Activation of protein kinase A and C prevents the recovery from persistent depolarization produced by oxygen and glucose deprivation in rat hippocampal neurons

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Abstract

Intracellular recordings were made from rat hippocampal CA1 neurons in rat brain slice preparations to investigate whether cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) and calcium/phospholipid-dependent protein kinase C (PKC) contribute to the membrane dysfunction induced by oxygen and glucose deprivation (OGD). Superfusion of oxygen- and glucose-deprived medium produced a rapid depolarization approximately 5 min after the onset of the superfusion. When oxygen and glucose were reintroduced immediately after the rapid depolarization, the membrane depolarized further (persistent depolarization) and reached 0 mV after 5 min from the reintroduction. The pretreatment of the slice preparation with PKA inhibitors, H-89 and Rp-cyclic AMPS, and an adenylate cyclase inhibitor, SQ 22,536, significantly restored the membrane toward the preexposure potential level after the reintroduction of oxygen and glucose in a concentration dependent manner. On the other hand, a phospholipase C inhibitor, U 73122, a PKC inhibitor, GF 109203X, and a non-selective protein kinase inhibitor, staurosporine, also significantly restored the membrane after the reintroduction. Moreover, an inositole-1, 4, 5-triphosphate receptor antagonist, 2-aminoethyl diphenylborinate, and calmodulin inhibitors, trifluoperazine and W-7, significantly restored the membrane after the reintroduction. While, neither an α subunit-selective antagonist for stimulatory G-protein, NF 449, a Ca$^{2+}$/calmoduline-dependent kinase II inhibitor, KN-62, nor a myosin light chain kinase inhibitor, ML-7, significantly restored the membrane after the reintroduction. These results suggest that the activation of PKA and/or PKC prevent the recovery from the persistent depolarization produced by OGD. The Ca$^{2+}$/calmoduline-stimulated adenylate cyclase may contribute to the activation of PKA.
Introduction

Several lines of evidence support the proposal that the activation of PKA and PKC are related to the neuronal cell death caused by brain ischemia. A marked increase in the cAMP level and a decrease in the cGMP level in the gerbil cerebral cortex is found after 1 to 30 min of occlusion of the bilateral common carotid arteries (in vivo ischemia) (Kobayashi et al. 1977). The cAMP level does not decrease during the early phase (5 min) of in vivo ischemia in the rat hippocampus and neocortex (Blomqvist et al. 1985). cAMP then increases during 60 min of recirculation after in vivo ischemia (Blomqvist et al. 1985; Kobayashi et al. 1977). The PKA activity in the rat hippocampus and neocortex shows no change even after 20 min global ischemia (Aronowski et al. 1992); however, reductions in [³H]cyclic AMP binding activity have been noted in the dendritic subfields, but not the pyramidal layer of the hippocampal CA1 area after 15 min of hemispheric ischemia in gerbils (Tanaka et al. 1997b).

A significant increase in total DAG content occurs in the mouse and rat brain after 3 min ischemia (Aveldano and Bazan 1975; Banschbach and Geison 1974). The PKC activity is unchanged in the adult rat brain immediately after 20 min of in vivo ischemia (Louis et al. 1991). Twelve isoforms (α, β1, β2, γ, δ, ε, η, θ, μ, ν, ζ, and ι isoforms) of PKC have been identified in mammalian tissues. The α, β, γ, and ζ isoforms of PKC are predominant in the cytosolic fraction of the cell and the δ isoform of PKC is predominant in the synaptosome-rich fraction, while the ε isoform of PKC is equally distributed both in the cytosolic and synaptosome-rich fractions in rat forebrain. The total amount of α, β, and γ isoforms of PKC does not change after 5 – 30 min of forebrain ischemia but these isoforms translocate to the synaptosome-rich and plasma membrane-rich fractions (Harada et al. 1999).
The β2 and ε isoforms of PKC increase and the total activity of the PKC isoforms increase after 15 min of oxygen and glucose deprivation (OGD) in rat cerebral cortex slices. The β1 and γ isoforms of PKC in cytosolic fractions decrease after 15 min of OGD, and the β1, β2, γ, and ε isoforms of PKC increase in membranous fraction (Selvatici et al. 2002).

In CA1 neurons of the rat hippocampal slices, superfusion with oxygen- and glucose-deprived medium produce a rapid depolarization approximately 5 min after the onset of the superfusion (Tanaka et al. 1997a). When oxygen and glucose are reintroduced immediately after rapid depolarization, the membrane depolarizes further (persistent depolarization) and reaches 0 mV (irreversible depolarization) after 5 min from the onset of the reintroduction: as a result, the neurons show no functional recovery (irreversible membrane dysfunction). Moreover, the persistent depolarization is Ca^{2+}-dependent process, which is mediated by the activation of ionotropic glutamate (Glu) receptors and the Ca^{2+}-induced Ca^{2+} release from intracellular Ca^{2+} store sites (Yamamoto et al. 1997). It is therefore possible that the Ca^{2+}/CaM-stimulated AC, CaMK II, and PKC are activated during persistent depolarization. Nevertheless, the critical contribution of PKA, CaMK II, and PKC to generating irreversible membrane dysfunction is still unclear.

In the present study, we examined whether or not PKA and PKC contribute to the irreversible membrane dysfunction induced by OGD. Inhibitors for PKA and PKC have been administered to the rat hippocampal slices and the potential changes in CA1 neurons during and after ischemic exposure have been compared between the slices with no administration (control) and the slices administered these drugs. In addition, to clarify the metabolic pathway for activation of PKA and PKC, which leads to the irreversible change, we have examined effects of an inhibitor for AC, PLC, CaM, or CaMK II on the potential
Changes following OGD.

Methods

All experiments were conducted in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Science of the Physiological Society of Japan and had the approval of the Institutional Animal Use and Care Committee in Kurume University. Wistar rats (male, 250 - 350 g; 8 – 12 weeks old) were rapidly decapitated under ether anaesthesia, and the forebrains removed and placed in chilled (4 - 6 °C) Krebs solution which was aerated with 95% O2-5% CO2. The composition of Krebs solution was (in mM): 117 NaCl, 3.6 KCl, 2.5 CaCl2, 1.2 MgCl2, 1.2 NaH2PO4, 25 NaHCO3, and 11 glucose. The hippocampus was dissected and then sliced (thickness of 400 µm) with a Vibratome (Oxford). A slice was placed on a nylon net in a recording chamber (volume, 500 µl) and immobilized with a titanium grid placed on the upper surface of the section. The preparation was completely submerged in the superfusing solution (temperature at 36.5 ± 0.5 °C, flow at 4-6 ml/min).

Intracellular recordings from CA1 neurons were made with glass micropipettes filled with K acetate (2 M). The electrode resistance was 50 - 90 MΩ. In conventional intracellular recordings, the apparent input resistance in CA1 neurons was monitored by passing small hyperpolarizing pulses (0.2 - 0.4 nA, 200 msec) through the recording electrode every 3 sec.

Slices were made 'ischemic' by superfusion with medium equilibrated with 95 % N2 - 5 % CO2 and deprived of glucose which was replaced with NaCl isoosmotically (oxygen- and glucose-deprived medium). When switching the superfusing media, there was a delay of
15 - 20 s before the new medium reached the chamber, due to the volume of the connecting tubing. As a result, the chamber was filled with the test solution approximately 30 sec after switching the solution. Oxygen and glucose were reintroduced to the slice preparation immediately after the generation of the rapid depolarization. We used one slice for one experiment since the responses to OGD could not be reproduced after the first superfusion of the oxygen- and glucose-deprived medium.

The drugs used were 3-(N-[dimethylamino]propyl-3-indolyl)-4-(3-indolyl)maleimide (GF109203X); 1-[6-[(17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122); 2-Aminoethyl diphenylborinate (2APB, all from Tocris Cookson Ltd.); staurosporine (from Boehringer Mannheim Biochemica); N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89); 1-[N, O-bis(1, 5-isoquinolinesulfonyl)-N-methyl-l-tyrosyl]-4-phenylpiperazine (KN-62, all from Seikagaku Corporation); 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine (SQ 22,536); Rp-adenosine 3’, 5’-cyclic monophosphorothioate triethylammonium salt (Rp-cAMPS); 10-[3-(4-Methylpiperazin-1-yl)propyl]-2-(trifluoro-methyl)-10H-phenothiazine dihydrochloride (Trifluoperazine dihydrochloride); N-(6-amino-hexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7); 1-(5-Iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride (ML-7); 4, 4’, 4”, 4”’-[carbonylbis [imino-5, 1, 3-benzenetriyl bis(carbonylimino)]tetrakis(benzene-1, 3-disulfonic acid) octasodium salt (NF 449); 1-(5-Isoquinolinylsulfonyl)homopiperazine dihydrochloride (HA-1077); 2-{1-[3-(Amidinothio)propyl]-1H-indol-3-yl}-3-(1-methylindol-3-yl) maleimide methanesulfonate salt (Ro 31-8220); 5-(2-Benzothiazolyl)-3-ethyl-2-[2-(methylphenylamino) ethenyl]-1-phenyl-1H-benzimidazolium iodide (Akt inhibitor IV, all from SIGMA). All
drugs were dissolved in the perfusate and applied by bath application. The slices were pretreated with media containing test compounds for 20 min before ischemic exposure. The latency of the rapid depolarization was measured from the onset of superfusion to onset of the rapid depolarization, estimated by extrapolating the slope of the rapid depolarization to the slope of the slow depolarization (Tanaka et al. 1997a). Recovery after the reintroduction of oxygen and glucose is defined as follows: no recovery, 30 - 60 min after reintroduction the membrane potential lay between 0 and -19 mV; complete recovery, the membrane potential was more negative than -60 mV; partial recovery, membrane potential repolarized to a value between -20 and -59 mV (Yamamoto et al. 1997).

All quantitative results were expressed as the means ± SD. The number of slices examined is given in parentheses. A one-way analysis of variance (ANOVA) with the Dunnett post hoc test was used to compare the data. Statistical significance was determined at the level of $P< 0.05$ unless otherwise indicated.

Results

Effects of PKC inhibitor on the irreversible depolarization produced by OGD

Hippocampal CA1 neurons with stable membrane potentials more negative than -60 mV were used for the following studies. The resting membrane potential and the apparent input resistance in CA1 neurons were $-71 \pm 6$ mV and $40 \pm 9 \, \text{M} \Omega \, (n = 283)$, respectively. Hippocampal slice preparations were used only once under control conditions for exposure to OGD, since the CA1 neurons in the slice preparations would be irreversibly damaged and the
responses during OGD and after reintroduction of oxygen and glucose could not be reproducible after the first exposure to OGD. The slice preparations were, therefore, pretreated with drugs and exposed to OGD only once. To elucidate the effects of PKC on the potential change after OGD, a broad-spectrum inhibitor for protein kinases, staurosporine, and a specific inhibitor for PKC, GF 109203X, were administered to the slice preparations. The administration of GF 109203X at concentrations >3 µM induced a depolarization of a few millivolts, whereas staurosporine (0.1 – 1 µM) or GF 109203X (1 – 3 µM) did not induce any potential change before ischemic exposure (Table 1). Figure 1A shows the typical potential changes in CA1 neurons during and after OGD in the absence (control) and presence of GF 109203X (3 µM) or staurosporine (0.1 µM). In the control condition, OGD produced a sequence of potential change consisting of an initial hyperpolarization, a slow depolarization, and a rapid depolarization (Fig. 1A top). When oxygen and glucose were reintroduced immediately after generation of the rapid depolarization, the membrane depolarized further (persistent depolarization) and reached 0 mV (irreversible depolarization). The membrane never showed a restoration to the potential level before exposure to oxygen- and glucose-deprived medium. The amplitude and the duration of the initial hyperpolarization in the control condition were –5.9 ± 2.6 mV and 2.8 ± 0.9 min (n = 75), respectively. The latency and the maximal slope of the rapid depolarization were 5.3 ± 1.0 min and 9.5 ± 4.0 mV/s (n = 75), respectively. The pretreatment of the slices with GF 109203X (3 µM) did not significantly change the amplitude and the duration of the initial hyperpolarization (~6.5 ± 4.8 mV and 3.0 ± 0.9 min (n = 8)), and the latency and the maximal slope of the rapid depolarization (5.3 ± 0.8 min and 8.0 ± 1.8 mV/s (n = 8)). The pretreatment of the slices with staurosporine (0.1 µM) did not significantly change the amplitude and the duration of the
initial hyperpolarization (−5.5 ± 2.4 mV and 2.8 ± 1.0 min (n = 11)), and the latency and the
maximal slope of the rapid depolarization (5.8 ± 1.1 min and 9.8 ± 1.9 mV/s (n = 11)). After
the reintroduction of oxygen and glucose, however, GF 109203X restored the membrane
potential either partially or completely to the preexposure level in a concentration-dependent
manner and staurosporine also restored the membrane potential (Fig. 1, A and B, and Table 1).
The membrane 30 min after the reintroduction significantly repolarized in the presence of GF
109203X (3 µM) or staurosporine (0.1 – 1 µM) in comparison with the absence of the drugs
(Fig. 1C and Table 1). The administration of an IP₃ receptor antagonist, 2APB at
concentrations ≥ 100 µM induced a slow continuous depolarization, whereas 2APB (50 µM)
or a PLC inhibitor, U 73122 (25 – 50 µM) did not induce any potential change before
ischemic exposure (Table 1). The pretreatment of the slices with U 73122 (25 – 50 µM) or
2APB (50 µM) did not significantly change the latency and the maximal slope of rapid
depolarization (data not shown). Both U 73122 and 2APB restored the membrane potential
either partially or completely to the preexposure level after the reintroduction of oxygen and
glucose (Fig. 1B). The membrane 30 min after the reintroduction significantly repolarized in
the presence of U 73122 (50 µM) or 2APB (50 µM) in comparison with the absence of the
drugs (Fig. 1C and Table 1). It is therefore possible that the activation of PKC contributes to
the generation of the irreversible depolarization.

Effects of AC and PKA inhibitor on the irreversible depolarization produced by
OGD

A PKC inhibitor, staurosporine, also inhibits PKA, PKG and tyrosine kinase,
therefore we next examined effects of adenylate cyclase (AC) and of PKA on the irreversible
depolarization produced by OGD. The administration of a membrane-permeable AC
inhibitor, SQ 22,536 (30 – 100 µM), membrane-permeable inhibitors for PKA, Rp-cAMPS
(30 – 100 µM) and H-89 (0.3 – 1 µM) did not change the resting membrane potential (Table
1). Figure 2A shows the typical potential changes in CA1 neurons during and after OGD in
the absence and presence of SQ 22,536 (100 µM) or H-89 (1 µM). The pretreatment of the
slices with SQ 22,536 (100 µM) did not significantly change the amplitude and the duration
of the initial hyperpolarization (– 5.9 ± 2.8 mV and 3.0 ± 1.0 min (n = 11)), and the latency
and the maximal slope of the rapid depolarization (5.6 ± 0.6 min and 8.2 ± 2.4 mV/s (n = 11)).
The pretreatment of the slices with H-89 (1 µM) did not significantly change the amplitude
and the duration of the initial hyperpolarization (– 5.6 ± 4.1 mV and 2.8 ± 1.0 min (n = 12)),
and the latency and the maximal slope of the rapid depolarization (5.9 ± 0.4 min and 10.0 ±
3.4 mV/s (n = 12)). After the reintroduction of oxygen and glucose, SQ 22,536, Rp-cAMPS
or H-89 restored the membrane potential either partially or completely to the preexposure
level in a concentration dependent manner (Fig. 2, A and B). The membrane 30 min after the
reintroduction significantly repolarized in the presence of SQ 22,536 (30 – 100 µM),
Rp-cAMPS (100 µM) or H-89 (1 µM) in comparison with the absence of the drugs (Fig. 2C
and Table 1). The pretreatment of the slices with an α subunit-selective antagonist for
Gs-protein, NF 449 (30 µM), did not significantly change the latency and the maximal slope
of rapid depolarization (data not shown). After the reintroduction of oxygen and glucose,
NF 449 restored the membrane potential partially (2 of 10 neurons) but the membrane did not
significantly repolarized toward the preexposure level in the presence of NF 449 (Fig. 3). It
is therefore possible that the cAMP, which produced by the activation of AC, mediates the
activation of PKA thus contributes to the generation of the irreversible depolarization. This activation of AC may not be mediated by the $\alpha$ subunit of Gs-protein.

Effects of Ca$^{2+}$/CaM inhibitors and other protein kinase inhibitors on the irreversible depolarization produced by OGD

We next examined effects of Ca$^{2+}$/CaM, myosin light chain kinase (MLCK) and CaMK II on the irreversible depolarization produced by OGD. The administration of Ca$^{2+}$/CaM antagonists, trifluoperazine (100 $\mu$M) and W-7 (50 $\mu$M) did not induce any potential change before ischemic exposure (Table 1). Figure 3A shows the typical potential changes in CA1 neurons during and after OGD in the absence and presence of trifluoperazine (100 $\mu$M) or W-7 (50 $\mu$M). The pretreatment of the slices with trifluoperazine (100 $\mu$M) did not significantly change the latency and the maximal slope of the rapid depolarization (6.0 ± 0.7 min and 10.0 ± 3.0 mV/s ($n = 9$)). The pretreatment of the slices with W-7 (50 $\mu$M) did not significantly change the latency and the maximal slope of the rapid depolarization (6.0 ± 0.3 min and 9.7 ± 2.8 mV/s ($n = 10$)). After the reintroduction of oxygen and glucose, trifluoperazine or W-7 restored the membrane potential either partially or completely to the preexposure level (Fig. 3, A and B). The membrane 30 min after the reintroduction significantly repolarized in the presence of trifluoperazine (100 $\mu$M) or W-7 (50 $\mu$M) in comparison with the absence of the drugs (Fig. 3C and Table 1). On the other hand, the administration of a CaMK II inhibitor, KN-62 (10 $\mu$M) or a MLCK inhibitor, ML-7 (10 $\mu$M) did not change the resting membrane potential (Table 1). The pretreatment of the slices with KN-62 (10 $\mu$M) or ML-7 (10 $\mu$M) did not significantly change the latency and the maximal
slope of rapid depolarization (data not shown). After the reintroduction of oxygen and glucose, KN-62 restored the membrane potential partially (2 of 10 neurons) to the preexposure level (Fig. 3B). The membrane did not significantly repolarized toward the preexposure level in the presence of KN-62 or ML-7 30 min after the reintroduction in comparison to the control condition (Fig. 3C and Table 1). These results indicate that the Ca\(^{2+}/CaM\) may contribute to generation of the irreversible depolarization, but the Ca\(^{2+}/CaM\) at least did not mediate the activation of CaMK II or MLCK to generate the irreversible depolarization. It is possible that the activation of Ca\(^{2+}/CaM\)-stimulated AC produces the activation of PKA.

A PKA inhibitor, H-89, at low concentration also inhibits the activity of Rho-associated protein kinase II (ROCK-II), p70 S6 kinase (S6K1) and mitogen-and stress-activated protein kinase-1 (MSK1), and the half maximal inhibitory concentration (IC\(_{50}\)) is similar in these enzymes including PKA. We next examined effects of other protein kinase inhibitors on the irreversible depolarization produced by OGD. The administration of an inhibitor for ROCK-II, HA-1077 (20 µM), an inhibitor for both S6K1 and MSK1, Ro 31-8220 (1 µM), or an inhibitor for Akt/protein kinase B (PKB), Akt inhibitor IV (15 µM), did not change the resting membrane potential (Table 1). The pretreatment of the slices with HA-1077 (20 µM), Ro 31-8220 (1 µM) or Akt inhibitor IV (15 µM) did not significantly change the latency and the maximal slope of rapid depolarization (data not shown). After the reintroduction of oxygen and glucose, the membrane potential was partially restored to the preexposure level in the presence of HA-1077 (4 of 11 neurons), Ro 31-8220 (4 of 10 neurons) or Akt inhibitor IV (1 of 12 neurons) (Fig. 4A). The membrane did not significantly repolarized toward the preexposure level in the presence of HA-1077, Ro
31-8220 or Akt inhibitor IV 30 min after the reintroduction in comparison to the control condition (Fig. 4B and Table 1). From these results, it is concluded that the activation of PKA induced by cAMP, which produced by Ca^{2+}/CaM-stimulated AC, generate the irreversible depolarization following OGD (cf. Fig. 5).

Discussion

In rat hippocampal CA1 neurons, PKA inhibitors, an adenylate cyclase (AC) inhibitor, and Ca^{2+}/CaM inhibitors, significantly restored the membrane potential after OGD toward the preexposure level in the majority of neurons tested. On the other hand, a CaMKII inhibitor, MLCK inhibitor, and Gsα subunit-selective antagonist, did not either accelerate or inhibit the generation of the rapid depolarization and the irreversible depolarization after OGD. These results indicate that the reaction mediated by PKA contributes to the generation of the irreversible depolarization (Fig. 5). The cAMP produced by Ca^{2+}/CaM-stimulated AC may contribute to the activation of PKA. PKC inhibitors, a PLC inhibitor, and an IP_{3} receptor antagonist, significantly restored the membrane potential in the majority of neurons tested. These results indicate that the activation of PKC also contributes to the generation of the irreversible depolarization induced by OGD.

Effects of PKA inhibitors on the irreversible depolarization after the reintroduction of oxygen and glucose

The present results show that PKA inhibitors, such as H-89 and Rp-cAMPS, provide
protection against the generation of the irreversible depolarization. The concentration of Rp-cAMPS (100 µM), which have been used in this study, is as same as the concentration that inhibits the PKA mediated potentiation of AMPA/kainate receptor-induced currents in cultured hippocampal neurons (Wang et al. 1991). The value of half maximal inhibitory concentration (IC₅₀) of H-89 for PKA, S6K1, MSK1 and ROCK-II is 135, 80, 120, and 270 nM, respectively (Davies et al. 2000). The concentration of H-89 (1 µM), which significantly restored the membrane to the preexposure level, was higher than the IC₅₀ value for PKA, S6K1, MSK1 and ROCK-II. The concentration of ROCK-II inhibitor, HA-1077 (20 µM) and that of S6K1 and MSK1 inhibitor, Ro 31-8220 (1 µM), which have been used in this study, is high enough form the IC₅₀ value of HA-1077 for ROCK-II (1.9 µM) and the IC₅₀ values of Ro 31-8220 for S6K1 (15 nM) and MSK1 (8 nM) (Davies et al. 2000). An IC₅₀ value of H-89 for Akt/PKB is 2.6 µM (Davies et al. 2000) and the concentration of H-89 (1 µM), which used in this study, was lower than the IC₅₀ value for Akt/PKB, therefore, H-89 may have little inhibitory effect on Akt/PKB. The concentration of Akt inhibitor IV (15 µM) is high enough for the IC₅₀ value for Akt/PKB (0.6 – 1.3 µM) (Kau et al. 2003). In the present study, the Akt inhibitor IV had no significant restoration of the membrane after OGD. These results indicate that the activation of PKA contributes to the generation of the irreversible depolarization after OGD.

Effects of AC inhibitors on the irreversible depolarization after the reintroduction of oxygen and glucose

The present results indicate that an AC inhibitor, SQ 22,536, also significantly
restores the membrane toward the preexposure level after OGD. The concentration of SQ 22,536 (100 μM), which have been used in this study, is as same as the concentration that inhibits the cAMP mediated reduction of the afterhyperpolarization induced by β-adrenoceptor activation in hippocampal CA1 pyramidal neurons (Madison and Nicoll 1986). The administration of a Gsα subunit-selective antagonist, NF 449, did not significantly restore the membrane after OGD. The concentration of NF 449 (30 μM) used in this study is high enough form the IC₅₀ value of NF 449 for inhibition of GTPγS binding to the Gsα subunit (0.14 μM) (Hohenegger et al. 1998). These results indicate that the cAMP produced by AC contributes to the activation of PKA after OGD, but Gsα subunit at least does not couple the activation of AC.

Effects of Ca²⁺/CaM inhibitors and other protein kinase inhibitors on the irreversible depolarization produced by OGD

Nine isoforms of membrane-bound AC (AC1 – AC9) has been found. The Gsα subunit activates all isoforms of AC, on the other hands, Ca²⁺/CaM also stimulates AC1 and AC8, and PKC stimulates AC1, AC2, AC3, AC5 and AC7 (Antoni 2000; Cooper 2003; Defer et al. 2000; Wang and Storm 2003; Xia and Storm 1997). The present results show that Ca²⁺/CaM inhibitors, trifluoperazine and W-7, provide protective effects against the irreversible depolarization after OGD. W-7 is an inhibitor of CaM, phosphodiesterase, and MLCK. The value of the IC₅₀ for these enzyme activities is approximately 30 – 50 μM (Cafouleas et al. 1982; Hidaka et al. 1981). Neither CaMK II inhibitor, KN-62, nor MLCK inhibitor, ML-7, show any significant protection against the irreversible depolarization after
OGD, suggesting that the involvement, if any, of CaMK II or MLCK in the irreversible depolarization is minimal. It is therefore possible that the Ca\(^{2+}\)/CaM stimulates AC for production of cAMP, and then the cAMP activates PKA. The reaction mediated by PKA again may contribute to the generation of the irreversible depolarization after OGD.

Site of action of PKA

Ionotropic glutamate receptors can be broadly classified as N-methyl-D-aspartate (NMDA) or \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors on the basis of selective agonist. Currents and Ca\(^{2+}\) influx induced by activation of AMPA/kainate receptor channel are potentiated by activation of PKA and are suppressed by a competitive PKA inhibitor in mouse hippocampal cultured neurons and in AMPA/kainate receptor channel expressed in oocyte membrane (Keller et al. 1992; Wang et al. 1991). NMDA induced currents and Ca\(^{2+}\) influx through activated NMDA receptor channels are also potentiated by activation of PKA in spinal dorsal horn neurons and in rat hippocampal cultured neurons (Cerne et al. 1993; Skeberdis et al. 2006). These results suggest that the enhancement of currents and Ca\(^{2+}\) influx through ionotropic glutamate receptor channels by the activation of PKA contribute to the generation of the irreversible depolarization after OGD in the present study. Since the pretreatment of the slices with a NMDA receptor antagonist, \(\text{dl-2-amino-5-phosphonopentanoic acid}\), or an AMPA/kainate receptor antagonist, 6-cyano-7-nitroquinoxaline-2, 3-dione, restore the membrane toward the preexposure level after superfusion of oxygen- and glucose-deprived medium in rat hippocampal CA1 neurons (Yamamoto et al. 1997).
In rat hypothalamic neurons, adrenomedullin-induced NO release is blocked by PKA inhibitors, H-89 and Rp-cAMP (Xu and Krukoff 2007). These results indicate that the increase in the [Ca\(^{2+}\)]\(_i\) and in the activity of PKA elevates NOS activity and NO production. It is also possible that the activation of PKA contribute to the generation of the irreversible depolarization through increase in production of NO in the present study. Since the NO scavengers, carboxy-2-phenyl-4, 4, 5, 5-tetramethyl-imidazole-1-oxyl-3-oxide and hemoglobin, and a NO synthase inhibitor, \(\text{N}^\text{G}\)-nitro-L-arginine restore the membrane toward the preexposure level after superfusion of oxygen- and glucose-deprived medium in rat hippocampal CA1 neurons (Onitsuka et al. 1998).

Effects of PKC inhibitors on the irreversible depolarization after the reintroduction of oxygen and glucose

The present results show that PKC inhibitors, GF 109203X and staurosporine, provide protection against the generation of the irreversible depolarization. The concentration of staurosporine (0.1 - 1 µM), which have been used in this study, is high enough from the IC\(_{50}\) values of staurosporine for PKC (2.7 nM) and PKA (7 nM) (Nakano et al. 1987; Tamaoki et al. 1986). The protective action of staurosporine against the irreversible depolarization, therefore, may partially be produced by inhibition of PKA. On the other hand, GF 109203X is specific inhibitor for PKC but not PKA (Davis et al. 1989). GF 109203X also inhibits mitogen-activated protein kinase-activated protein (MAPKAP) kinase-1\(\beta\) (IC\(_{50}\) = 50 nM) and S6K1 (IC\(_{50}\) = 100 nM) (Alessi 1997). Ro 31-8220 selectively inhibits MAPKAP kinase-1\(\beta\) (IC\(_{50}\) = 3 nM) and S6K1 (IC\(_{50}\) = 15 nM) (Davies et al. 2000).
In the present study, Ro 31-8220 (1 µM) did not significantly restore the membrane toward the preexposure level after OGD, suggesting that GF 109203X inhibits PKC. A PLC inhibitor, U 73122, and an IP₃ receptor antagonist, 2APB, provide significant protection against the generation of the irreversible depolarization, supporting the conclusion that the reaction mediated by the activation of PKC may produce the irreversible depolarization after OGD.

The PKC activator, 4-β-phorbol-12, 13-dibutyrate (PDBu) enhances the NMDA-induced currents in NMDA receptor channels expressed in oocyte membranes, and PKC inhibitor blocks the enhancement of NMDA-induced currents produced by the application of PDBu or a metabotropic glutamate receptor agonist, trans-ACPD (Kelso et al. 1992). In rat hippocampal CA1 neurons, trans-ACPD enhances NMDA-induced currents via activation of PKC (Anikszteji et al. 1992), and a PKC activator, 4-β-phorbol-12-myristate-13-acetate, enhances NMDA-induced currents (Lu et al. 1999). These results suggest that the enhancement of currents through NMDA receptor channels by activation of PKC contribute to the generation of the irreversible depolarization after OGD. Since the pretreatment of the slices with NMDA receptor antagonist, DL-2-amino-5-phosphonopentanoic acid, restore the membrane toward the preexposure level after superfusion of oxygen- and glucose-deprived medium (Yamamoto et al. 1997).

In conclusion, the activation of PKA and/or PKC prevents the recovery from the persistent depolarization produced by OGD. The Ca²⁺/calmoduline-stimulated adenylate cyclase may contribute to the activation of PKA.

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Fig. 1  The effects of a phospholipase C (PLC) inhibitor, an inositol 1,4,5-trisphosphate (IP₃) receptor antagonist, and protein kinase C (PKC) inhibitors on the potential changes produced by oxygen and glucose deprivation (OGD) in rat hippocampal CA1 neurons. OGD was administered between downward arrow and upward arrow. Dotted line shows the preexposure level in this and subsequent figures. 

A: typical changes in the membrane potential during and after OGD in the absence of the drugs (top), and in the presence of GF 109203X (middle), or staurosporine (bottom). Downward deflections are hyperpolarizing electrotonic potentials elicited by anodal current pulses (in range: 0.2 – 0.4 nA for 200 ms every 3 s) in this and subsequent figures. The resting potentials were -73, -72, and -75 mV from top to bottom. B: the effects of a PLC inhibitor (U 73122), an IP₃ receptor antagonist (2APB), and PKC inhibitors (GF 109203X and staurosporine) on the percent of neurons exhibiting recovery. Open column, shaded column, and closed column indicates no, partial, and complete recovery, respectively. C: the effects of a PLC inhibitor, an IP₃ receptor antagonist, and PKC inhibitors on the potential recovery 30 min after the reintroduction of oxygen and glucose. Each column shows the mean ± SD in this and subsequent figures. In the presence of U 73122, 2APB, GF 109203X or staurosporine, the membrane potential repolarized toward the preischemic exposure level after the reintroduction of oxygen and glucose. *, P < 0.05; **, P < 0.01; one-way ANOVA with Dunnett post hoc comparisons.

Fig. 2  The effects of an adenylate cyclase inhibitor and protein kinase A (PKA) inhibitors on the potential changes produced by OGD in rat hippocampal CA1 neurons.
A: typical changes in the membrane potential during and after OGD in the absence (top), and in the presence of SQ 22,536 (middle), or H-89 (bottom). The resting membrane potentials were -73, -72, and -75 mV from top to bottom. B: the effects of an adenylate cyclase inhibitor (SQ 22,536) and PKA inhibitors (Rp-cAMPS and H-89) on the percent of neurons exhibiting recovery. Open column, shaded column, and closed column indicates no, partial, and complete recovery, respectively. C: the effects of an adenylate cyclase inhibitor and PKA inhibitors on the potential recovery 30 min after the reintroduction of oxygen and glucose. In the presence of SQ 22,536, Rp-cAMPS or H-89, the membrane potential repolarized toward the preischemic exposure level in a concentration-dependent manner after the reintroduction of oxygen and glucose. *, P < 0.05; **, P < 0.01; one-way ANOVA with Dunnett post hoc comparisons.

Fig. 3 The effects of a Gsα subunit inhibitor, Ca\(^{2+}\)/calmodulin inhibitors, a Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMK II) inhibitor, and a myosin light chain kinase (MLCK) inhibitor on the potential changes produced by OGD in rat hippocampal CA1 neurons.

A: typical changes in the membrane potential during and after OGD in the absence (top), and in the presence of trifluoperazine (middle), or W-7 (bottom). The resting membrane potentials were -75, -73, and -75 mV from top to bottom. B: the effects of Ca\(^{2+}\)/calmodulin inhibitors (trifluoperazine and W-7), a CaMK II inhibitor (KN-62), a MLCK inhibitor (ML-7), and a Gsα subunit inhibitor (NF 449) on the percent of neurons exhibiting recovery. Open column, shaded column, and closed column indicates no, partial, and complete recovery, respectively. C: the effects of Ca\(^{2+}\)/calmodulin inhibitors, a CaMK II inhibitor, a MLCK
inhibitor, and a Gsα subunit inhibitor on the potential recovery 30 min after the reintroduction of oxygen and glucose. In the presence of trifluoperazine or W-7, the membrane potential repolarized toward the preischemic exposure level after the reintroduction of oxygen and glucose. *, *P < 0.05; one-way ANOVA with Dunnett post hoc comparisons.

Fig. 4  The effects of an inhibitor for Rho-associated protein kinase II (ROCK-II), an inhibitor for p70 S6 kinase (S6K1) and mitogen-and stress-activated protein kinase-1 (MSK1), and an inhibitor for Akt/PKB on the potential changes produced by OGD in rat hippocampal CA1 neurons.

A: the effects of an inhibitor for ROCK-II (HA-1077), an inhibitor for S6K1 and MSK1 (Ro 31-8220), and an inhibitor for Akt/PKB (Akt inhibitor IV) on the percent of neurons exhibiting recovery. Open column, shaded column, and closed column indicates no, partial, and complete recovery, respectively. B: the effects of an inhibitor for ROCK-II, an inhibitor for S6K1 and MSK1, and an inhibitor for Akt/PKB on the potential recovery 30 min after the reintroduction of oxygen and glucose.

Fig. 5  Putative mechanisms that contribute to the generation of the irreversible depolarization induced by OGD.

OGD induces an accumulation of extracellular glutamate (Glu) to activate ionotropic Glu receptors (NMDA- and/or AMPA/kainate-type receptors). The activation of ionotropic Glu receptors induces a depolarization, which increases permeability of Ca2+ at the NMDA-type receptor channels and voltage-gated Ca2+ channels. These mechanisms increase the intracellular Ca2+ concentration ([Ca2+]i) and trigger the Ca2+-induced Ca2+ release (CICR).
from the intracellular Ca\textsuperscript{2+} store sites (e.g. endoplasmic reticulum). On the other hand, accumulated extracellular Glu activates the metabotropic Glu receptor, which activates phospholipase C (PLC) via the dissociation of Gq-protein. When the PLC is activated, the PLC produces diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP\textsubscript{3}) from membranous phospholipids, then DAG activate protein kinase C (PKC) and IP\textsubscript{3} induces increase in the [Ca\textsuperscript{2+}], by releasing Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+} store sites via activation of IP\textsubscript{3} receptor. Elevated [Ca\textsuperscript{2+}], triggers the activation of adenylate cyclase (AC) and nitric oxide synthase (NOS) via Ca\textsuperscript{2+}/calmodulin. The AC produces cyclic AMP (cAMP) and the cAMP activates cAMP-dependent protein kinase (PKA). Reactions mediated by PKA and PKC contribute to the generation of the irreversible depolarization. The activation of PKA enhances NMDA- and AMPA/kainate-type Glu receptors, and the activation of PKC enhances NMDA-type Glu receptor. The activation of PKA augments the activity of NOS. NO produced by activation of NOS yields peroxynitrate to damage the cytoskeleton and induce peroxydation of the lipid, thus causing the irreversible depolarization. Thin solid arrows: putative routs for intracellular signal transduction systems. Thick solid arrows: putative routs for intracellular signal transduction systems, which mainly contribute to the irreversible depolarization produced by OGD. Dashed arrows: putative routes for intracellular signal transduction systems, which contribute relatively little to the generation of the irreversible depolarization. A plus sign near the thin solid arrow indicate the enhancement of the activity.
Table 1. The effects of inhibitors for protein kinases and enzymes, which activate the protein kinases, on the potential immediately before OGD and the potential 30 min after reintroduction of oxygen and glucose (recovery potential).

<table>
<thead>
<tr>
<th>Enzyme Blocker</th>
<th>Potential immediately before OGD, mV</th>
<th>Recovery Potential, mV</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>-69.9 ± 5.2</td>
<td>-3.3 ± 5.3 (75)</td>
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<tr>
<td>U73122 PLC</td>
<td>25 µM: -70.3 ± 5.2, 50 µM: -70.9 ± 6.1</td>
<td>25 µM: -9.1 ± 21.2 (7), 50 µM: -22.8 ± 15.6 (12)**</td>
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<tr>
<td>2APB IP3 receptor</td>
<td>50 µM: -72.6 ± 6.7</td>
<td>-25.7 ± 9.7 (10)*</td>
</tr>
<tr>
<td>GF109203X PKC</td>
<td>1 µM: -72.1 ± 3.6, 3 µM: -70.6 ± 4.5</td>
<td>-18.5 ± 18.8 (8), -35.1 ± 15.4 (8)**</td>
</tr>
<tr>
<td>Staurosporine PKC and PKA</td>
<td>0.1 µM: -70.3 ± 6.5, 1 µM: -70.7 ± 3.4</td>
<td>-44.5 ± 31.6 (11)<strong>, -33.6 ± 30.4 (11)</strong></td>
</tr>
<tr>
<td>SQ 22,536 adenylate cyclase</td>
<td>30 µM: -71.2 ± 6.8, 100 µM: -66.4 ± 5.8</td>
<td>-20.0 ± 12.7 (9), -31.2 ± 15.0 (11)**</td>
</tr>
<tr>
<td>Rp-cAMP PKA</td>
<td>30 µM: -70.9 ± 7.2, 100 µM: -72.7 ± 7.0</td>
<td>-7.4 ± 5.8 (8), -28.6 ± 17.5 (10)**</td>
</tr>
<tr>
<td>H-89 PKA</td>
<td>0.3 µM: -75.6 ± 6.6, 1 µM: -69.4 ± 5.5</td>
<td>-12.0 ± 10.3 (9), -44.0 ± 25.0 (12)**</td>
</tr>
<tr>
<td>Trifluoperazine Ca²⁺/Calmodulin</td>
<td>100 µM: -68.1 ± 5.7</td>
<td>-21.7 ± 25.2 (9)*</td>
</tr>
<tr>
<td>W-7 Ca²⁺/Calmodulin</td>
<td>50 µM: -72.8 ± 6.5</td>
<td>-21.7 ± 30.3 (10)*</td>
</tr>
<tr>
<td>KN-62 CaMK II</td>
<td>10 µM: -70.4 ± 3.8</td>
<td>-9.4 ± 11.1 (10)</td>
</tr>
<tr>
<td>ML-7 MLCK</td>
<td>10 µM: -70.3 ± 3.9</td>
<td>-5.0 ± 1.1 (10)</td>
</tr>
<tr>
<td>NF 449 Gsα subunit</td>
<td>30 µM: -73.6 ± 8.1</td>
<td>-9.1 ± 10.8 (10)</td>
</tr>
<tr>
<td>HA-1077 ROCK-II</td>
<td>20 µM: -72.3 ± 5.1</td>
<td>-16.2 ± 15.2 (11)</td>
</tr>
<tr>
<td>Ro 31-8220 S6K1 and MSK1</td>
<td>1 µM: -72.0 ± 5.6</td>
<td>-13.0 ± 14.3 (10)</td>
</tr>
<tr>
<td>Akt inhibitor IV Akt/PKB</td>
<td>15 µM: -72.6 ± 6.4</td>
<td>-6.6 ± 8.4 (12)</td>
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