Reactive oxygen species and nitric oxide mediate plasticity of neuronal calcium signaling

Olena Yermolaieva*, Nathan Brot†, Herbert Weissbach‡, Stefan H. Heinemann§, and Toshinori Hoshi*¶

*Department of Physiology and Biophysics, University of Iowa, Bowen Science Building, Iowa City, IA 52242; †Hospital for Special Surgery, Cornell University Medical Center, New York, NY 10021; ‡Department of Biological Sciences, Florida Atlantic University, Boca Raton, FL 33431; and §Arbeitsgruppe Molekulare und Zelluläre Biophysik am Klinikum der Friedrich-Schiller-Universität Jena, Drackendorfer Strasse 1, D-07747 Jena, Germany

Contributed by Herbert Weissbach, November 10, 1999

Reactive oxygen species (ROS) and nitric oxide (NO) are important participants in signal transduction that could provide the cellular basis for activity-dependent regulation of neuronal excitability. In young rat cortical brain slices and undifferentiated PC12 cells, paired application of depolarization/agonist stimulation and oxidation induces long-lasting potentiation of subsequent Ca^{2+} signaling that is reversed by hypoxia. This potentiation critically depends on NO production and involves cellular ROS utilization. The ability to develop the Ca^{2+} signal potentiation is regulated by the developmental stage of nerve tissue, decreasing markedly in adult rat cortical neurons and differentiated PC12 cells.

Reactive oxygen/nitrogen species (ROS/RNS) often are considered as tissue-damaging agents in connection with oxidative stress, aging, and neurodegenerative diseases (1), especially in the presence of elevated cytosolic Ca^{2+} (2). For instance, during reperfusion injury, excess oxidants may induce Ca^{2+} excitoxicity (3). However, oxidants such as superoxide anion (O_2^-), peroxynitrite ($ONOO^-$), and hydrogen peroxide (H_2O_2) are synthesized by the cell during its normal activity (4). Moreover, brain-imaging techniques (5) demonstrate that functionally active neurons show increased metabolic activity and oxygen consumption, resulting in higher levels of ROS/RNS. For example, visual stimulation causes the consumption of glucose and oxygen to rise in the human visual cortex (6, 7). This interdependence between the neuronal metabolic state and functional activity indicates a possible physiological role for ROS/RNS as potential regulators of neuronal activity. The idea of possible involvement of ROS and free radicals in electrical and developmental neuronal plasticity has been suggested recently (2, 8); however, no direct evidence at the cellular level has been presented.

Like other posttranslational modifications, such as phosphorylation, oxidation of amino acid residues in proteins promoted by ROS/RNS alters properties of a number of cellular proteins involved in neuronal excitability and Ca^{2+} signaling, from voltage-gated K^+ and Ca^{2+} channels to ryanodine receptor and calmodulin (9–14). Modulation of cytosolic Ca^{2+}-signaling pathways in neurons by ROS therefore could induce dramatic and long-term changes in the cellular excitability and neuronal activity. For example, increased cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_c$) often lead to activation of Ca^{2+}-dependent gene expression of regulatory factors such as the cAMP response element (CRE) and the CREB-binding protein, which often are considered to play roles in long-term information storage (15). The free radical and the second messenger nitric oxide (NO) also exerts its long-term effects on plasticity and development of nerve tissue interacting with Ca^{2+} signaling (16, 17). An extremely prolonged increase in $[Ca^{2+}]_c$, however, may lead to neuronal cell death (18), particularly during ischemia/reperfusion and oxidative stress (19). Therefore, oxidation promoted by ROS and free radicals potentially could alter the elements involved in Ca^{2+} homeostasis and long-term excitability to influence neuronal information storage at the single-cell, synaptic, and higher levels. In this report, we demonstrate that temporal pairing of depolarization/chemical stimulation and oxidation promoted by ROS potentiates cytosolic Ca^{2+} signaling in an activity- and NO-dependent manner and that this intracellular Ca^{2+} signal plasticity is developmentally regulated.

Materials and Methods

PC12 cells (CLONTECH) were grown in DMEM containing 85% horse serum, 10% FBS, 5% 2 mM L-glutamine, 100 mg/ml G418, 100 units/ml penicillin G, and 100 mg/ml streptomycin, at 10% CO_2, on poly-L-lysine-covered glass coverslips. Cells were loaded with 1 μM fura-2 AM (Molecular Probes) for 1 h, washed, and mounted in the chamber of the spectrofluorimeter (F-4500; Hitachi, Tokyo). The standard recording medium contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 mM glucose, and 10 mM Hepes, pH 7.4 (NaOH). High-K^+ medium contained an additional 30 mM KCl in place of NaCl. The chamber was constantly perfused with the recording medium. Measurements of $[Ca^{2+}]_c$ were performed essentially as described (20). To induce oxidation by O_2, the recording solution was bubbled with 100% O_2 for 10 min immediately before application. Application of H_2O_2 and O_2 at the concentrations used in this study had no significant effects on the fura-2 isobestic signal at 360 nm and did not alter the fura-2 responses to Ca^{2+}. For NO measurements, PC12 cells were incubated for 1 h at room temperature with 10 μM DAF-2 DA (Calbiochem) in the standard recording medium and washed, and NO levels were measured essentially as described by Kojima *et al.* (21). Basal level of NO production was measured before stimulation and subtracted from the measurements. For ROS/RNS measurements, PC12 cells were incubated for 30 min in the medium containing 5 μM dihydrorhodamine 123 (Calbiochem) and washed, and ROS/RNS production was measured in the spectrofluorimeter (λ_{ex} = 500 nm, λ_{em} = 530 nm). Basal level of ROS/RNS production was subtracted from the measurements.

Experiments with rats were carried out in accordance with the National Institutes of Health and the University of Iowa Animal Care and Use Guidelines. Rats (Long–Evans male; Harlan, Indianapolis) were decapitated, and the brain was removed rapidly and transferred to ice-cold saline containing 124 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 23 mM $NaHCO_3$, and 10 mM glucose, pH 7.4 (95% O_2/5% CO_2). The coronal slices were prepared by cutting the rostral end of the neocortex on a Brinkmann chopper. Slices were loaded with 5 μM fura-2 AM for Ca^{2+} measurements. The medium was bubbled with 20% O_2 for normoxia, 95% O_2 for oxidation, and 0% O_2 for hypoxia.

Abbreviations: ROS, reactive oxygen species; RNS, reactive nitrogen species; NGF, nerve growth factor; NOS, nitric oxide synthase; MsrA, methionine sulfoxide reductase; NaNP, sodium nitroprusside.

¶To whom reprint requests should be addressed. E-mail: hoshi@hoshi.org.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

The amount of met(O) in the tissues was determined by treating the extracted protein with cyanogen bromide, which destroys methionine but not methionine sulfoxide (22, 23), and then subjecting the treated and nontreated samples to HCl hydrolysis as described (24). Under this condition, met(O) is quantitatively reduced to methionine and the amount of met in the cyanogen-treated sample divided by the met content in the untreated sample represents the fraction of met(O) in the extract.

All reagents were from Sigma unless otherwise stated. The experiments were carried out at room temperature (23°C).

Results

To address the role of ROS/RNS in intracellular Ca^{2+} signaling contributing to neuronal information storage, we examined how pairing of depolarization/chemical stimulation and oxidation alters subsequent intracellular Ca^{2+} signals induced by depolarization. The rat pheochromocytoma (PC12) neurosecretory cell line shows electrical excitability and developmental regulation by nerve growth factor (NGF) (25), and these cells were used as a model neuron system in this study. Repeated applications of high-K^+-induced depolarization alone to either differentiated or undifferentiated PC12 cells elicited Ca^{2+} signals that are similar in amplitude, with no prolonged change in the basal $[Ca^{2+}]_c$ (Fig. 1*A*). An application of 0.03% H_2O_2 evoked a small Ca^{2+} transient, but did not affect the subsequent Ca^{2+} responses to K^+ depolarization (Fig. 1*B*). However, a single, combined application of depolarization and oxidation by H_2O_2 (0.03%) to undifferentiated PC12 cells markedly potentiated the subsequent Ca^{2+} signals in response to K^+ depolarization (Figs. 1*C* and 2*A*). After this single, paired application of depolarization and oxidation, subsequent depolarization-induced Ca^{2+} signals developed faster and their amplitudes were dramatically greater (Fig. 2*A*). Typically, the Ca^{2+} signal was enhanced by 150–200% after the paired stimulation (Fig. 2 *A* and *B*, before and after). In addition, paired application of depolarization and oxidation induced a sustained elevation of the basal $[Ca^{2+}]_c$ (Fig. 2*A*). This potentiation of cytosolic Ca^{2+} response in both the depolarization-induced signal and the basal level was maintained as long as the data collection continued, up to 2.5 h, indicating that a single pairing of depolarization and oxidation could have a very long-lasting impact on $[Ca^{2+}]_c$. Similar Ca^{2+} response potentiation also was obtained by paring depolarization and O_2 (95%) instead of H_2O_2 (not shown).

The depolarization-induced $[Ca^{2+}]_c$ signal potentiation induced by pairing of depolarization and oxidation was reversed by a brief hypoxia treatment (10 min) (Fig. 2*A*), providing an insight into physiological changes in neuronal cells induced by brain ischemic episodes. Interestingly, PC12 cells differentiated by NGF (100 ng/ml) did not show any sign of such potentiation, although a small decrease in the amplitude of the Ca^{2+} signal after hypoxia often was observed (Fig. 2 *C* and *D*). Moreover, the sustained elevation of $[Ca^{2+}]_c$ observed after paired application of oxidation and depolarization in undifferentiated PC12 cells also was absent in the NGF-differentiated PC12 cells (Fig. 2*C*). Thus, potentiation of the $[Ca^{2+}]_c$ signal induced by temporal pairing of oxidation and depolarization is regulated both by hypoxia and cellular differentiation.

The potentiation of the Ca^{2+} signal induced by paired application of depolarization and oxidation is likely to be a widespread phenomenon in excitable cells. The Ca^{2+} signals from rat cortical neurons demonstrated strikingly similar potentiation. Cells from young rats (9–30 days old) showed a long-lasting, sustained elevation of basal $[Ca^{2+}]_c$ after a single, paired application of depolarization and oxidation with either H_2O_2 or O_2 (Fig. 2*E*). A 2- to 3-fold increase in subsequent depolarization-induced Ca^{2+} signal was observed (compare the first and third responses to K^+ depolarization, Fig. 2*E*). The neuronal rather

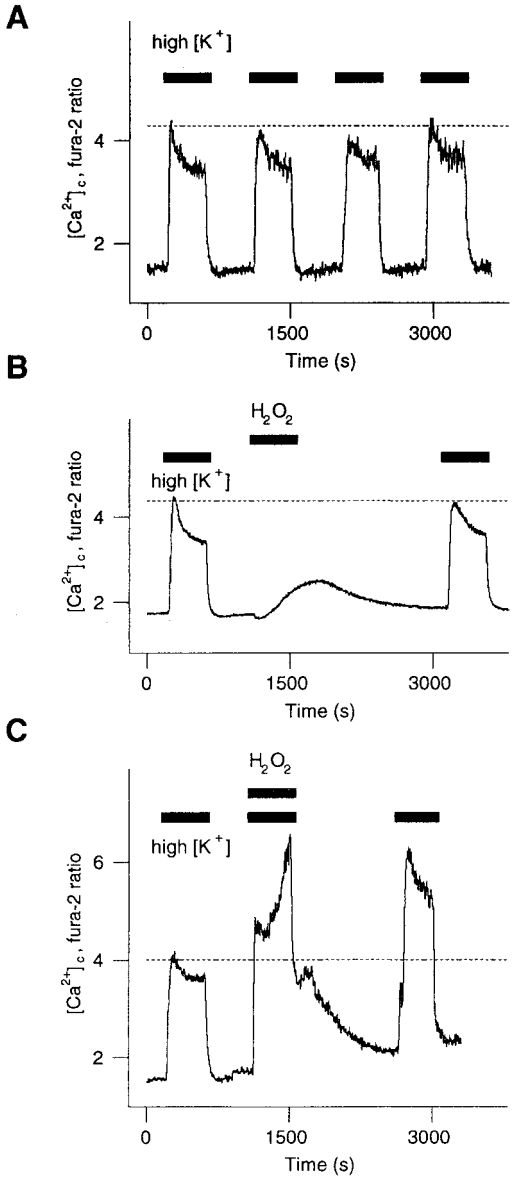

Fig. 1. Concurrent depolarization and oxidation potentiate Ca^{2+} signaling. (*A*) Fura-2-loaded undifferentiated PC12 cells were subjected to a series of 400-s K^+-depolarizations ($[K^+]_o$ = 35 mM) applied every 15 min. (*B*) Control K^+ depolarization followed by application of 0.03% H_2O_2 and the subsequent K^+ depolarization. (*C*) Control K^+ depolarization followed by combined application of high $[K^+]$ and H_2O_2 and the following K^+ depolarization.

than glial origin of this phenomenon was supported by the observation that incubation of the slices for 30 min with a glial-specific metabolic poison fluorocitrate (26) did not affect the Ca^{2+} signal potentiation by oxidation.

Similar to NGF-differentiated PC12 cells, the Ca^{2+} signal potentiation in the brain cells from young adult rats was much less pronounced, practically disappearing in the cells from the animals more than 40 days old (Fig. 2*F*). This observation suggests that the Ca^{2+} signal potentiation phenomenon may play roles in normal brain development. It is interesting to note that, in rat cortex development, the period between postnatal days 9 and 35 is considered to be the critical period for neuronal plasticity (27).

Oxidation potentiates the Ca^{2+} signaling when paired not only with depolarization but also, and even more dramatically, with

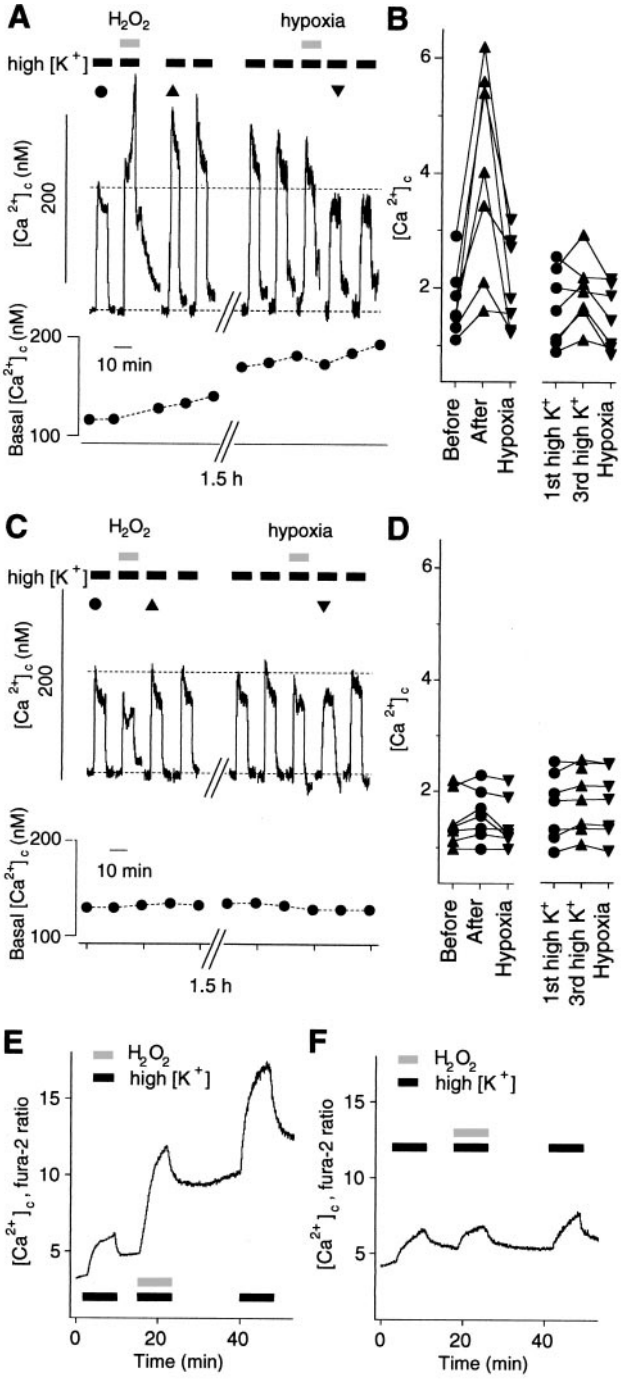

Fig. 2. Oxidation potentiates Ca^{2+} signaling in undifferentiated PC12 cells and young rat cortex. (*A*) After the control depolarization (●), the depolarizing stimulus was combined with oxidation (0.03% H_2O_2). Subsequent potentiated responses to K^+ depolarization (▲) were maintained for more than 1.5 h and reversed after 600-s hypoxia (▼). Changes in the basal Ca^{2+} level are shown in the lower trace. (*B*) Undifferentiated PC12 cells: amplitude of the K^+ depolarization before (●) and after (▲) potentiation and reversed by hypoxia (▼) from the series of seven experiments as in *A*. (*C*) Similar experiments as in *A* on NGF-differentiated PC12 cells. (*D*) NGF-treated PC12 cells; series of seven experiments as in *B*. (*E*) Young (12-day-old) rat cortical slice was loaded with fura-2 and $[Ca^{2+}]_c$ was monitored in response to K^+ depolarization, depolarization and oxidation, and subsequent K^+ depolarization. Depicted is a representative trace from the series of six experiments with rats between 9 and 30 days old; $[Ca^{2+}]_c$ is shown as fura-2 signal ratio at 340 nm and 380 nm. (*F*) Adult (52-day-old) rat cortical slice (experiment as in *E*) from the series of five experiments on rats between 40 and 110 days old.

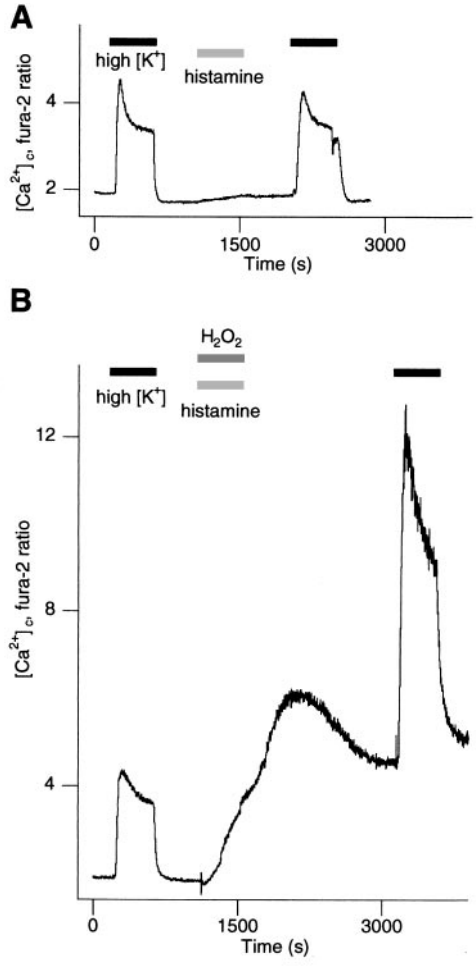

Fig. 3. Histamine and oxidation potentiate Ca^{2+} signaling. $[Ca^{2+}]_c$ was monitored in undifferentiated PC12 cells with fura-2. (*A*) Control K^+ depolarization followed by application of 100 μM histamine and subsequent K^+ depolarization. (*B*) $[Ca^{2+}]_c$ responses to K^+ depolarization before, during, and after combined application of 100 μM histamine and 0.03% H_2O_2. Representative results are from the series of four experiments.

chemical agonist stimulation. In PC12 cells, the neuromediator histamine evokes neurotransmitter release via activation of histamine receptors. In particular, activation of H2 receptors leading to stimulation of adenylyl cyclase activity and NO synthesis is implicated in regulation of cellular growth and differentiation (28). Histamine (100 μM) alone induced a very small Ca^{2+} signal in PC12 cells without affecting subsequent depolarization-induced Ca^{2+} signals (Fig. 3*A*), similar to application of H_2O_2 alone (Fig. 1*B*). However, when histamine and H_2O_2 were applied simultaneously, a long-lasting increase in $[Ca^{2+}]_c$ was observed (Fig. 3*B*). Furthermore, after one-time paring of histamine application and oxidation, Ca^{2+} signals in response to depolarization were dramatically greater, typically by 200–300% (Fig. 3*B*), and this potentiation lasted up to 2 h. Similar potentiation was observed when histamine and O_2 were applied concurrently (not shown). As found with pairing of depolarization and oxidation, the key requirement to induce the Ca^{2+} signal potentiation was the timing of pairing between chemical simulation of cells and oxidation. Only when these two stimuli were presented together was long-lasting enhancement of the Ca^{2+} signal observed.

H_2O_2 is a membrane-permeable oxidant, and, once in the intracellular compartment, it may react with oxidizable groups

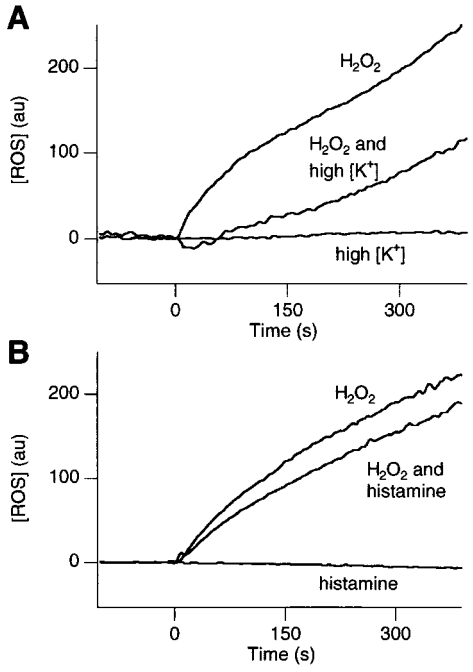

Fig. 4. ROS production by PC12 cells during oxidation and stimulation. Undifferentiated PC12 cells were loaded with dihydrorhodamine. (*A*) ROS concentration during application of 0.03% H_2O_2, K^+ depolarization, and the combined application. (*B*) ROS concentration during application of 0.03% H_2O_2, 100 μM histamine, and the combination of both. Representative results are from the series of four experiments for each treatment.

directly or react with biologically prevalent metal ions such as iron and copper (Fenton reaction) to form other strong oxidants such as the hydroxyl radical (4). We directly confirmed intracellular ROS/RNS production by using the fluorescent dye dihydrorhodamine (29) (Fig. 4). As expected, H_2O_2 application alone increased the level of ROS. Neither depolarization nor application of histamine alone induced any obvious change in the ROS level. However, when applied in combination with H_2O_2, these stimuli significantly reduced the increase in ROS caused by H_2O_2. Though seemingly unexpected, this result is in line with the possibility that ROS are consumed vigorously during depolarization/agonist stimulation to oxidize, directly or indirectly, the elements involved in cellular Ca^{2+} homeostasis. Consistent with this idea, in the presence of the reducing agent DTT (5 mM), paired application of depolarization and H_2O_2 did not increase the amplitude of subsequent responses to K^+ depolarization (Fig. 5*C*).

Application of histamine to PC12 cells leads to enhanced NO synthesis (28). Thus, we examined whether NO may be involved in potentiation of Ca^{2+} signaling. Using the fluorescent dye DAF-2 (21), we measured NO production in PC12 cells during depolarization or histamine application. Application of 100 μM histamine induced a significant increase in NO production (Fig. 5*A*). During depolarization, however, the NO signal decreased, indicating that cellular NO consumption may have increased. We cannot totally exclude the possibility that the NO synthase activity decreased, although, considering the stimulation of NOS activity by Ca^{2+} and calmodulin (30), the $[Ca^{2+}]_c$ rise during depolarization would be more likely to enhance the NOS activity. The important role of NO in oxidation-mediated Ca^{2+}-signaling potentiation is demonstrated further in Fig. 5*B*, where we were able to mimic the potentiation induced by paired application of histamine and H_2O_2 by using the NO donor sodium nitroprusside (NaNP). Application of 100 μM NaNP on its own failed to

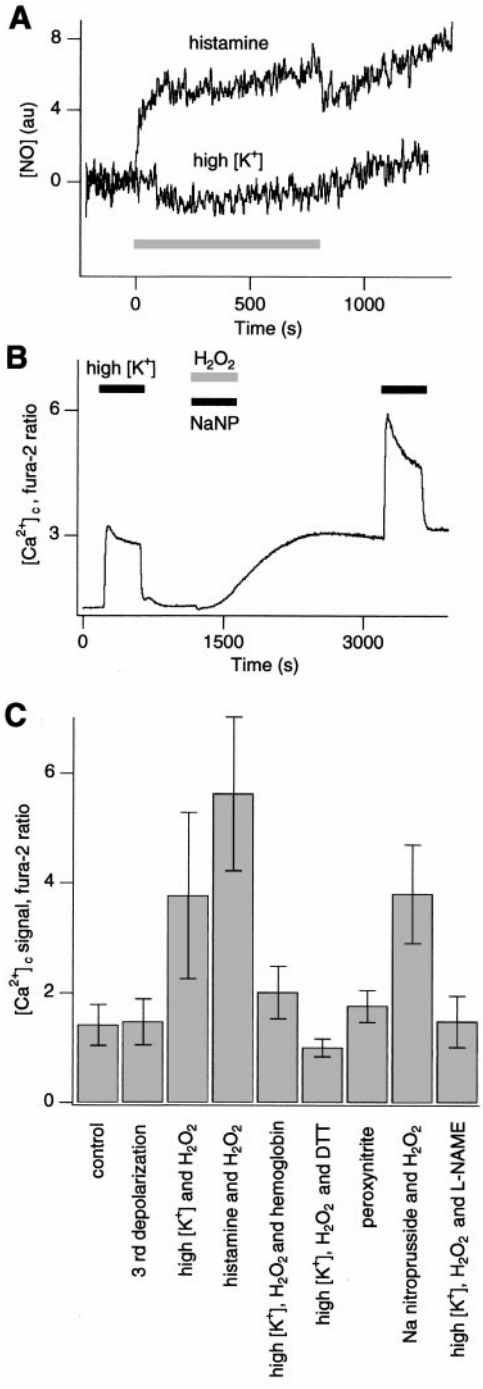

Fig. 5. NO is involved in potentiation of Ca^{2+} signaling. (*A*) NO level measured by using DAF-2 in PC12 cells during application of either 100 μM histamine or K^+ depolarization (shown by bar). Representative results are from the series of four experiments for each treatment. (*B*) Ca^{2+} signal in PC12 cells in response to K^+ depolarization before, during, and after simultaneous application of 100 μM NaNP and 0.03% H_2O_2. (*C*) Ca^{2+} signal amplitude in undifferentiated PC12 cells in response to K^+ depolarization after the treatment listed. Control, K^+ depolarization in naïve cells. Concentrations used were 35 mM K^+, 0.03% H_2O_2, 100 μM histamine, 0.1 mM hemoglobin, 5 mM DTT, 100 μM peroxynitrite, 100 μM NaNP, and 12 mM L-NAME. For each experiment, $n = 4$–7.

elevate basal $[Ca^{2+}]_c$, and no subsequent potentiation of Ca^{2+} signaling was observed ($[Ca^{2+}]_c$ rise during K^+ depolarization amplitude: 1.4 ± 0.3, control, and 1.4 ± 0.4, after NaNP applica-

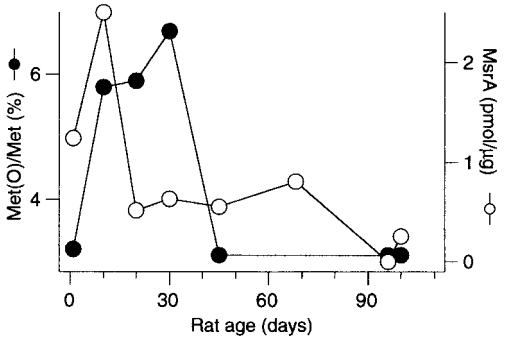

Fig. 6. Methionine-reducing system of the nerve cells throughout development and differentiation. Shown are oxidized methionine-to-total methionine ratio percentage (●) and MsrA activity (○) (pmol/μg protein) in the rat brain between postnatal days 1 and 100.

tion). However, when H_2O_2 was applied concurrently, potentiation of subsequent Ca^{2+} responses as well as the sustained rise in $[Ca^{2+}]_c$ were similar to the results obtained with histamine (Fig. 3*B*). Furthermore, in the presence of the NO scavenger hemoglobin (0.1 mM), paired application of histamine and oxidation did not potentiate the Ca^{2+} signal, and nitro-L-arginine methyl ester (L-NAME) (12 mM), the nitric oxide synthase (NOS) inhibitor, also abolished the Ca^{2+} signal potentiation (Fig. 5*C*). Taken together, these results suggest that pairing of depolarization/chemical stimulation and oxidation potentiates Ca^{2+} signaling in a NO-dependent manner. NO is often considered to react with O_2^- to form the strong oxidant and nitrating agent peroxynitrite, which is implicated in ROS-mediated tissue injury (31). Direct application of peroxynitrite, however, did not cause any significant potentiation of the depolarization-Ca^{2+} signal (Fig. 5*C*). Stimulation of NO production and the presence of ROS/RNS therefore appear to be the key requirements for enhancement of the Ca^{2+} transient and the sustained elevation of $[Ca^{2+}]_c$, which, in turn, could trigger a number of pathways leading to plasticity of Ca^{2+} signaling in neuronal cells.

ROS/RNS readily promote oxidation of cysteine and methionine residues in proteins. Methionine is oxidized to methionine sulfoxide [met(O)], and its reduction is catalyzed by the enzyme peptide methionine sulfoxide reductase (MsrA; ref. 32). Depending on the activity level of MsrA, the effect of methionine oxidation could be short or long lasting. Methionine oxidation-reduction has been shown to alter the properties of voltage-gated K^+ channels (11) and also implicated in up-regulation of voltage-gated Ca^{2+} channels (12). Thus, under oxidative conditions, the voltage-dependent pathway of Ca^{2+} entry into the excitable cell may be altered significantly. Furthermore, calmodulin, which plays critical roles in a large number of intracellular Ca^{2+}-dependent processes including Ca^{2+} homeostasis, is regulated by methionine oxidation in an age-dependent way (33). Our results presented earlier show that the Ca^{2+} signal potentiation is preferentially observed in undifferentiated PC12 cells and in young neurons (see Fig. 2). This suggests that the MsrA activity and/or met(O) content of the tissue may be regulated developmentally in a similar fashion. Thus, we examined whether methionine oxidation is involved in the Ca^{2+} signal potentiation by measuring the tissue met(O) content and the MsrA enzymatic activity (22, 24). The changes in the MsrA activity and the relative met(O) concentration in the rat brain during the first 100 days are shown in Fig. 6. Both the MsrA activity and the met(O) concentration increased during the first 10 postnatal days, and then the MsrA activity level decreased markedly, whereas relative met(O) decreased only after day 30, which is thought to represent the end of the critical time for the cortex development (27). Undoubtedly, considering the complexity of the cellular Ca^{2+} homeostasis, multiple factors contribute to the observed oxidative Ca^{2+} signal potentiation and its regulation by development. The results presented suggest, however, that methionine oxidation and MsrA are likely to play an important role.

Discussion

We have demonstrated here that, in rat cortical neurons and a model PC12 cell line, one-time pairing of oxidation and depolarization/chemical stimulus dramatically enhances subsequent cellular Ca^{2+} signaling, representing a form of activity-dependent modulation of cellular excitability. The temporal pairing of depolarization and oxidation stimuli appears critical to potentiate the Ca^{2+} response, thus providing a physiological link between functional activity and the metabolic state of the neuronal cell through modulation of the Ca^{2+} homeostasis. Oxidation-mediated Ca^{2+} signal plasticity is observed preferentially in the developmentally young cells, disappearing after differentiation in culture and also after a specific brain development stage. Interestingly, hyperoxia has been shown to induce differentiated neuronal phenotype in PC12, possibly via production of ROS (34). This is consistent with our results that ROS/RNS are directly involved in activity-dependent neuronal differentiation.

The molecular mechanism of oxidative potentiation of Ca^{2+} signaling most likely involves a number of intracellular proteins involved in Ca^{2+} homeostasis. Oxidation of amino acid residues in ion channels is expected to alter cellular excitability and Ca^{2+} signaling (for review, see ref. 9). Oxidation of intracellular Ca^{2+} release channel ryanodine receptor and its resulting increased activity (13) may account for the sustained increase in basal $[Ca^{2+}]_c$ observed during potentiation in our experiments (Fig. 2*A*). Calmodulin is also another prime target of oxidation. During development and aging, methionine residues in calmodulin undergo oxidation to met(O), resulting in the decreased ability of calmodulin to transduce Ca^{2+} signals (33). Oxidation of calmodulin would be expected to greatly contribute to the activity-dependent potentiation of Ca^{2+} signaling, as well as alter regulatory effects of calmodulin on ion channels, activation of plasma membrane Ca^{2+} pump, activation of NOS, and gene expression (35–38). Oxidative agents could have direct effects on gene transcription (39), and elevation of $[Ca^{2+}]_c$ during potentiation also may function as a trigger for gene transcription via the CREB-binding protein CBP (40, 41), contributing to long-term cellular plasticity.

The crucial role for NO in oxidative potentiation of Ca^{2+} signaling triggered by pairing of electrical/agonist stimulation and oxidation demonstrated here provides an insight into the cellular mechanisms involved in neuronal developmental plasticity. NO could influence its effectors by stimulating the cGMP-dependent signaling pathway and/or by acting as a weak radical. This study does not directly address how these two mechanisms contribute to the Ca^{2+} potentiation. Acting as a radical, NO may directly affect the properties of voltage-dependent K^+ channels and thus alter cellular excitability and Ca^{2+} signaling (42, 43). NO also has been implicated in cellular plasticity including neuronal differentiation and neurite outgrowth and, as recently suggested, in cortex development (44–46). In the rat cortex, NO synthesis has been shown to reach its maximum in the second postnatal week and start to decrease after day 20 (47, 48). Our findings that the Ca^{2+} potentiation depends on NO (Fig. 5) and becomes less pronounced with age (Fig. 2) and that increased MsrA activity and met(O) content are developmentally transient (see Fig. 6) suggest that oxidative Ca^{2+} potentiation may be closely linked with cortex development. It is also noteworthy that NO has been shown to amplify Ca^{2+}-induced gene transcription and trigger growth arrest during differentiation of PC12 cells

when NO application coincided with Ca^{2+} influx (16, 49), which closely resembles our experimental paradigm reported here.

Activity-, NO-, and ROS-dependent potentiation of Ca^{2+} signaling and the resulting increased neuronal excitability could provide a basis for information storage at the cellular level. The observation that this oxidative Ca^{2+} potentiation can be reversed by brief hypoxia (Fig. 2*A*) might provide an insight into understanding the damaging effects of oxygen deprivation on brain function. This study presents the phenomenon of oxidative potentiation of neuronal excitability and Ca^{2+} signaling and establishes its connection with neuronal differentiation through neuronal development factors such as NO, Ca^{2+}, and NGF, as well as the redox activity of the tissue (MsrA expression). We suggest that the above potentiation of Ca^{2+} signaling may contribute to a long-lasting increase in neurotransmitter release by active neurons and, thus, may be involved in synaptic modulation and higher brain functions.

This paper is dedicated to the late L. Fulson. We thank Dr. J. Thommandru for PC12 cell cultures. This work was supported in part by National Institutes of Health Grants GM57654, HL14388, and DFG He2993/1.

1. Beckman, K. B. & Ames, B. N. (1998) *Physiol. Rev.* **78,** 547–581.
2. Mattson, M. P. (1996) *Perspect. Dev. Neurobiol.* **3,** 79–91.
3. Giroux, C. & Scatton, B. (1996) *Eur. Neurol.* **36,** 61–64.
4. Acworth, I. N., McCabe, D. A. & Maher, T. J. (1997) in *Antioxidants, Oxidants and Free Radicals*, eds. Baskin, S. & Salem, H. (Taylor and Francis, London), pp. 23–25.
5. Davis, T. L., Kwong, K. K., Weisskoff, R. M. & Rosen, B. R. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 1834–1839.
6. Chen, W., Novotny, E. J., Zhu, X. H., Rothman, D. L. & Shulman, R. G. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 9896–9900.
7. Vafaee, M. S., Marrett, S., Meyer, E., Evans, A. C. & Gjedde, A. (1998) *Acta Neurol. Scand.* **98,** 85–89.
8. Auerbach, J. M. & Segal, M. (1997) *J. Neurosci.* **17,** 8695–8701.
9. Kourie, J. I. (1998) *Am. J. Physiol.* **275,** C1–C24.
10. Ruppersberg, J. P., Stocker, M., Pongs, O., Heinemann, S. H., Frank, R. & Koenen, M. (1991) *Nature (London)* **352,** 711–714.
11. Ciorba, M. A., Heinemann, S. H., Weissbach, H., Brot, N. & Hoshi, T. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 9932–9937.
12. Li, A., Segui, J., Heinemann, S. H. & Hoshi, T. (1998) *J. Neurosci.* **18,** 6740–6747.
13. Xu, L., Eu, J. P., Meissner, G. & Stamler, J. S. (1998) *Science* **279,** 234–237.
14. Gao, J., Yin, D. H., Yao, Y., Sun, H., Qin, Z., Schoneich, C., Williams, T. D. & Squier, T. C. (1998) *Biophys. J.* **74,** 1115–1134.
15. Silva, A. J., Kogan, J. H., Frankland, P. W. & Kida, S. (1998) *Annu. Rev. Neurosci.* **21,** 127–148.
16. Peunova, N. & Enikolopov, G. (1993) *Nature (London)* **364,** 450–453.
17. Lev-Ram, V., Jiang, T., Wood, J., Lawrence, D. S. & Tsien, R. Y. (1997) *Neuron* **18,** 1025–1038.
18. McGinnis, K. M., Wang, K. K. & Gnegy, M. E. (1999) *J. Neurochem.* **72,** 1853–1863.
19. Chakraborti, T., Das, S., Mondal, M., Roychoudhury, S. & Chakraborti, S. (1999) *Cell. Signal.* **11,** 77–85.
20. Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450.
21. Kojima, H., Nakatsubo, N., Kikuchi, K., Kawahara, S., Kirino, Y., Nagoshi, H., Hirata, Y. & Nagano, T. (1998) *Anal. Chem.* **70,** 2446–2453.
22. Villa, S., De Faszio, G. & Canosi, U. (1989) *Anal. Biochem.* **177,** 161–164.
23. Joppich-Kuhn, R., Corkill, J. A. & Giese, R. W. (1982) *Anal. Biochem.* **119,** 73–77.
24. Brot, N., Werth, J., Koster, D. & Weissbach, H. (1982) *Anal. Biochem.* **122,** 291–294.
25. Obermeier, A., Bradshaw, R. A., Seedorf, K., Choidas, A., Schlessinger, J. & Ulrich, A. (1995) *Ann. N. Y. Acad. Sci.* **766,** 1–17.
26. Fonnum, F., Johnsen, A. & Hassel, B. (1997) *Glia* **21,** 106–113.
27. Fox, K. (1995) *Neuron* **15,** 485–488.
28. Del Valle, J. & Gantz, I. (1997) *Am. J. Physiol.* **273,** G987–G996.
29. Crow, J. P. (1997) *Nitric Oxide* **1,** 145–157.
30. Bredt, D. S. & Snyder, S. H. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 682–685.
31. Stadtman, E. R. & Berlett, B. S. (1998) *Drug Metab. Rev.* **30,** 225–243.
32. Moskovitz, J., Weissbach, H. & Brot, N. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 2095–2099.
33. Gao, J., Yin, D., Yao, Y., Williams, T. D. & Squier, T. C. (1998) *Biochemistry* **37,** 9536–9548.
34. Katoh, S., Mitsui, Y., Kitani, K. & Suzuki, T. (1997) *Biochem. Biophys. Res. Commun.* **241,** 347–351.
35. Zhang, S., Ehlers, M. D., Bernhardt, J. P., Su, C. T. & Huganir, R. L. (1998) *Neuron* **21,** 443–453.
36. Peterson, B. Z., DeMaria, C. D. & Yue, D. T. (1999) *Neuron* **22,** 549–558.
37. Hellermann, G. R. & Solomonson, L. P. (1997) *J. Biol. Chem.* **272,** 12030–12034.
38. Bito, H., Deisseroth, K. & Tsien, R. W. (1996) *Cell* **87,** 1203–1214.
39. Zheng, M. & Storz, G. (1998) *Science* **279,** 1718–1721.
40. Dolmetsch, R. E., Lewis, R. S., Goodnow, C. C. & Healy, J. I. (1997) *Nature (London)* **386,** 855–858.
41. Hu, S.-C., Chirvia, J. & Ghosh, A. (1999) *Neuron* **22,** 799–808.
42. Ciorba, M. A., Heinemann, S. H., Weissbach, H., Brot, N. & Hoshi, T. (1999) *FEBS Lett.* **442,** 48–52.
43. Taglialatela, M., Pannaccione, A., Iossa, S., Castaldo, P. & Annunziato, L. (1999) *Mol. Pharmacol.* **56**, in press.
44. Dawson, T. M., Sasaki, M., Gonzalez-Zulueta, M. & Dawson, V. L. (1998) *Prog. Brain Res.* **118,** 3–11.
45. Hindley, S., Juurlink, B. H., Gysbers, J. W., Middlemiss, P. J., Herman, M. A. & Rathbone, M. P. (1997) *J. Neurosci. Res.* **47,** 427–439.
46. Cramer, K. S., Leamey, C. A. & Sur, M. (1998) in *Progress in Brain Research*, eds. Mize, R. R., Dawson, T. M. & Friedlander, M. J. (Elsevier Science, Amsterdam), pp. 101–114.
47. Luth, H. J., Hedlich, A., Hilbig, H., Winkelmann, E. & Mayer, B. (1995) *J. Hirnforschung* **36,** 313–328.
48. Bravo, H., Inzunza, O., Fernandez, V. & Sanhueza, M. (1997) *Neurosci. Lett.* **234,** 103–106.
49. Peunova, N. & Enikolopov, G. (1995) *Nature (London)* **375,** 68–73.