measured by means of confocal fluorescence microscopy of fluo-3-loaded cells. Simultaneously, the oxidation of ethidine to the ethidium cation, which is specific for the superoxide anion, was used to determine O_2^- elevations (Bindokas et al., 1996). Micrographs are representative examples in which the right panel of a pair illustrates the typical general increase in fluorescence evoked by high-frequency stimulation. *C*, *D*, Weak, low-frequency stimuli (5 Hz, 18 or 180 sec) elicited only low, sustained $[Ca^{2+}]_i$ plateaus (*C*, bottom traces) and did not promote O_2^- generation (*D*, bottom traces). In contrast, strong, high-frequency stimulation (50 Hz/18 sec or 100 Hz/18 sec) evoked high-amplitude Ca^{2+} spikes (*C*, top traces), which returned to baseline within 1–2 min, and remained at basal levels for at least 45 min. It also evoked a sustained increase in cytosolic O_2^- (*D*, top traces). The sharp, transient rise in ethidium fluorescence occurring at the onset of stimulation and persisting for ~2 min is an artifact arising, at least in part, because temporary, stimulus-induced collapse of the mitochondrial membrane potential retards mitochondrial uptake and subsequent fluorescence quenching of the ethidium cation (Budd et al., 1997).

means of MetaMorph software (Universal Imaging Corporation, Downingtown, PA). For Ca^{2+} and O_2^- measurements, data were averaged from four to six independent preparations, with 5–10 cells in each experiment. $[Ca^{2+}]_i$ is presented as F/F_0, where F_0 equals the average fluorescent intensity before stimulation. O_2^- production was calculated as $F - F_0$, where F_0 equals the background fluorescent intensity at a given time point estimated by extrapolating the slope of the baseline during the prestimulus period.

Chemicals. Manganese (III) tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) and FK506 were purchased from Calbiochem (San Diego, CA). Rotenone, cyanide, oligomycin, *N-tert*-butyl-α-(2-sulfo-phenyl)-nitrone (SPBN), *N*-acetylcysteine (NAc), diethyldithiocarbamic acid (DETC), okadaic acid (OA), and TTX were obtained from Sigma-Aldrich.

Results

Superoxide production is selectively induced by high-frequency stimulation

In cultured rat CA1/CA3 hippocampal neurons, confocal fluorescence microscopy was used to simultaneously measure electrically evoked changes in concentrations of $[Ca^{2+}]_i$ and O_2^- (Fig. 1*A*,*B*). Neurons cultured on glass coverslips were mounted between a pair of electrodes and field stimulated at three different frequencies, namely, 5, 50, and 100Hz. With low-frequency stimulation (5 Hz), short (18 sec) and long (180 sec) stimuli induced only a small $[Ca^{2+}]_i$ rise, which was sustained for the duration of the stimulus (Fig. 1*C*). Neither stimulus affected O_2^- levels (Fig. 1*D*). In contrast, high-frequency stimulation (50 Hz/18 sec or 100 Hz/18 sec) evoked a high-amplitude Ca^{2+} spike that was graded with stimulus frequency (Fig. 1*C*). Large $[Ca^{2+}]_i$ elevations activated by 50 Hz/18 sec or 100 Hz/18 sec were associated with the onset of significantly increased O_2^- production (Fig. 1*D*). Increased production of O_2^- was sustained for several minutes, although $[Ca^{2+}]_i$ had essentially returned to baseline within ~1 min after stimulation. These observations indicate that strong but physiological Ca^{2+} entry evoked by high-frequency stimulation is coupled to enhanced O_2^- production. This is expected to reset the intracellular redox steady state in favor of oxidative species, presuming that the activity of ROS scavengers, such as SOD, remains constant. This appears to occur without long-term injury, as indicated by maintained Ca^{2+} homeostasis and cell structure and viability 24 hr after stimulation (see Materials and Methods).

Superoxide enhances calcium-dependent kinase activity

Quantitative confocal fluorescence microscopy of immunostained cells was used to investigate the stimulus-induced activation of CaMKII and PKC; the activity of the former is phosphorylation dependent, whereas that of the latter is not. Because CaMKII undergoes a characteristic autophosphorylation that is a good measure of its activity (Lisman et al., 2002), cultured neurons were stained with an antibody that recognizes an appropriately autophosphorylated (Thr-286) form of CaMKII (pCaMKII). This antibody showed that pCaMKII was diffusely localized within the cytoplasm of resting neurons (Fig. 2*A*). Stimulation with 100 Hz/18 sec, but not 5 Hz/180 sec, promoted an increase in pCaMKII, as indicated by increased fluorescence from cytoplasmic regions (Fig. 2*A*,*C*, white vs gray bars).

Protein kinase C was assayed by taking advantage of the fact that activity-dependent translocation of the PKCα isoform to the plasma membrane is essential for its activity (Newton, 1997). Therefore, a change in immunofluorescence intensity of an antibody specific for PKCα phosphorylated at Ser-657 (pPKCα) at or near the plasma membrane served as a good indicator of enhanced PKC activity. In resting neurons, pPKCα immunoreactivity was diffusely distributed throughout the cell (Fig. 2*B*), but after high-frequency stimulation (100 Hz/18 sec), it was strongly associated with the plasma membrane (Fig. 2*B*). Quantitative analysis of pPKCα immunoreactivity over the entire cell body region of hippocampal neurons showed that, as expected, the global signal did not change under any stimulation condition (data not shown). Measurements of pPKCα that were limited to only a marginal shell under the plasma membrane revealed that stimulation with 100 Hz/18 sec, but not 5 Hz/180 sec, led to the accumulation of pPKCα in subplasmalemmal regions (Fig. 2*B*,*D*, white vs gray bars).

A panel of drugs affecting O_2^- stability was used to investigate whether O_2^- influenced Ca^{2+}-dependent kinase activities. Reduction of O_2^- levels by 100 μM SPBN or 5 mM NAc (both ROS scavengers), as well as by 25 μM MnTMPyP (an SOD mimetic), blunted the increases in CaMKII and pPKCα activities that are normally induced by 100 Hz/18 sec (Fig. 2*C*,*D*, bottom panels, compare gray bars, black bars). Conversely, inhibition of O_2^- degradation by 5 mM DETC (an SOD inhibitor) extended the lifetime of O_2^- and thereby enhanced CaMKII and pPKCα activities induced by 100 Hz/18 sec. Note that Ca^{2+}-activated CaMKII and pPKCα activities were only partially depressed by SPBN, NAc, and MnTMPyP, consistent with the idea that O_2^- production mainly serves as a modulator of Ca^{2+}-dependent activation.

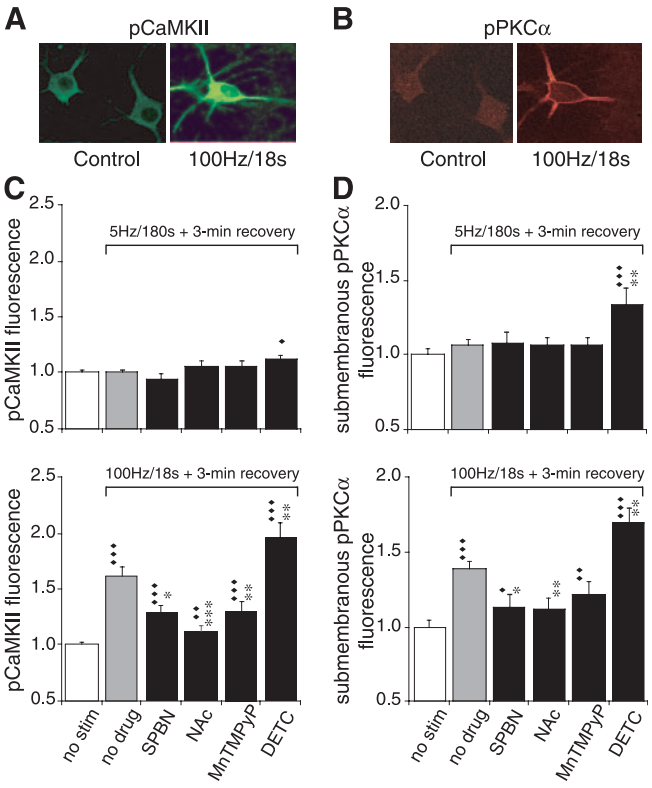

Figure 2. Superoxide levels affect protein kinase activity. *A*, *B*, Cultured hippocampal neurons were immunostained with antibodies recognizing an autophosphorylated (Thr-286) form of CaMKII and a phosphorylated (Ser-657) form of PKCα (pPKCα) that translocates to the plasma membrane during activation. Representative confocal images illustrate changes induced by high-frequency field stimulation. Elevated CaMKII activity was characterized by a general increase in cytoplasmic fluorescence intensity (*A*, right), whereas activation of pPKCα led to its accumulation at or near the plasma membrane (*B*, right). *C*, Quantitative immunocytochemistry revealed that stimulation with 100 Hz/18 sec (bottom), but not 5 Hz/180 sec (top), promoted CaMKII autophosphorylation, presumably reflecting an increase in kinase activity (compare white bars, gray bars). Increased activation induced by 100 Hz/18 sec (bottom) was significantly reduced by ROS scavengers (SPBN and NAc) and by an SOD mimetic (MnTMPyP) but was enhanced by an SOD inhibitor (DETC), indicating that CaMKII activity was dependent on cellular O_2^- levels. With 5 Hz/180 sec (top), only DETC affected CaMKII activity, which occurs because weak stimulation does induce some O_2^- production. Normally the small amount produced is efficiently degraded by endogenous SOD, but, in the presence of SOD inhibitors, O_2^- persists longer and can be detected. Diamonds and asterisks indicate statistical significance relative to control neurons and to neurons stimulated without drugs, respectively. $*^{\blacklozenge}p < 0.05$; $**^{\blacklozenge\blacklozenge}p < 0.01$; $***^{\blacklozenge\blacklozenge\blacklozenge}p < 0.001$). *D*, Experiments analogous to those in *C* were used to quantify pPKCα translocation to the plasma membrane. Analysis of pPKCα immunoreactivity over the entire cell body region showed that the global signal did not change quantitatively under any stimulation condition (data not shown). However, poststimulus measurements restricted to subplasma membrane regions revealed a pattern of O_2^--dependent kinase activity that was essentially similar to that of CaMKII.

With low-frequency stimulation (5 Hz/180 sec), SPBN, NAc, and MnTMPyP had no effect, whereas SOD inhibition (DETC) promoted kinase activity (Fig. 2*C*,*D*, top panels). The likely explanation for the DETC effect is that some O_2^- is produced even in response to weak Ca^{2+} entry and under basal conditions as well, but the amount is small enough to be efficiently degraded by SOD and other antioxidants.

Superoxide that is effective for promoting kinase activity is produced by mitochondria

We next sought to identify the major source(s) of stimulus-induced O_2^- production and establish its effect on kinase activation. Mitochondrial respiration stands out as a strong candidate, insofar as it has been shown previously that mitochondria are the main source of O_2^- that stabilizes nuclear pCREB after similarly strong stimulation in the same cell preparation (Hongpaisan et al., 2003). Rotenone and cyanide block electron transport at complex I and complex IV, respectively, in the mitochondrial inner membrane (Nicholls and Budd, 2000); we found previously that, in intact hippocampal neurons under our culture conditions, both of these drugs effectively inhibit depolarization-evoked increases in mitochondrial O_2^- production (Hongpaisan et al., 2003). Both drugs also block mitochondrial ATP production while accelerating its consumption by reversal of the ATP synthase, but the latter effect is greatly reduced in the presence of oligomycin (2 μM). Furthermore, inhibiting mitochondrial ATP production in neurons stimulates a large compensatory increase in glycolytic ATP synthesis (Nicholls and Budd, 2000). We previously examined ATP depletion in the same cell preparation using as a benchmark the ATP-dependent translocation of Ca^{2+}/calmodulin to the nucleus (Deisseroth et al., 1996) and found that ATP was adequately maintained over exposure times of at least 15 min (Hongpaisan et al., 2003).

Rotenone and oligomycin (2 μM each) or cyanide and oligomycin (1 mM and 2 μM) marginally enhanced $[Ca^{2+}]_i$ elevations induced by 100 Hz/18 sec stimuli (Fig. 3*A*), which is expected because these agents partially collapse the mitochondrial membrane potential, thus retarding mitochondrial uptake of cytosolic Ca^{2+} (Nicholls and Budd, 2000). Simultaneous measurements of O_2^- levels after high-frequency stimulation revealed that both inhibitors completely blocked mitochondrial O_2^- generation (Fig. 3*B*); inhibition persisted for many minutes after the blockers had been washed out and the stimulus and its associated Ca^{2+} transient had terminated. [The artifactual rise in ethidium fluorescence during and shortly after stimulation (Fig. 3*B*) is explained in the legend to Figure 1.] It should be noted that the effects of respiratory inhibitors, especially rotenone (without oligomycin), on ROS production appear to vary depending on experimental conditions and preparations (Votyakova and Reynolds, 2001), although it seems clear experimentally that, in our cells, both rotenone and cyanide effectively shut down O_2^- production.

At 3 min after stimulation, weak stimulation (5 Hz/180 sec) with or without respiratory blockers had no effect on CaMKII or pPKCα activities (Fig. 3*C*). However, after high-frequency stimulation (100 Hz/18 sec) both blockers significantly attenuated the increases in CaMKII autophosphorylation and pPKCα translocation seen in the absence of respiratory blockers (Fig. 3*D*). The results indicate that enhanced generation of mitochondrial O_2^- induced by high-frequency stimulation is effective in upregulating CaMKII and PKC activities.

To further establish that these pharmacological effects were specifically attributable to mitochondrial O_2^-, and not another ROS or from another source, cultured neurons were transfected with a mitochondrial Mn-SOD plasmid. In cells overexpressing Mn-SOD and GFP (Fig. 3*E*, bottom row of micrographs), both field stimulation (100 Hz/18 sec) and depolarization with 90 mM K^+ [the latter is an alternative strong but physiological stimulus that also promotes mitochondrial O_2^- generation (Hongpaisan et al., 2003)] depressed both CaMKII and pPKCα activities (Fig. 3*E*, right panel). This observation supports the conclusion that O_2^-, and not another ROS such as H_2O_2, is mainly responsible for kinase upregulation. In addition, immunocytochemical staining for Mn-SOD revealed a distinct, punctate staining pattern that was particularly evident in transfected cells (data not shown). Such a pattern is characteristic of mitochondria and, considering

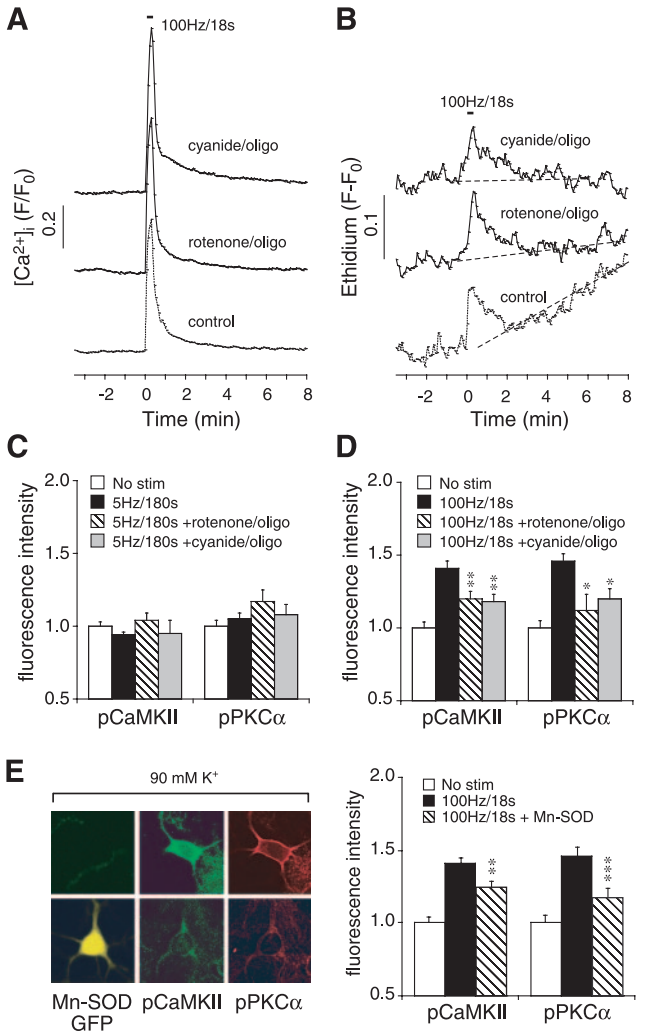

Figure 3. Superoxide that affects protein kinase activity is produced by mitochondria. *A*, *B*, High-frequency stimulation (100 Hz/18 sec) in the presence of rotenone–oligomycin, a mixture that inhibits mitochondrial respiration at complex I without significant ATP depletion, slightly enhanced $[Ca^{2+}]_i$ elevations (*A*) and completely inhibited mitochondrial O_2^- production (*B*). An essentially similar effect is seen in the presence of cyanide–oligomycin, which blocks respiration further down the chain at complex IV. *C*, Low-frequency stimulation (5 Hz/180 sec) with or without respiratory inhibitors had no effect on CaMKII autophosphorylation or pPKCα translocation. *D*, The normal increases in CaMKII and pPKCα activity that follow high-frequency stimulation (100 Hz/18 sec; compare white bars, black bars) were suppressed by either mixture of respiration blockers, indicating that Ca^{2+} entry-dependent mitochondrial O_2^- normally upregulates these kinases. *E*, The normal increases in CaMKII and pPKCα activity induced by strong Ca^{2+} entry after either high-frequency stimulation (100 Hz/18 sec) (right panel, black bars) or depolarization with 90 mM K^+ (left panel, top row) were depressed in neurons overexpressing mitochondrial Mn-SOD/GFP (left panel, bottom row), which is expected because these cells scavenged O_2^- more efficiently (right panel, hatched bars). Symbols indicating statistical significance are as defined in Figure 2.

that the Mn isoform of SOD is expected to target to mitochondria, is consistent with the idea that mitochondria are a major source of active O_2^-. Nonetheless, it is certainly possible that some fraction of Mn-SOD is expressed in non-mitochondrial locations.

Calcium accumulation by spatially peripheral mitochondria is sufficient for superoxide enhancement of kinase activity

In many cells, stimulus-dependent mitochondrial Ca^{2+} accumulation preferentially occurs within a few micrometers of the plasma membrane, that is, in organelles close to sites of Ca^{2+} entry (Pivovarova et al., 1999; Montero et al., 2000; Hongpaisan et al., 2001). Therefore, the next set of experiments addressed whether subplasmalemmal mitochondria might play a privileged role in the O_2^--mediated enhancement of CaMKII and PKC activities. The membrane-permeable Ca^{2+} chelators BAPTA-AM and EGTA-AM were used to distinguish spatially peripheral mitochondria from the general population of these organelles. Because BAPTA has a much faster on-rate for Ca^{2+} binding than EGTA, it is much more effective for reducing $[Ca^{2+}]_i$ elevations within a few micrometers of Ca^{2+} entry sites (Deisseroth et al., 1996). Stimulation with 100 Hz/18 sec in the presence of EGTA induced a small increase in global $[Ca^{2+}]_i$, similar in magnitude to that induced by 5 Hz stimulation in the absence of EGTA (compare Figs. 4*A*, 1*C*). Under these conditions, O_2^- production was also inhibited but not completely (Fig. 4*B*). In contrast, BAPTA, as expected, essentially eliminated both the $[Ca^{2+}]_i$ elevation and O_2^- production normally induced by 100 Hz/18 sec (Fig. 4*A*,*B*). These observations indicate that a significant $[Ca^{2+}]_i$ increase in subplasmalemmal regions mainly is sufficient to increase O_2^- production.

The effects of BAPTA and EGTA on CaMKII and PKC were also investigated. BAPTA completely blocked stimulus-evoked increases in CaMKII and pPKCα activities, whereas EGTA was ineffective (Fig. 4*C*). Similar to cells that were not loaded with exogenous buffers (Fig. 3*D*), increased activity of both kinases in EGTA-loaded cells was suppressed by blocking mitochondrial O_2^- production with rotenone–oligomycin (Fig. 4*C*, rightmost bar of each set). Together, the results indicate that Ca accumulation by peripheral mitochondria is necessary and sufficient for O_2^--mediated enhancement of CaMKII and PKC activities.

Two distinct mechanisms for superoxide enhancement of kinase activity

Phosphorylated CaMKII is known to be dephosphorylated directly by the protein phosphatases PP1 and PP2A and indirectly by PP2B (or calcineurin) (Kasahara et al., 1999; Soderling and Derkach, 2000; Winder and Sweatt, 2001). Considering the general sensitivity of protein phosphatases to oxidizing agents (Wang et al., 1996; Winder and Sweatt, 2001; Dröge, 2002), as well as previous evidence linking phosphatase activity to CREB levels (Bito et al., 1996; Hongpaisan et al., 2003), we used pharmacological approaches to investigate the possibility that O_2^- inhibition of phosphatases underlies enhanced protein kinase activity. Specifically, different concentrations of OA were used to differentiate the activity of PP2A [which is strongly inhibited by low (20 nM) OA] from PP1 [which is unaffected by low OA but inhibited at higher (1 μM) concentrations]; PP2B was blocked by FK506 (1 μM).

Under conditions in which O_2^- production is normal, i.e., in the absence of respiratory blockers, phosphatase inhibitors had little or no effect on the expected high-frequency stimulus-induced increase in CaMKII autophosphorylation, although blockade of PP2A (low OA) did result in a small elevation (Fig. 5*A*, left bar group). When mitochondrial respiration is blocked by rotenone–oligomycin, a suppression of stimulus-induced increases in CaMKII autophosphorylation is normally observed (Figs. 3*D*, 5*A*; in the latter, compare gray bars of left and middle groups). This suppression was not just abolished by all three inhibitors (Fig. 5*A*, middle bar group); it was converted to an enhancement compared with high-frequency stimulation without drugs (Fig. 5*A*, compare black bars, left and middle bar groups). The results are consistent with the following scheme: mitochondrial O_2^- normally generated during high-frequency

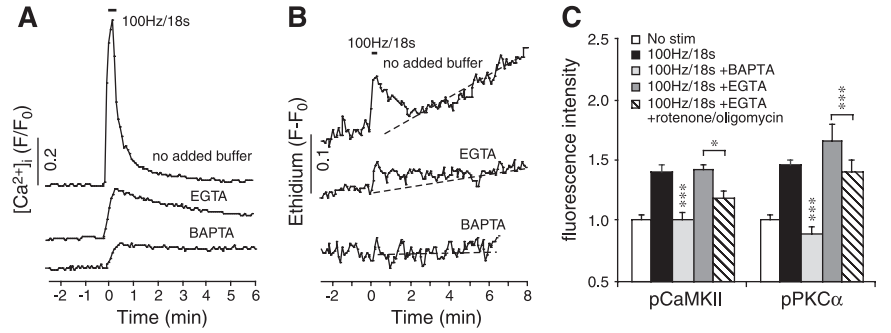

Figure 4. Superoxide that affects protein kinase activity is produced by subplasmalemmal mitochondria. *A*, Global Ca^{2+} transients induced by 100 Hz/18 sec were essentially suppressed in neurons loaded with the Ca^{2+} chelators BAPTA-AM (1 mM) or EGTA-AM (3 mM). *B*, Normal increases in O_2^- induced by 100 Hz/18 sec were completely abolished in neurons loaded with BAPTA, which is very effective for reducing Ca^{2+} elevations near influx channels. In contrast, stimulus-induced O_2^- production in the presence of EGTA, which is far less effective near the plasma membrane, was significantly but not completely abolished. The results suggest that a $[Ca^{2+}]_i$ increase in subplasma membrane regions is important for O_2^- production. *C*, At 3 min after stimulation, BAPTA completely blocked stimulus-induced increases in CaMKII and pPKCα activities, whereas EGTA was ineffective. In EGTA-loaded cells, stimulated increases in kinase activities were still sensitive to rotenone–oligomycin. Symbols indicating statistical significance are as defined in Figure 2.

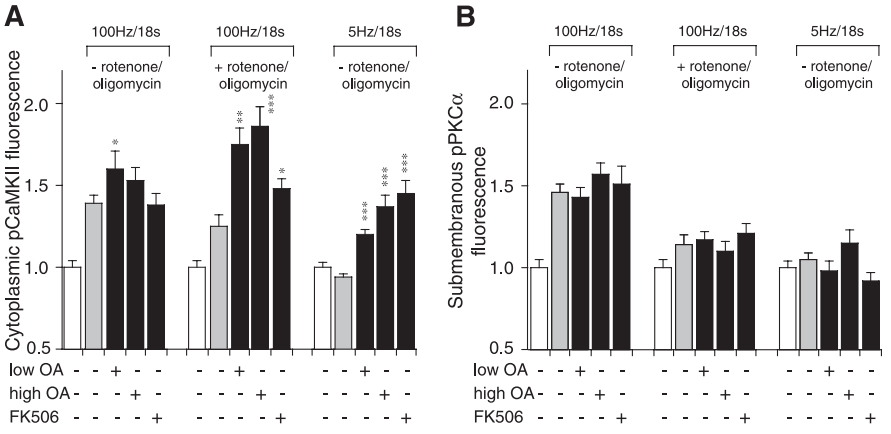

Figure 5. Mechanisms of kinase upregulation by mitochondrial superoxide. *A*, Inhibition of protein phosphatases by mitochondrial O_2^- promotes CaMKII activation. Superoxide-enhanced CaMKII autophosphorylation activated by high-frequency stimulation (100 Hz/18 sec) was not affected by inhibiting PP1 (high OA, 1 μM) or calcineurin (FK506) and was only weakly elevated by inhibiting PP2A (low OA, 20 nM) (left bar group). The low level of OA-sensitive PP2A activity observed here indicates that mitochondrial O_2^- suppressed but did not abolish phosphatase activity and is consistent with the idea that the effect of O_2^- is mainly modulatory. All three blockers not only reversed the suppression of CaMKII autophosphorylation normally observed in the presence of rotenone–oligomycin (compare white bars, gray bars; see also Fig. 3*D*) but even enhanced autophosphorylation compared with 100 Hz/18 sec stimulation without drugs (middle bar group); blocking PP1 was particularly effective. In neurons activated by weak stimulation (5 Hz/180 sec), pharmacological block of any of these phosphatases leads to enhancement of CaMKII phosphorylation (right bar group) because, under these conditions, protein phosphatases are not already suppressed by mitochondrially produced superoxide. Symbols indicating statistical significance are as defined in Figure 2. *B*, None of the three protein phosphatase inhibitors had an effect on pPKCα activation, regardless of stimulation protocol or the presence of respiratory inhibitors. Therefore, mitochondrial O_2^- must activate pPKCα (see Fig. 3*D*) by a mechanism(s) other than phosphatase inhibition.

stimulation suppresses the activities of protein phosphatases. Consequently, phosphatase blockers have minimal effect because these enzymes are already essentially inhibited. When mitochondrial respiration is inhibited, however, the O_2^- block is relieved; now, CaMKII autophosphorylation is opposed by the activity of all three phosphatases, leading to a lower steady-state level of phosphorylated CaMKII (Fig. 3*D*). Under such conditions, CaMKII phosphorylation will be sensitive to, and enhanced by, phosphatase inhibition, as observed (Fig. 5*A*, middle bar group).

This scheme further predicts that CaMKII autophosphorylation levels should be low under conditions, for example, of weak stimulation (5 Hz/180 sec) (Fig. 2*C*), that minimize respiration and O_2^- production, and therefore relieve the stimulus-dependent O_2^- block on phosphatase activity. In these circumstances, CaMKII autophosphorylation should be upregulated in the presence of phosphatase blockers, as observed (Fig. 5*A*, right bar group)

In resting cells, protein kinase C is already in a phosphorylated, phosphatase-resistant state and, as already mentioned, depends on translocation, not further phosphorylation, for stimulus-dependent activity enhancement (Newton, 1997). As expected, pharmacological inhibition each of the three protein phosphatases had no effect on the translocation of phosphorylated PKCα to the plasma membrane in low frequency-stimulated neurons or in high frequency-stimulated neurons in the presence or absence of rotenone–oligomycin (Fig. 5*B*). In addition, global levels of pPKCα, i.e., measured over the nucleus plus cytoplasm including subplasmalemmal regions, after 100 Hz/18 sec stimulation were not affected when O_2^- levels were enhanced by DETC or suppressed by SPBN, NAc, and MnTMPyP (data not shown), consistent with a mechanism that depends on translocation.

Repetitive strong calcium entry induces prolonged superoxide generation and PKA activation

In general, stable, long-term potentiation of hippocampal neurons, lasting for several hours or even days, is typically produced by multiple, repetitive trains of high-frequency stimuli (Impey et al., 1996, 1998; Wu et al., 2001; Lisman et al., 2002). We therefore examined O_2^- production after a repetitive, closely spaced high-frequency stimulus train (100 Hz/18 sec, three trains at 1 min intervals) and found that such a stimulation protocol evoked the expected set of $[Ca^{2+}]_i$ spikes in parallel with O_2^- elevations that were not markedly different from those induced by a single high-frequency stimulus (Fig. 6*A*,*B*; compare Figs. 6*B*, top trace, 1*D*, top trace). In contrast, a similar stimulus train that was more widely spaced (100 Hz/18 sec, three trains at 5 min intervals) elicited larger increases in O_2^- that were prolonged and appeared to be additive (Fig. 6*A*,*B*, bottom traces).

PKA, which plays an important role in plastic responses such as LTP and gene expression (Soderling and Derkach, 2000), is activated by multiple trains of high-frequency stimuli (Impey et al., 1996). Because the data presented here show that similar stimulus trains also preferentially upregulate mitochondrial O_2^- production (Fig. 6*B*, bottom trace), the effect of mitochondrial O_2^- on PKA activity was investigated. Ca^{2+} influx increases cAMP production and promotes release of the catalytic subunits of PKA from inactive heterotetramers, which are now free to phosphorylate target proteins in a variety of locations, including the nucleus (Hagiwara et al., 1993; Griffioen and Thevelein, 2002). Tak-

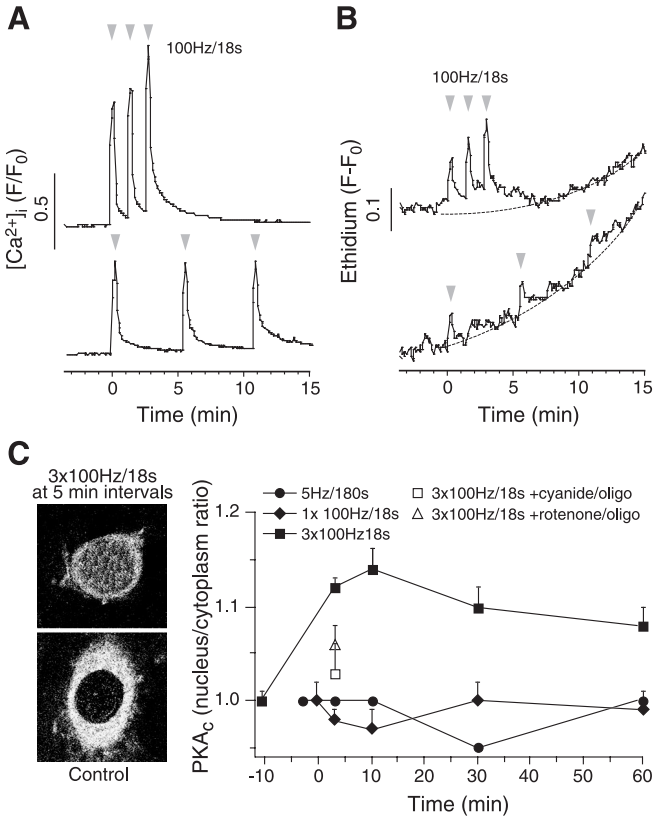

Figure 6. PKA activity is enhanced by the high superoxide levels reached during repetitive strong calcium entry. *A*, *B*, A train of three strong (100 Hz/18 sec) stimuli at 1 min intervals evoked the expected Ca^{2+} transients and O_2^- elevation (top traces). These responses were not markedly different from those induced by a single stimulus. A similar train (100 Hz/18 sec) at 5 min intervals evoked comparable Ca^{2+} spikes but led to further enhancement and prolongation of O_2^- elevations (bottom traces). *C*, A train of three strong (100 Hz/18 sec) stimuli at 5 min intervals (filled squares), but not single 100 Hz/18 sec or 5 Hz/180 sec pulses (filled diamonds and circles, respectively), led to a long-term increase in the fraction of dissociated catalytic Cα subunit that translocated to the nucleus, as estimated by quantitative immunocytochemistry (representative confocal images, left panels). Presumably, this reflects increased PKA activity. The increase in nuclear PKA immunofluorescence was sensitive to inhibition of mitochondrial O_2^- production by rotenone–oligomycin or cyanide–oligomycin (open symbols).

ing the nuclear import of the dissociated Cα subunit of PKA (PKA_C,) as a measure of plasticity-related PKA activity, we determined by quantitative immunocytochemistry the fraction of PKA_C that was translocated to the nucleus. Over the hour after stimulation with three 100 Hz/18 sec trains at 5 min intervals, PKA_C immunoreactivity in the nucleus increased relative to that in the cytoplasm (Fig. 6*C*). A similar increase in nuclear PKA_C immunoreactivity was not triggered by single high-frequency (100 Hz/18 sec) or low-frequency (5 Hz/180 sec) pulses (Fig. 6*C*).

Direct evidence for O_2^- modulation of PKA activity was obtained using pharmacological approaches analogous to those described for CaMKII. The increase in PKA activity induced by three episodes of 100 Hz/18 sec stimulation at 5 min intervals was blocked by inhibition of mitochondrial O_2^- production with rotenone–oligomycin or cyanide–oligomycin (Fig. 6*C*). In addition, only the SOD inhibitor DETC, which increases O_2^- levels, promoted PKA activity in response to a single 100 Hz/18 sec stimulus, whereas O_2^--suppressing reagents had no effect (Fig. 7*A*). With a single 5 Hz/180 sec stimulus, all drugs were ineffective (data not shown). In contrast, the increased PKA activity induced by repetitive strong stimuli (Fig. 6*C*) was further enhanced by DETC but was strongly inhibited by reagents (SPBN, NAc, and MnTMPyP) that reduce O_2^- levels (Fig. 7*B*). These results indicate that mitochondrially derived O_2^- production enhances PKA activity, but this effect requires prolonged and larger O_2^- elevations compared with CaMKII and pPKCα upregulation.

To determine whether the mechanism of PKA upregulation paralleled those for CaMKII or PKC, we used the same panel of protein phosphatase inhibitors used previously, e.g., in Figure 5. Inhibition of PP1 and PP2A by OA had no effect on PKA activity induced by three well spaced 100 Hz/18 pulses, whereas blocking PP2B with FK506 showed a slight but statistically insignificant tendency to enhance the increase in PKA activity (Fig. 7*C*, left bar group). When mitochondrial O_2^- production was blocked by rotenone–oligomycin, however, the inhibitor sensitivity of phosphatases was unmasked (Fig. 7*C*, right bar group); the effects are qualitatively similar to those observed for CaMKII (Fig. 5). Although the mechanism underlying these observations is not clear, we speculate that mitochondrial O_2^- may modulate PKA activity, at least in part, by inhibiting the dephosphorylation of RII regulatory subunits, which therefore promotes the proper localization and availability of catalytic subunits.

Discussion

In this study, we present data that point to a novel set of pathways for modulating the activity of the regulatory protein kinases CaMKII, PKC, and PKA. The existence of these pathways, commonly linking the strength of Ca^{2+} entry to mitochondrial production of ROS and consequently to the optimized upregulation of these kinases, strongly suggests that mitochondrial respiration can play an important role in modulating synaptic activity-dependent plastic responses, such as enhanced gene expression and LTP. Considering that the mechanism described here is analogous to a pathway for upregulating the activity of the nuclear transcription factor CREB (Bito et al., 1996; Hongpaisan et al.,

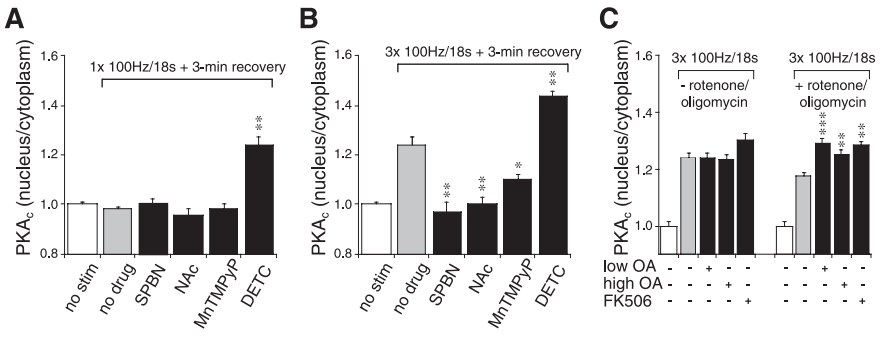

Figure 7. Mitochondrial superoxide-mediated inhibition of phosphatases enhances PKA activity. *A*, During a single 100 Hz/18 sec stimulus, reagents that scavenge O_2^- did not affect PKA activity. The SOD inhibitor DETC, for reasons explained in the legend to Figure 2, promoted PKA activity. *B*, Increased PKA activity induced by three 100 Hz/18 sec pulses at 5 min intervals was essentially inhibited by agents that reduced O_2^- levels but was enhanced by DETC. This pattern is similar to those for CaMKII and pPKCα after a single 100 Hz/18 sec stimulus (Fig. 2*C*,*D*). *C*, Inhibiting PP1, PP2A, or PP2B had little or no effect on PKA elevations induced by multiple stimuli (3 100 Hz/18 sec pulses at 5 min intervals) (left bar group). When mitochondrial respiration is blocked, however, phosphatase inhibitor sensitivity is unmasked (right bar group). As with CaMKII (Fig. 5*A*), enhancement of PKA activity can be explained by mitochondrial O_2^- inhibition of deactivating protein phosphatases. Symbols indicating statistical significance are as defined in Figure 2.

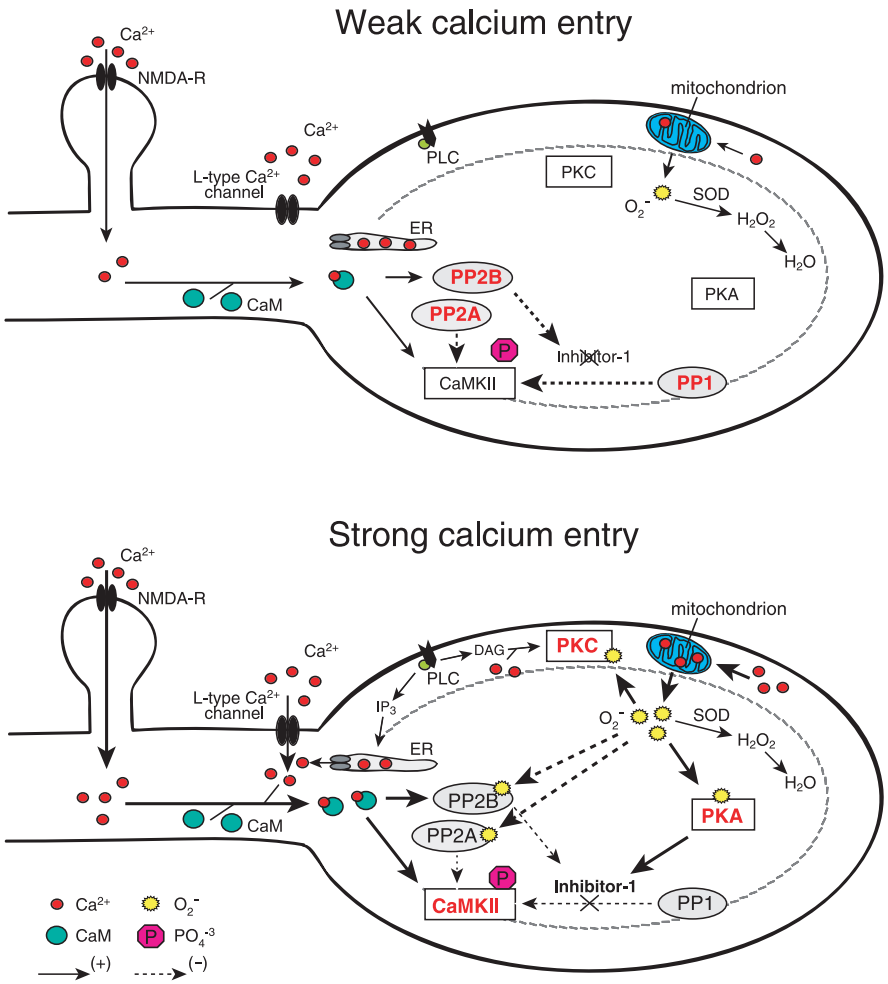

Figure 8. Diagrams comparing the effects of weak and strong calcium entry on kinase activity. After activity-induced elevation of cytosolic Ca^{2+}, Ca^{2+}/CaM-dependent autophosphorylation of CaMKII is accelerated to a degree that depends on the size, location, and timing of the elevation, but CaMKII dephosphorylation is also promoted by the stimulatory effect of Ca^{2+}/CaM-dependent PP2B (calcineurin), acting indirectly through the PP2B-dependent repressor protein Inhibitor 1, on PP1. [PP2A is also phosphorylated and activated by a Ca^{2+}-dependent kinase, but, in this case, the kinase is casein kinase 2α (Heriche et al., 1997).] When Ca^{2+} entry is submaximal (top), the activity of PP1, together with basal PP2A activity, establishes a steady-state level of active CaMKII that is relatively low. In addition, the stimulus is too weak to significantly activate PKA. Weak Ca^{2+} entry also stimulates some mitochondrial O_2^- production, but the amount is small enough that it is rapidly degraded by SOD. Red lettering indicates enhanced activity; solid arrows, stimulatory effects; dashed arrows, inhibitory effects. Line weights correlate with relative strength; crossed arrows indicate blocking effects. After strong Ca^{2+} entry (bottom), CaMKII is upregulated because mitochondria in high-Ca^{2+} microdomains close to subplasmalemmal sites of Ca^{2+} entry accumulate large amounts of Ca^{2+} and increase their production of O_2^-, which in turn deactivates PP2B and PP2A and suppresses the dephosphorylation of CaMKII. This leads to an elevated steady-state level of phosphorylated CaMKII. Similar O_2^--modulated, phosphatase-dependent mechanisms upregulate PKA, which in addition further elevates CaMKII because the suppressor protein Inhibitor 1, which blocks PP1 and therefore CaMKII deactivation, is phosphorylated (activated) by PKA (Soderling and Derkach, 2000). In contrast to CaMKII and PKA, pPKCα translocation and subsequent upregulation does not depend on phosphatase activity. The mechanism by which O_2^- contributes to Ca^{2+}-mediated pPKCα activation is not known, but one possibility envisions direct modulation by mitochondrial O_2^--induced thiol oxidation (Knapp and Klann, 2000). DAG, Diacylglycerol; ER, endoplasmic reticulum; PLC, phospholipase C.

2003) and is also consistent with preliminary evidence indicating that similar mechanisms affect ERK/MAPK cascades (extracellular signal-regulated kinase/mitogen-activated protein kinase) (Hongpaisan et al., 2002), we anticipate that kinase upregulation by Ca^{2+}-dependent mitochondrial ROS may prove to be a general feature of activity-dependent plasticity. If this is the case, it represents yet another way that mitochondria, acting as Ca^{2+} sensors, play a role in signal transduction (Friel, 2000; Rizzuto et al., 2000).

The scheme described here is novel in two respects. First, it is generally accepted that, in non-neuronal cell types, physiological stimuli can lead to transient elevation of ROS, particularly O_2^- and H_2O_2, which in turn function as messengers for a variety of signaling cascades (Griendling et al., 2000; Hancock et al., 2001; Dröge, 2002). In neurons, however, in which ROS are generally considered to be injurious molecules, evidence for any signaling role is sparse and only just emerging (for review, see Knapp and Klann, 2002). Second, and although mitochondrial respiration is usually a significant source of ROS, mitochondrial ROS are generally thought not to be involved in cell signaling (Hancock et al., 2001; Dröge, 2002). Again, however, recent studies indicate that, in some circumstances, mitochondrial O_2^- does play such a role; examples include gene expression mediated by the nuclear factors NF-κB and CREB (Josse et al., 1998; Werner and Werb, 2002; Hongpaisan et al., 2003). Present results add to the growing body of evidence indicating that ROS signaling is significant in neurons and that, in this case, mitochondria are a major, perhaps even the predominant, source of signaling radicals. Overexpressing Mn-SOD appears to target this enzyme mainly to mitochondria; thus, the consequent kinase-suppressing effects of elevated Mn-SOD provide additional support for the idea that the active ROS is mitochondrial in origin.

Manipulating Mn-SOD activity also addresses the question of which ROS is the dominant effector molecule. Because O_2^- is rapidly reduced to peroxide, H_2O_2 is often considered functionally more important than O_2^- (Inoue et al., 2003). Indeed, present pharmacological experiments correlating O_2^- inhibition and O_2^- scavenging with kinase suppression could equally well reflect the activity of O_2^- or of downstream H_2O_2. However, enhancing SOD activity by overexpression or by application of a mimetic should accelerate the conversion of O_2^- to H_2O_2. Thus, the enhanced oxidative activity observed under these conditions argues in favor of O_2^-. Second, and as mentioned previously (Hongpaisan et al., 2003), strong stimulation produced no response from the H_2O_2 indicator 2′7′-dichlorodihydrofluorescein. On balance, it appears that, at least in hippocampal cells, the evidence favors O_2^- as the principal ROS modulator of Ca^{2+}-dependent signaling cascades.

Mitochondrial Ca^{2+} uptake is graded with stimulus strength and duration. Typically, sustained $[Ca^{2+}]_i$ increases into the micromolar range are necessary to induce the elevation of free intramitochondrial Ca^{2+} that is required for maximal activation of Ca^{2+}-sensitive mitochondrial dehydrogenases (Hajnoczky et al., 1995) and therefore of ATP and, presumably, O_2^- production. Beyond dependence on $[Ca^{2+}]_i$, there is growing awareness that

intracellular Ca^{2+} dynamics has a significant spatial component, at least in part attributable to the fact that the Ca^{2+}-handling characteristics of cell organelles are themselves strongly dependent on their spatial location and local environment (Friel, 2000). Mitochondrial Ca^{2+} uptake, in particular, is notably more robust in those organelles situated close to a Ca^{2+} source (Rizzuto et al., 1998; Montero et al., 2000), a fact that, in the case of neurons, favors uptake by mitochondria near plasma membrane Ca^{2+} channels (Hongpaisan et al., 2001). Our experiments using EGTA versus BAPTA to tease out the response of spatially peripheral mitochondria (Deisseroth et al., 1996) indicate, as previously inferred (Bito et al., 1996), that this theme is recapitulated in stimulated hippocampal neurons. Restricting strong Ca^{2+} loading to peripheral mitochondria has the effect of spatially localizing Ca^{2+} signals, including $[Ca^{2+}]_i$- and endoplasmic reticulum-dependent signals (Hongpaisan et al., 2001), to subplasmalemmal regions. Such compartmentalization may well represent a strategy for optimizing the regulation of plasma membrane-associated assemblies, e.g., PKC- or MAPK-related complexes. The idea that subplasmalemmal localization reflects the privileged nature of such regions is reinforced by the existence of additional mechanisms to optimize subplasmalemmal signaling. As one pertinent example, certain non-mitochondrial routes of O_2^- production, notably the NADPH oxidase pathway, depend on membrane proteins that by their nature maximize O_2^- at the plasma membrane (Knapp and Klann, 2002).

There appear to be at least two mechanisms by which elevated O_2^- influences kinase activity. For kinases whose activity is regulated by phosphorylation, the apparent enhancing effect of elevated O_2^- is in fact a suppression of dephosphorylation by one or more of the protein phosphatases PP1, PP2A, and PP2B, all of which are directly or indirectly inhibited by O_2^- (Klann and Thiels, 1999; Soderling and Derkach, 2000; Winder and Sweatt, 2001). This mechanism, which has been characterized previously in the Ca^{2+} entry-dependent stimulation of CREB phosphorylation (Bito et al., 1996; Hongpaisan et al., 2003), is essentially a negative regulation that resets the steady state of target kinases at a higher level (Lonze and Ginty, 2002). The observation that this general mechanism operates to stabilize the active, phosphorylated forms of pCREB (and probably nuclear CaMKIV), CaMKII, PKA, and ERK1/2 suggests that it is globally important. In contrast to the phosphatase-dependent scheme just described, both O_2^- and H_2O_2 are known, in many cell types including hippocampal neurons, to directly activate not only PKC but also protein tyrosine kinases and members of the MAPK family (Dröge, 2002; Knapp and Klann, 2002).

Summary of kinase activation pathways

The pathways involved in Ca^{2+}-dependent kinase activation are summarized in Figure 8. After low-frequency stimulation, weak Ca^{2+} entry raises global $[Ca^{2+}]_i$ levels and mobilizes Ca^{2+}/CaM only modestly. Although CaMKII autophosphorylation may be stimulated to some extent, small Ca^{2+} elevations favor phosphatase deactivation of CaMKII through the constitutive action of PP2A and by indirectly upregulating, through PP2B-dependent dephosphorylation (suppression) of Inhibitor 1, the dephosphorylation of CaMKII by PP1 (Soderling and Derkach, 2000). Furthermore, small $[Ca^{2+}]_i$ increases do not significantly promote mitochondrial Ca^{2+} uptake and ATP synthesis, so that any O_2^- produced as a byproduct of basal respiration is completely destroyed by SOD and other intrinsic antioxidants. Effective ROS scavenging under basal or weak stimulus conditions explains why ROS modulation of kinase activity is stimulus strength dependent rather than constitutive.

In contrast to weak Ca^{2+} entry, even a brief high-frequency stimulation is sufficient to induce strong Ca^{2+} entry via NMDA receptors and L-type Ca^{2+} channels (Bading, 2000), thereby elevating Ca^{2+}/CaM and promoting both stimulating (CaMKII autophosphorylation) and suppressing (PP2B dephosphorylation) forks of the main CaMKII regulatory pathways (Tokuda and Hatase, 1998; Winder and Sweatt, 2001; Lisman et al., 2002). Consequently, CaMKII activity is not fully developed.

So far, mitochondrial function has not been invoked, but independent Ca^{2+}-sensitive processes draw this organelle into the scheme. Strong Ca^{2+} entry leads to large $[Ca^{2+}]_i$ elevations in subplasma membrane regions of cytoplasm, which activates robust Ca^{2+} uptake and O_2^- generation by mitochondria located in these regions. Mitochondrial O_2^- suppresses PP2A and PP2B directly, and PP1 indirectly, as described. The net result is a further elevation and stabilization, an optimization, of active CaMKII because its dephosphorylation is suppressed. These same principles apply to other kinases that are modulated by phosphorylation, e.g., PKA, whose activity is increased by a mechanism (not illustrated for simplicity) that is analogous to that for CaMKII in the sense that phosphatase inhibition (in this case of RII dephosphorylation) underlies the effect (Griffioen and Thevelein, 2002). Strong Ca^{2+} entry also activates pPKCα, but here the stimulatory effect of O_2^- is thought to occur via a conformation change induced by thiol oxidation (Knapp and Klann, 2000). Regardless of mechanistic details, however, pPKCα upregulation illustrates an entirely different pathway for coupling ROS elevations to Ca^{2+} signals.

Last, it is necessary to note that the ROS-dependent pathways discussed here are primarily, if not exclusively, modulatory. The "fine tuning" nature of this form of regulation is apparent in the size of the responses, typically 50–100%. That said, the broad applicability of these pathways suggests that they are of global significance. In particular, the general strategy for upregulating the activity of phosphorylation-dependent proteins, namely, inhibition by mitochondrial O_2^- of phosphatase-dependent inactivation, is not limited to kinases but appears to apply to certain transcription factors, e.g., pCREB (Bito et al., 1996; Hongpaisan et al. 2003) and to some steps in the ERK/MAPK cascade (Hongpaisan et al., 2002), implying that this is a general and wide-ranging mechanism for modulating Ca^{2+} signaling.

References

Bading H (2000) Transcription-dependent neuronal plasticity the nuclear calcium hypothesis. Eur J Biochem 267:5280–5283.

Bindokas VP, Jordan J, Lee CC, Miller RJ (1996) Superoxide production in rat hippocampal neurons: selective imaging with hydroethidine. J Neurosci 16:1324–1336.

Bito H, Deisseroth K, Tsien RW (1996) CREB phosphorylation and dephosphorylation: a Ca^{2+}- and stimulus duration-dependent switch for hippocampal gene expression. Cell 87:1203–1214.

Bito H, Deisseroth K, Tsien RW (1997) Ca^{2+}-dependent regulation in neuronal gene expression. Curr Opin Neurobiol 7:419–429.

Brocard JB, Tassetto M, Reynolds IJ (2001) Quantitative evaluation of mitochondrial calcium content in rat cortical neurones following a glutamate stimulus. J Physiol (London) 531:793–805.

Budd SL, Castilho RF, Nicholls DG (1997) Mitochondrial membrane potential and hydroethidine-monitored superoxide generation in cultured cerebellar granule cells. FEBS Lett 415:21–24.

Deisseroth K, Bito H, Tsien RW (1996) Signaling from synapse to nucleus: postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity. Neuron 16:89–101.

De Koninck P, Schulman H (1998) Sensitivity of CaM kinase II to the frequency of Ca^{2+} oscillations. Science 279:227–230.

Dröge W (2002) Free radicals in the physiological control of cell function. Physiol Rev 82:47–95.
Friel DD (2000) Mitochondria as regulators of stimulus-evoked calcium signals in neurons. Cell Calcium 28:307–316.
Griendling KK, Sorescu D, Lassègue B, Ushio-Fukai M (2000) Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. Arterioscler Thromb Vasc Biol 20:2175–2183.
Griffioen G, Thevelein JM (2002) Molecular mechanisms controlling the localisation of protein kinase A. Curr Genet 41:199–207.
Hagiwara M, Brindle P, Harootunian A, Armstrong R, Rivier J, Vale W, Tsien R, Montminy MR (1993) Coupling of hormonal stimulation and transcription via the cyclic AMP-responsive factor CREB is rate limited by nuclear entry of protein kinase A. Mol Cell Biol 13:4852–4859.
Hajnoczky G, Robb-Gaspers LD, Seitz MB, Thomas AP (1995) Decoding of cytosolic calcium oscillations in the mitochondria. Cell 82:415–424.
Hancock JT, Desikan R, Neill SJ (2001) Role of reactive oxygen species in cell signalling pathways. Biochem Soc Trans 29:345–350.
Hardingham GE, Bading H (1999) Calcium as a versatile second messenger in the control of gene expression. Microsc Res Tech 46:348–355.
Hardingham GE, Bading H (2003) The yin and yang of NMDA receptor signalling. Trends Neurosci 26:81–89.
Hardingham GE, Arnold FJ, Bading H (2001) A calcium microdomain near NMDA receptors: on switch for ERK-dependent synapse-to-nucleus communication. Nat Neurosci 4:565–566.
Heriche JK, Lebrin F, Rabilloud T, Leroy D, Chambaz EM, Goldberg Y (1997) Regulation of protein phosphatase 2A by direct interaction with casein kinase 2α. Science 276:952–955.
Hongpaisan J, Pivovarova NB, Colegrove SL, Leapman RD, Friel DD, Andrews SB (2001) Multiple modes of calcium-induced calcium release in sympathetic neurons II: a $[Ca^{2+}]_i$- and location-dependent transition from endoplasmic reticulum Ca accumulation to net Ca release. J Gen Physiol 118:101–112.
Hongpaisan J, Winters CA, Andrews SB (2002) Mitochondrial and non-mitochondrial pathways for superoxide regulation of ERK1/2. Soc Neurosci Abstr 28:43.8.
Hongpaisan J, Winters CA, Andrews SB (2003) Calcium-dependent mitochondrial superoxide modulates nuclear CREB phosphorylation in hippocampal neurons. Mol Cell Neurosci 24:1103–1115.
Impey S, Mark M, Villacres EC, Poser S, Chavkin C, Storm DR (1996) Induction of CRE-mediated gene expression by stimuli that generate long-lasting LTP in area CA1 of the hippocampus. Neuron 16:973–982.
Impey S, Obrietan K, Wong ST, Poser S, Yano S, Wayman G, Deloulme JC, Chan G, Storm DR (1998) Cross talk between ERK and PKA is required for Ca^{2+} stimulation of CREB-dependent transcription and ERK nuclear translocation. Neuron 21:869–883.
Inoue M, Sato EF, Nishikawa M, Park AM, Kira Y, Imada I, Utsumi K (2003) Mitochondrial generation of reactive oxygen species and its role in aerobic life. Curr Med Chem 10:2495–2505.
Josse C, Legrand-Poels S, Piret B, Sluse F, Piette J (1998) Impairment of the mitochondrial electron chain transport prevents NF-κB activation by hydrogen peroxide. Free Radic Biol Med 25:104–112.
Kasahara J, Fukunaga K, Miyamoto E (1999) Differential effects of a calcineurin inhibitor on glutamate-induced phosphorylation of Ca^{2+}/calmodulin-dependent protein kinases in cultured rat hippocampal neurons. J Biol Chem 274:9061–9067.
Klann E, Thiels E (1999) Modulation of protein kinases and protein phosphatases by reactive oxygen species: implications for hippocampal synaptic plasticity. Prog Neuropsychopharmacol Biol Psychiatry 23:359–376.
Knapp LT, Klann E (2000) Superoxide-induced stimulation of protein kinase C via thiol modification and modulation of zinc content. J Biol Chem 275:24136–24145.
Knapp LT, Klann E (2002) Role of reactive oxygen species in hippocampal long-term potentiation: contributory or inhibitory? J Neurosci Res 70:1–7.
Lisman J, Schulman H, Cline H (2002) The molecular basis of CaMKII function in synaptic and behavioural memory. Nat Rev Neurosci 3:175–190.
Lonze BE, Ginty DD (2002) Function and regulation of CREB family transcription factors in the nervous system. Neuron 35:605–623.
Lu Z, McLaren RS, Winters CA, Ralston E (1998) Ribosome association contributes to restricting mRNAs to the cell body of hippocampal neurons. Mol Cell Neurosci 12:363–375.
Montero M, Alonso MT, Carnicero E, Cuchillo-Ibanez I, Albillos A, Garcia AG, Garcia-Sancho J, Alvarez J (2000) Chromaffin-cell stimulation triggers fast millimolar mitochondrial Ca^{2+} transients that modulate secretion. Nat Cell Biol 2:57–61.
Newton AC (1997) Regulation of protein kinase C. Curr Opin Cell Biol 9:161–167.
Nicholls DG, Budd SL (2000) Mitochondria and neuronal survival. Physiol Rev 80:315–360.
Oancea E, Meyer T (1998) Protein kinase C as a molecular machine for decoding calcium and diacylglycerol signals. Cell 95:307–318.
Pivovarova NB, Hongpaisan J, Andrews SB, Friel DD (1999) Depolarization-induced mitochondrial Ca accumulation in sympathetic neurons: spatial and temporal characteristics. J Neurosci 19:6372–6384.
Rizzuto R, Pinton P, Carrington W, Fay FS, Fogarty KE, Lifshitz LM, Tuft RA, Pozzan T (1998) Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. Science 280:1763–1766.
Rizzuto R, Bernardi P, Pozzan T (2000) Mitochondria as all-round players of the calcium game. J Physiol (Lond) 529:37–47.
Sengpiel B, Preis E, Krieglstein J, Prehn JH (1998) NMDA-induced superoxide production and neurotoxicity in cultured rat hippocampal neurons: role of mitochondria. Eur J Neurosci 10:1903–1910.
Soderling TR, Derkach VA (2000) Postsynaptic protein phosphorylation and LTP. Trends Neurosci 23:75–80.
Tokuda M, Hatase O (1998) Regulation of neuronal plasticity in the central nervous system by phosphorylation and dephosphorylation. Mol Neurobiol 17:137–156.
Votyakova TV, Reynolds IJ (2001) $\Delta\Psi_m$-dependent and -independent production of reactive oxygen species by rat brain mitochondria. J Neurochem 79:266–277.
Wang X, Culotta VC, Klee CB (1996) Superoxide dismutase protects calcineurin from inactivation. Nature 383:434–437.
Werner E, Werb Z (2002) Integrins engage mitochondrial function for signal transduction by a mechanism dependent on Rho GTPases. J Cell Biol 158:357–368.
Whisler RL, Goyette MA, Grants IS, Newhouse YG (1995) Sublethal levels of oxidant stress stimulate multiple serine/threonine kinases and suppress protein phosphatases in Jurkat T cells. Arch Biochem Biophys 319:23–35.
Winder DG, Sweatt JD (2001) Roles of serine/threonine phosphatases in hippocampal synaptic plasticity. Nat Rev Neurosci 2:461–474.
Wu GY, Deisseroth K, Tsien RW (2001) Activity-dependent CREB phosphorylation: convergence of a fast, sensitive calmodulin kinase pathway and a slow, less sensitive mitogen-activated protein kinase pathway. Proc Natl Acad Sci USA 98:2808–2813.