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DOR activation inhibits anoxic/ischemic Na⁺ influx through Na⁺ channels via PKC mechanisms in the cortex

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Abstract

Activation of delta-opioid receptors (DOR) is neuroprotective against hypoxic/ischemic injury in the cortex, which is at least partially related to its action against hypoxic/ischemic disruption of ionic homeostasis that triggers neuronal injury. Na⁺ influx through TTX-sensitive voltage-gated Na⁺ channels may be a main mechanism for hypoxia-induced disruption of K⁺ homeostasis, with DOR activation attenuating the disruption of ionic homeostasis by targeting voltage-gated Na⁺ channels. In the present study we examined the role of DOR in the regulation of Na⁺ influx in anoxia and simulated ischemia (oxygen-glucose deprivation) as well as the effect of DOR activation on the Na⁺ influx induced by a Na⁺ channel opener without anoxic/ischemic stress and explored a potential PKC mechanism underlying the DOR action. We directly measured extracellular Na⁺ activity in mouse cortical slices with Na⁺ selective electrodes and found that (1) anoxia-induced Na⁺ influx occurred mainly through TTX-sensitive Na⁺ channels; (2) DOR activation inhibited the anoxia/ischemia-induced Na⁺ influx; (3) veratridine, a Na⁺ channel opener, enhanced the anoxia-induced Na⁺ influx; this could be attenuated by DOR activation; (4) DOR activation did not reduce the anoxia-induced Na⁺ influx in the presence of chelerythrine, a broad-spectrum PKC blocker; and (5) DOR effects were blocked by PKCβII peptide inhibitor, and PKCθ pseudosubstrate inhibitor, respectively. We conclude that DOR activation inhibits anoxia-induced Na⁺ influx through Na⁺ channels via PKC (especially PKCβII and PKCθ isoforms) dependent mechanisms in the cortex.

Keywords

Delta-opioid receptor; Ionic homeostasis; Na⁺ influx; Na⁺ channel; Neuroprotection; Hypoxia/ischemia

Introduction

Finding new strategies against hypoxic/ischemic injury has been a long-term battle and attracted much attention from both scientists and clinicians in diversified fields (Fan et al.,

2006; Fazzina et al., 2010; Jin et al., 2008; Lin et al., 2009; Ma et al., 2005; Pallast et al., 2010; Sun et al., 2008). One of the most important notions is that ionic disruption may initiate hypoxic/ischemic injury. Excessive disruption of ionic homeostasis is the initial and key step in hypoxic/ischemic neuronal injury and death (Chao and Xia, 2010; Sung et al., 2008). Large Na^+ influx induces cellular injury manifested as acute functional and morphological changes, e.g., loss of electrophysiological response to stimulus, cell swelling, bleb formation and membrane injury. Removal of extracellular Na^+ or blockade of Na^+ entry prevents hypoxic/ischemic neuronal damage and death (Chao and Xia, 2010). Our previous work has demonstrated that delta-opioid receptor (DOR) activation is neuroprotective against hypoxic/ischemic stress (see review by Chao and Xia, 2010); this finding is also supported by several lines of new evidence from many independent laboratories (Borlongan et al., 2009; Gao et al., 2010; Gwak et al., 2010; Turner and Johnson, 2011; Yang et al., 2011). In our mechanistic exploration, we found, besides the increase in pro-survival signaling and the enhanced anti-oxidative capacity (Feng et al., 2009), the stabilization of disrupted ionic homeostasis via the interaction of DOR with voltage-gated Na^+ channels in the early phase of the stress underlies the DOR neuroprotection against hypoxia/ischemia (Chao et al., 2008, 2009; Kang et al., 2009). For example, we observed that after being exposed to prolonged hypoxia during postnatal development, cortical neurons are more sensitive to subsequent stress, which is largely attenuated by Na^+ channel blocker tetrodotoxin (TTX). Intriguingly, an increased Na^+ channel density/current (Gu and Haddad, 2003; Gu et al., 2008; Xia et al., 2000) and decreased DOR density (Xia et al., 2001) occurred in the exposed brain. Furthermore, we observed that in a mutant brain with epileptic seizures, cortical neurons are hyper-excitable with an up-regulation of voltage-gated Na^+ channels (Xia et al., 2003) and down-regulation of DOR expression (Zhao et al., 2005). Our preliminary study directly showed that DOR protects against anoxic disruption of Na^+ homeostasis in the cortex via Na^+ channel regulation (Kang et al., 2009). However, it is unknown if DOR activation can inhibit Na^+ influx induced by ischemic stress or other factors that lead to Na^+ influx without hypoxic/ischemic stress. This is an important issue to address for a better understanding of DOR's role in the regulation of ionic homeostasis and for potential applications of DOR-mediated neuroprotection in clinical settings.

Moreover, the mechanisms underlying how DOR regulates Na^+ homeostasis in the cortex is unknown. We previously observed that protein kinase C (PKC) is involved in DOR protection against neuronal injury in prolonged hypoxia (Ma et al., 2005) and against anoxic K^+ derangement in short-term anoxia (Chao et al., 2007b). It is unclear, however, if the same mechanism mediates the regulation of Na^+ homeostasis under hypoxic/ischemic stress. More importantly, there is no information available for PKC isozymes involved in the DOR neuroprotection. PKC represents a family of at least 10 different ubiquitous serine/threonine isozymes that are expressed in the brain and regulate a broad spectrum of cellular functions. PKC has been implicated in several nervous system diseases, including cerebral ischemia (Battaini, 2001; Bright and Mochly-Rosen, 2005; Chou and Messing, 2005). Various PKC isozymes mediate different and sometimes opposing functions after activation by the same stimulus (Chen et al., 2001; Wang et al., 2004). PKC isozymes such as PKC β II and PKC θ belong to different subgroups of PKC, and some of them have been linked with the functioning of Na^+ channel (Vijayaragavan et al., 2004). The expression/activities of these isozymes during hypoxia/ischemia are different (Krupinski et al., 1998; Li et al., 2006; Selvatici et al., 2003, 2006; Sieber et al., 1998), and they may play different roles in cell protection. For example, PKC β II has been shown to be neuroprotective (Cordey and Pike, 2006; Li et al., 2005), while little information is available as for the role of PKC θ in neuroprotection (Fauconnier et al., 2011). DOR belongs to a family of G-protein-coupled metabotropic receptors, whose effect is mediated by G proteins and G-protein-dependent cytoplasmic second messengers involving protein kinases (Law et al., 2000; Ma et al., 2005;

Narita et al., 2006). Voltage-gated Na⁺ channels are important targets modulated by metabotropic receptors via G protein/protein kinases, including PKC (Chen et al., 2006; Dascal and Lotan, 1991; West et al., 1991). Therefore, it is possible that PKC functions as a “signal bridge” between DOR and Na⁺ channels, and mediates the effects of DOR on Na⁺ channels in hypoxia/ischemia.

In the present study, we investigated if DOR activation inhibits Na⁺ influx induced by anoxia, simulated ischemia (oxygen-glucose deprivation, OGD) and by veratridine, a Na⁺ channel opener without hypoxia/ischemia and if PKC, especially PKCβII and PKCθ isoforms, is involved in DOR protection against the disruption of Na⁺ homeostasis.

Materials and methods

Slice preparation

The experiments were approved by the Animal Care and Use Committee of Yale University School of Medicine, which is accredited by the American Association for Accreditation for Laboratory Animal Care. Slices of the frontoparietal cortex were prepared as described in our previous studies (Chao et al., 2007a,b). Transverse cortical slices (400 μm) were cut from the brains of 24–32 day old male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) with a vibrotome containing carbogen (95% O₂, 5% CO₂) saturated ice-cold standard artificial cerebrospinal fluid (ACSF), and then transferred to an incubation holder placed in a beaker containing 150 ml ACSF vigorously aerated with carbogen at ~35 °C. Standard ACSF consisted of (in mM) NaCl 125, KCl 3.1, NaHCO₃ 26, CaCl₂ 2.4, MgSO₄ 1.3, NaH₂PO₄ 1.25, and dextrose 10 at pH 7.4. After an equilibration period of at least 90 min in carbogen saturated ACSF at ~35 °C, slices were used for recording. The recordings were made in the outer layer (corresponding to layer II and III) of the cortex.

Induction of anoxia or oxygen-glucose deprivation (OGD) in cortical slices

A slice was transferred to the recording chamber (Model RC-22C, Warner Instrument Co., Hamden, CT) which was perfused with carbogen saturated ACSF (35.5±0.5 °C) at a flow rate of ~3 ml/min. Slices were completely submerged 0.5–1 mm below the ACSF surface in the tissue chamber and kept under normoxic conditions for at least 15 min at ~35.5 °C before the experimental measurements were taken.

Anoxia was induced by switching from the control superfusate (95% O₂, 5% CO₂) to one continuously bubbled with 95% N₂ and 5% CO₂. Each slice was subjected to a single period of anoxia that continued for about 1.5 min after the onset of anoxic depolarization (as assessed by an abrupt drop in extracellular [Na⁺] that usually occurs within 10 min after the onset of anoxia), or for a period of 20 min if anoxic depolarization did not occur. OGD was produced by exposing slices to D-glucose-deficient ACSF (D-glucose was substituted by equimolar mannitol) continuously aerated with 95% N₂ and 5% CO₂, as what was done in anoxic induction.

Measurements of extracellular [Na⁺]

Extracellular Na⁺ concentrations ([Na⁺]_o) were measured using Na⁺-sensitive microelectrodes. Na⁺-sensitive microelectrodes were prepared as described previously (Kang et al., 2009; Müller and Somjen, 2000). Glass capillary-pulled electrodes were silanized by exposure to hexamethyldisilazane for Na⁺ electrode, and subsequently baked at about 180 °C for at least 2 h. The microelectrode tips were then broken back to ~2 μm. The internal filling solution (150 mM NaCl+10 mM HEPES) was injected from the back into the electrode. A cocktail mixture of internal filling fluid for Na⁺ electrodes (10% Na⁺ ionophore VI, 89.5% 2-nitrophenyl octyl ether, and 0.5% potassium tetraphenylborate), with a height

of about 1 mm was sucked into the microelectrode tips. The reference electrode was a Ag/AgCl bridge electrode embedded in 2% agar in 3 M KCl. Calibrations were carried out by detecting the responses generated in NaCl solutions (5, 10, 20, 50, 80, 100, 120, 150 mM NaCl) in triplicate. For each concentration, the average of voltage changes in three separate tests was used as the final voltage change. Over this range, the electrode response was near ideal, showing a logarithmic relationship to $[Na^+]_o$.

Electrical signals were monitored on an oscilloscope, recorded by a DC amplifier (Model IE-210, LPF 200, Warner Instrument Co., Hamden, CT) and digitized by an Axon mini-digitizer acquisition system (Model miniDigi 1A, Axon Instruments, Union City, CA) at a sampling rate of 100 Hz. The following parameters are used for assessment of Na^+ activities: (1) the maximal drop in extracellular Na^+ concentration induced by anoxia; (2) the time of occurrence of the maximal drop in extracellular Na^+ concentration induced by anoxia (T_{max}), which refers to the time interval from the beginning of anoxia/OGD to the time point where the maximal $[Na^+]_o$ drop occurs; and (3) the recovery duration of the return of reduced Na^+ levels to the pre-anoxic/OGD level during reoxygenation.

After recording of a stable baseline for at least 5 min, the slices were subject to experimental treatments. The electrophysiological recordings were continuously performed for at least 60 min.

Drug administration

Drugs were applied to cortical slices by switching from control superfusate to one containing drugs, which was controlled by a six-channel valve-controlled solution perfusion system (Model VC-6, Warner Instrument Co., Hamden, CT). All drugs were perfused for 20 min before the induction of anoxia, and continued till the end of anoxic induction.

Chemicals

Na^+ ionophore VI (Fluka 71739), 2-nitrophenyl octyl ether (Fluka 73732), sodium tetraphenyl borate (Fluka 72018), hexamethyldisilazane (Fluka 52619), naltrindole, veratridine, cell permeable protein kinase β II peptide inhibitor I trifluoroacetate salt (PKC β II PI), and mannitol were purchased from Sigma Chemicals Co. (St. Louis, MO). Tetrodotoxin was purchased from Tocris Cookson Inc. (Ellisville, MI). Chelerythrine chloride, myristoylated protein kinase C θ pseudosubstrate inhibitor (PKC θ PI, Myr-LHQRRGAIKQAKVHHVKC-NH₂), were purchased from EMD Biosciences Inc. (San Diego, CA); UFP 512 (H-Dmt-Tic-NH-CH(CH₂-COOH)-Bid), a specific and potent DOR agonist (Balboni et al., 2002), was synthesized by our research team.

Statistics

All data are expressed as mean $\pm$ SEM and the number of experiments (n) refers to the number of slices investigated. Except indicated in the text, one way analysis of variance followed by Newman Keuls test was used for multiple pairwise tests, and two-tailed, unpaired Student's *t*-test was used for comparison of the two experimental groups. Changes were identified as significant if $p < 0.05$.

Results

TTX blocked anoxia-induced Na^+ influx

As has been shown previously (Kang et al., 2009), anoxia induced a sudden drop in $[Na^+]_o$ from the basal level (approximately 152 mM) to 48.5 ± 2.9 mM in 17 out of 20 cortical slices (85% of response rate). Three slices did not respond to anoxia but showed a sudden drop in $[Na^+]_o$ in response to OGD, indicating that they have good viability. To certify whether the

voltage-gated Na^+ channels were involved in the anoxia-induced Na^+ influx, we perfused slices with TTX (1 μM). Our results showed that 7 out of 11 slices did not show any appreciable changes in $[\text{Na}^+]_o$ in response to anoxia with TTX perfusion (Fig. 1) ($p=0.0056$, Chi-Square test). OGD test showed these slices had good viability because they had an obvious sudden drop in $[\text{Na}^+]_o$ in response to OGD. Of the remaining 4 slices (36% of response rate), two showed a reduced drop in $[\text{Na}^+]_o$ induced by anoxia, and two did not have obvious changes in anoxia-induced $[\text{Na}^+]_o$ drop. These results suggest that voltage-gated Na^+ channels in the cortex principally mediate anoxia-induced Na^+ influx.

DOR activation attenuated anoxia or OGD-induced Na^+ influx

Our preliminary observation has shown that DOR activation inhibits anoxia-induced Na^+ influx in the cortex (Kang et al., 2009). To further confirm our observation, we perfused slices with UFP 512, a potent and specific DOR agonist (Balboni et al., 2002; Chao et al., 2007a), in different concentrations to examine the effects on anoxic Na^+ influx. With perfusion of UFP 512, the percentage of slices with no $[\text{Na}^+]_o$ drop in response to anoxia was increased. For example, 26% of the slices (5/19 and 6/23 respectively) had no response to anoxia under perfusion of 1 and 5 μM of UFP 512 respectively ($p=0.382$ and 0.373 respectively, Chi-Square test), and this percentage is significantly increased to 50% (8/16) in perfusion of 10 μM of UFP 512 ($p=0.024$, Chi-Square test). For the slices that showed a sudden drop in $[\text{Na}^+]_o$ in response to anoxia, perfusion of UFP 512 (1 and 5 μM) significantly attenuated the drop of $[\text{Na}^+]_o$ in a concentration-dependent fashion. For example, UFP 512 at 1 μM and 5 μM significantly attenuated the drop of $[\text{Na}^+]_o$ ($p<0.05$ and 0.01 respectively). In the group of 5 μM of UFP 512, UFP 512 also accelerated the recovery of the anoxia-induced drop of $[\text{Na}^+]_o$ ($n=17$, $p=0.05$). The time of occurrence of the maximal drop of $[\text{Na}^+]_o$ was not significantly different in both groups (Fig. 2). For the other 8 slices with a sudden $[\text{Na}^+]_o$ drop, increasing UFP 512 to 10 μM did not produce any significant decreases in the maximal Na^+ drop, occurrence of the maximal Na^+ drop, and recovery from anoxic Na^+ drop.

Since in clinical situations, such as stroke, concurrent lack of both oxygen and glucose often occurs, we extended our studies from oxygen deprivation alone to examining the effect of DOR activation on cortical slices deprived of both oxygen and glucose, to mimic ischemia. Similar to anoxia in most slices, OGD induced a sudden drop in $[\text{Na}^+]_o$ to a greater extent in all the slices investigated ($n=7$) (Fig. 3). For example, OGD induced an approximately 14% greater drop in $[\text{Na}^+]_o$ than anoxia, with about a 47% shorter period of the occurrence of a sudden drop of $[\text{Na}^+]_o$ and 0.8 times prolonged recovery from an OGD-induced drop of $[\text{Na}^+]_o$, suggesting OGD induced a more pronounced Na^+ influx in the cortex. With perfusion of UFP 512 (5 μM), OGD-induced Na^+ influx was markedly attenuated ($n=7$) (Fig. 3). For example, an OGD-induced drop of $[\text{Na}^+]_o$ decreased from 117.6 ± 2.1 mM to 101.3 ± 4.6 mM ($p=0.0075$). The recovery from Na^+ drop was accelerated (5.0 ± 0.5 min vs. 11.3 ± 1.2 min, $p=0.0004$). The occurrence of the maximal drop in $[\text{Na}^+]_o$ had no significant difference with UFP 512 perfusion ($n=7$) (4.5 ± 0.3 min vs. 4.3 ± 0.2 min, $p=0.56$).

To further ascertain the attenuating effect of DOR activation on anoxic Na^+ influx, we asked whether the effect of UFP 512 could be reversed by naltrindole (NTI), a DOR antagonist. Since our previous data have shown that 1 μM of NTI blocked DOR protection against anoxia-induced disruption of K^+ homeostasis in the cortex (Chao et al., 2007b), we also tested NTI at 1 μM in this work. Moreover, because UFP 512 at 5 μM is more effective in inhibiting Na^+ influx (Fig. 2), we perfused slices with 5 μM of UFP 512 and examined the effect of 1 μM of NTI. The results showed that perfusion with NTI (1 μM) alone, significantly increased the anoxia-induced drop of $[\text{Na}^+]_o$ ($p<0.05$) (Fig. 4 E), with a significantly shortened T_{max} ($p<0.001$) (Fig. 4 F) and a prolonged recovery from anoxic Na^+ drop ($p<0.01$) (Fig. 4 G) ($n=15$), suggesting that there is a tonic activity of endogenous

DOR in the cortex. In the presence of NTI (1 μ M), UFP 512 (5 μ M) could not inhibit anoxia-induced Na^+ influx anymore. The anoxia-induced drop in $[\text{Na}^+]_o$ increased from 82.9 ± 6.3 mM in UFP 512 alone (n=17) to 115.0 ± 0.9 mM in the group of UFP 512 plus naltrindole (n=17) ($p < 0.001$). The occurrence of the maximal drop in $[\text{Na}^+]_o$ was also shortened (10.0 ± 0.8 min in UFP 512 alone vs. 6.0 ± 0.7 min in UFP 512+NTI, n=17; respectively) ($p < 0.001$) and the shortened recovery from anoxic Na^+ drop by UFP 512 (4.5 ± 0.5 min) was also prolonged by NTI (11.7 ± 0.9 min) ($p < 0.001$) (Fig. 4).

These results suggest that DOR activation inhibits anoxia/OGD-induced Na^+ influx in the cortex.

DOR activation decreased veratridine-enhanced Na^+ influx

Veratridine is a Na^+ channel opener that increases Na^+ channel permeability and prevents their inactivation (Fekete et al., 2009; Ulbricht, 1998). Perfusion of 1 μ M veratridine did not produce any obvious changes in $[\text{Na}^+]_o$ in all the slices investigated (n=17) during 20 min of normoxia, but enhanced the anoxia-induced Na^+ influx by 9% ($p < 0.05$) (Figs. 5C, E). Under perfusion with 1 μ M veratridine, a very short period of anoxia (3.2 ± 0.2 min vs. 9.1 ± 0.8 min of anoxia in control, $p < 0.01$) was sufficient to induce a major decrease in $[\text{Na}^+]_o$, and the recovery from anoxic Na^+ drop was also prolonged 100% more than that of the control ($p < 0.001$) (Fig. 5). Perfusion of 5 μ M UFP 512 (n=15) greatly attenuated veratridine-enhanced anoxic Na^+ influx. The anoxia-induced drop in $[\text{Na}^+]_o$ was significantly decreased (93.7 ± 5.4 mM vs. 112.3 ± 2.1 mM in veratridine, $p < 0.01$), and the recovery from anoxia-induced Na^+ drop was shortened (4.1 ± 0.5 min vs. 13.6 ± 1.9 min in veratridine, $p < 0.001$). There was a definite trend towards delayed occurrence of the maximal drop in $[\text{Na}^+]_o$ due to anoxia although no statistical significance was found (4.1 ± 0.5 min vs. 3.2 ± 0.2 min in veratridine, $p = 0.07$) (n=15) (Fig. 5).

Increasing the concentration of veratridine to 5 and 10 μ M led to a significant drop in $[\text{Na}^+]_o$ within 3.5 min of perfusion even in normoxia (Fig. 6). For example, perfusion of 5 and 10 μ M veratridine, induced the decrease in $[\text{Na}^+]_o$ to the same levels (n=20, respectively) and a maximal drop of $[\text{Na}^+]_o$ was observed in 3.2 ± 0.2 min and 1.8 ± 0.1 min of perfusion, respectively, with a relatively prolonged recovery from a veratridine-induced drop of $[\text{Na}^+]_o$ (12.4 ± 1.2 min and 15.7 ± 1.5 min respectively) (Fig. 6). Perfusion of 5 μ M of UFP 512 significantly inhibited veratridine-induced Na^+ influx in 5 μ M (n=18) but not of 10 μ M veratridine (n=17). As shown in Fig. 6, with perfusion of UFP 512 (5 μ M), a 5 μ M veratridine-induced drop in $[\text{Na}^+]_o$ was decreased from 107.9 ± 1.1 mM to 97.5 ± 3.1 mM ($p = 0.004$), the occurrence of Na^+ drop was postponed (4.6 ± 0.5 min vs. 3.2 ± 0.2 min, $p = 0.01$), and the recovery from Na^+ drop was accelerated (8.7 ± 1.0 min vs. 12.4 ± 1.2 min, $p = 0.03$). In contrast, perfusion of UFP 512 (5 μ M) could not inhibit 10 μ M veratridine-induced Na^+ influx. With perfusion of UFP 512 (5 μ M), a 10 μ M veratridine-induced drop in $[\text{Na}^+]_o$ had a slight decrease but with no statistical significance ($p = 0.11$), the occurrence of Na^+ drop also did not have an obvious change ($p = 0.12$) and the recovery from Na^+ drop had a tendency of being shortened but with no statistical significance ($p = 0.07$) (Fig. 6).

Effect of PKC (and its isoforms) inhibition on DOR attenuation of anoxia-induced Na^+ influx

Our previous studies have shown that PKC is involved in DOR signaling in DOR neuroprotection (Ma et al., 2005). We have also shown that DOR protection against anoxic K^+ derangement in the cortex, which involves Na^+ influx via voltage-gated Na^+ channels (Chao et al., 2008, 2009; Kang et al., 2009), is mediated by the PKC pathway (Chao et al., 2007b). Voltage-gated Na^+ channels are important targets modulated by metabotropic receptors via G protein/protein kinases, particularly PKC (Chen et al., 2006; Dascal and

Lotan, 1991; West et al., 1991). Therefore, we asked if DOR attenuation of anoxic Na^+ influx is mediated by PKC activation. If so, what type(s) of PKC isoform is a key element?

Effect of chelerythrine on DOR attenuation of anoxia-induced Na^+ influx

To examine PKC effects on DOR attenuation of anoxic Na^+ influx, we perfused cortical slices with chelerythrine (CHEL), a broad-spectrum PKC blocker, and then checked the effect of DOR. As shown in Fig. 7, perfusion of CHEL (10 μM) alone had no effect on the anoxic drop in $[\text{Na}^+]_o$ ($p>0.05$, $n=17$). However, T_{max} for Na^+ drop was significantly shortened ($p<0.001$), and the recovery duration from anoxic Na^+ drop to baseline was greatly prolonged ($p<0.01$, $n=17$). These results suggest that TTX-sensitive Na^+ influx involves PKC activity. In the presence of CHEL (10 μM), perfusion of UFP 512 (5 μM) did not reduce anoxia-induced Na^+ influx. For example, the attenuation of Na^+ drop with DOR activation disappeared (no statistical significance was found in comparison with CHEL, control and UFP 512). T_{max} of Na^+ drop was 4.8 ± 0.5 min ($p<0.001$ vs. 10.0 ± 0.8 min in UFP 512, $p>0.05$ vs. 4.2 ± 0.5 min in CHEL alone) and the recovery duration from anoxic Na^+ drop to baseline was 8.6 ± 0.7 min ($p<0.01$ vs. 4.5 ± 0.5 min in UFP 512, $p>0.05$ vs. 9.2 ± 1.0 min in CHEL alone) ($n=16$) (Fig. 7). These results suggest DOR effects are PKC-mediated.

Effect of protein kinase C β II peptide inhibitor (PKC β II PI) on DOR attenuation of anoxia-induced Na^+ influx

PKC translocates from the cytosol to the membrane-bound site and thus display its function (Kraft and Anderson, 1983; Mochly-Rosen, 1995). To determine whether PKC β II isoform mediates the DOR attenuation of anoxic Na^+ influx, we perfused cortical slices with PKC β II PI. As shown in Fig. 8, 0.1 μM of PKC β II PI alone did not produce significant changes in either anoxia-induced maximal drop in $[\text{Na}^+]_o$ ($p>0.05$ vs. control) or recovery from Na^+ drop ($p>0.05$) ($n=16$) (Fig. 8). T_{max} for Na^+ drop was significantly shortened ($p<0.01$ vs. control, $n=16$) (Fig. 8). Co-perfusion with PKC β II PI (0.1 μM) and UFP 512 (5 μM) ($n=14$) no longer reduced the anoxia-induced drop in $[\text{Na}^+]_o$ (105.9 ± 1.9 mM in co-perfusion vs. 82.9 ± 6.3 mM in UFP 512 alone, $p<0.01$, and 106.4 ± 3.1 mM in PKC β II PI alone, $p>0.05$), or hasten the recovery from Na^+ drop (5.0 ± 0.5 min vs. 6.4 ± 0.9 min in PKC β II PI alone, $p>0.05$), nor did it shorten T_{max} for Na^+ drop (6.7 ± 0.8 min vs. 10.0 ± 0.8 min in UFP 512 alone, $p<0.05$, and 5.3 ± 0.6 min in PKC β II PI alone, $p>0.05$) (Fig. 8). These findings suggest that PKC β II isoform is involved in signaling of DOR effects on anoxia-induced Na^+ influx through TTX-sensitive Na^+ channels in the cortex.

Effect of protein kinase C θ pseudosubstrate inhibitor (PKC θ PI) on DOR attenuation of anoxia-induced Na^+ influx

To determine whether PKC θ isoform mediates the DOR attenuation of anoxic Na^+ influx, we perfused cortical slices with PKC θ PI. Similar to PKC β II PI perfusion, PKC θ PI (0.1 μM) alone did not produce significant changes in the anoxia-induced maximal drop in $[\text{Na}^+]_o$ ($p>0.05$ vs. control) ($n=16$). However, the occurrence of anoxia-induced Na^+ drop was shortened ($p<0.001$ vs. control) while the recovery from Na^+ drop was quickened ($p<0.05$). In co-perfusion with PKC θ PI (0.1 μM) and UFP 512 (5 μM), the anoxia-induced maximal drop of $[\text{Na}^+]_o$ was 99.6 ± 4.5 mM, and the maximal drop of $[\text{Na}^+]_o$ appeared in 5.8 ± 0.6 min of anoxia induction, and the recovery from anoxia-induced Na^+ drop was in 6.6 ± 0.9 min ($n=15$). In comparison with UFP 512 alone, there was a significant increase in Na^+ influx ($p<0.05$), and a significant reduction in the occurrence of Na^+ drop ($p<0.001$) ($n=15$) (Fig. 9). Though there was no statistical significance between PKC θ PI plus UFP 512 and UFP 512 alone, in terms of recovery from an anoxia-induced maximal Na^+ drop ($p=0.053$), there was an obvious trend to slow the recovery duration with co-perfusion of PKC θ PI (0.1 μM) and UFP 512 (5 μM) ($n=15$) (Fig. 9).

Discussion

We have systemically investigated the DOR protection against anoxia-induced Na^+ influx in the cortex and further clarified that voltage-gated Na^+ channels principally mediate anoxia-induced Na^+ influx in the cortex. We also show that DOR activation inhibits OGD or Na^+ channel opener induced Na^+ influx in the cortex. Moreover, we show that a critical signaling mechanism, i.e., the PKC, especially PKC β II and PKC θ isoforms, mediates the DOR action on Na^+ influx through Na^+ channels.

Role of voltage-gated Na^+ channels in anoxic/ischemic Na^+ influx in the cortex

Multiple pathways including voltage-gated Na^+ channels have been proposed to mediate hypoxic/ischemic Na^+ influx in neurons. However there is still dispute over the role of voltage-gated Na^+ channels in hypoxic/ischemic Na^+ entry (see review by Chao and Xia, 2010) despite the fact that blockade of these channels has been shown to attenuate/postpone anoxic depolarization, and prevent hypoxia/ischemia-induced neuronal injury and death (Banasiak et al., 2004; Müller and Somjen, 2000). Our results in this study, using direct measurement of extracellular Na^+ , indicate that the anoxia-induced $[\text{Na}^+]_o$ drop is attenuated or completely abolished in most slices investigated in the presence of TTX ($p=0.0056$ Chi-Square test). Together with our previous studies with Na^+ channel blocker and opener as well as Na^+ substitution in the cortex (Chao et al., 2008, 2009; Kang et al., 2009), these results strongly support a key role for voltage-gated Na^+ channels in anoxic/ischemic Na^+ influx into neurons in the cortex.

DOR activation attenuates anoxic/ischemic Na^+ influx via Na^+ channels in the cortex

We and others have demonstrated that DOR activation is neuroprotective against hypoxic/ischemic insults (Borlongan et al., 2009; Gao et al., 2010; Gwak et al., 2010; Turner and Johnson, 2011; Yang et al., 2011; also see review by Chao and Xia, 2010). One of the underlying mechanisms is associated with its capability to stabilize hypoxia/ischemia-induced disruption of K^+ homeostasis which, in a large part, relies on Na^+ entry via TTX-sensitive voltage-gated Na^+ channels (Chao et al., 2008, 2009). However, it is not clear whether DOR activation directly inhibits hypoxic/ischemic Na^+ entry via Na^+ channels. Most recently, our preliminary data, though with limited numbers of brain slices, suggest that DOR activation could reduce anoxic Na^+ influx in the mouse cortex (Kang et al., 2009). To ascertain this notion and validate the important conclusion, we systemically characterized the DOR effect by investigating the role of DOR in the anoxic Na^+ influx in the cortex with different concentrations of DOR agonist and along with a Na^+ channel opener and blocker. We also extend our scope from the anoxic condition to simulated ischemic condition. Our results clearly indicate that DOR activation attenuates Na^+ influx via Na^+ channels in the cortex not only in the condition of anoxia but also in that of ischemia, a clinically relevant situation in stroke.

The high concentration of UFP 512 may induce a relatively complex effect under certain conditions. We noticed that 10 μM of UFP 512 completely abolished the Na^+ response to anoxia in 50% of the slices. This rate is much higher than that at a lower concentration (1–5 μM), suggesting a better inhibitory efficacy. Surprisingly, there were half of the slices that had no appreciable reduction in hypoxic response. In these cases, other membrane proteins, besides DOR, may be activated, thus disturbing the function of DOR protection. For example, μ -opioid receptors (MOR) may be activated because UFP 512, like all other DOR agonists, has a low affinity for MOR though with the highest specificity in the currently available DOR agonists (Balboni et al., 2002). MOR is reported harmful/toxic but not protective to neurons in certain patho-physiological conditions including hypoxia/ischemia (Ammon-Treiber et al., 2005; Chao et al., 2007a; Chen et al., 2005; Kofke et al., 1999; Liao

et al., 2003; Yin et al., 1999). Therefore, the net outcomes of UFP 512 at a high concentration are determined by the balance between DOR protection and potential MOR toxicity.

DOR activation attenuates veratridine-induced Na⁺ influx

Na⁺ channel opener veratridine in a low concentration enhanced the anoxia-induced Na⁺ influx, while a higher concentration (5 μ M) of veratridine induced, even in normoxia, a considerably large drop of [Na⁺]_o in a very short period, which is comparable with the change that occurred in anoxia. These results further confirmed the role of Na⁺ channels in the hypoxia/ischemia-induced Na⁺ influx. Since DOR activation attenuated both the veratridine-enhanced anoxic change and veratridine-induced Na⁺ influx, DOR signal may involve a similar mechanism, i.e., targeting on Na⁺ channels. However, DOR activation could not affect the change in Na⁺ influx induced by 10 μ M of veratridine although 5 and 10 μ M of veratridine induced a similar extent of Na⁺ drop. This phenomenon may be explained by several possibilities. First, veratridine at more than 5 μ M might maximally open Na⁺ channels since the number of Na⁺ channels on the membrane is limited. However, a higher concentration (e.g., 10 μ M) may cause Na⁺ channels to stay open (Ulbricht, 1998), being resistant to DOR action. Alternatively, veratridine at higher concentrations may lead to some secondary effects (Fekete et al., 2009; Ulbricht, 1998), which in turn might abolish the effect of DOR activation on Na⁺ influx.

Our data suggest that Na⁺ channel ligands and other factors other than hypoxic/ischemic stress, if excessive, may disrupt Na⁺ homeostasis and thus cause neuronal injury. Indeed, a large Na⁺ influx may occur in certain conditions such as trauma (Agrawal and Fehlings, 1996; Iwata et al., 2004), and chemical/pollutant poisoning (Chao et al., 2012). No matter what is the cause, excessive Na⁺ influx triggers a disruptive change in other ions across the membrane and causes potentially injurious consequences to neurons, e.g., acute functional and morphological changes including loss of electrophysiological response to stimulus, cell swelling, bleb formation and membrane injury (Chao and Xia, 2010). Therefore, DOR-mediated protection against the disruption of Na⁺ homeostasis may potentially have a broad implication in neuroprotection either in hypoxic/ischemic or other conditions.

PKC involvement in DOR attenuation of disruption of Na⁺ homeostasis

The interaction between DOR and Na⁺ channels has been implicated in our previous studies (Xia et al., 2000, 2001, 2003; Zhao et al., 2005). Our recent work in *Xenopus* oocytes with co-expressed DOR and Na⁺ channels clearly showed that DOR activation/expression inhibit Na⁺ channel function (Kang et al., 2009). However, it is poorly understood at present how DOR regulates the activity of these Na⁺ channels. The PKC family of serine/threonine kinases regulates diverse cellular functions and has been implicated in hypoxic/ischemic stress (Bright and Mochly-Rosen, 2005; Chou and Messing, 2005). PKC has been shown to mediate DOR signaling and DOR neuroprotection (Chao et al., 2007b; Law et al., 2000; Ma et al., 2005; Narita et al., 2006). Intriguingly, activation of PKC has been demonstrated to modulate the activities of Na⁺ channels by phosphorylation of certain sites of Na⁺ channel subunits leading to the inhibition of Na⁺ currents (Chen et al., 2006; Dascal and Lotan, 1991; West et al., 1991), and preventing hypoxia/ischemia-associated neuronal loss and dendrite spine and synapse damage (Sun et al., 2008). This mechanism may contribute to intracellular Na⁺ overload through voltage-dependent Na⁺ channels (Banasiak et al., 2004; Churchwell et al., 1996; Friedman and Haddad, 1994; Hasbani et al., 1998; Koike et al., 2000). Therefore, PKC is very likely the link between DOR and Na⁺ channels, regulating Na⁺ entry through Na⁺ channels during anoxia/ischemia. The present study showed that DOR attenuation of anoxia-induced TTX-sensitive Na⁺ influx could be reversed by a broad-spectrum PKC blocker, chelerythrine. Fazzina et al. (2010) reported that activation of PKC

with phorbol ester significantly reduced brain water concentration in the infarction area (brain edema) in a rodent stroke model, which was associated with the reduced shift of Na^+ that normally accompanies cell swelling and brain edema (Ayata and Ropper, 2002; Churchwell et al., 1996). In addition, a recent study indicated that activation of opioid receptors including DOR significantly decreases water contents (brain edema) in OGD-exposed hippocampal slices (Yang et al., 2011). Our previous work indicated that DOR protection against anoxic K^+ derangement, which in a large part relies on Na^+ influx via TTX-sensitive voltage-gated Na^+ channels (Chao et al., 2008, 2009), is also PKC-dependent (Chao et al., 2007b). Taken together, these findings support our hypothesis that DOR activation attenuates anoxic Na^+ influx through Na^+ channels via a PKC-dependent pathway in the cortex.

The PKC family of serine/threonine kinases consists of at least 10 different isozymes that exist in the brain (Bright and Mochly-Rosen, 2005). Based on their structure and regulatory properties, these isozymes have been classified into three groups: conventional PKCs (α , βI , βII , γ) that are activated by diacylglycerol (DAG) and Ca^{2+} , novel PKCs (δ , ϵ , θ , η) that are activated by DAG but not Ca^{2+} , and atypical PKCs (ζ , λ/ι) that are insensitive to both DAG and Ca^{2+} (Chou and Messing, 2005). Although various PKC isozymes mediate different and sometimes opposing functions after activation by the same stimulus (Chen et al., 2001; Wang et al., 2004), we chose PKC βII and PKC θ isozymes to explore their role in DOR attenuation of anoxic Na^+ influx via Na^+ channels. This is based on the following reasons: (1) these isozymes belong to different groups, i.e., PKC βII belongs to conventional PKCs while PKC θ belong to novel ones and their role in mediating DOR effect on Na^+ influx is unknown despite PKC βII having been linked with the functioning of Na^+ channel (Vijayaragavan et al., 2004); (2) the roles of these isozymes in cell protection are unclear because little information is available regarding the role of PKC θ in neuroprotection (Fauconnier et al., 2011) though activation of PKC βII has been shown to be neuroprotective (Cordey and Pike, 2006; Li et al., 2005); (3) their changes in expression/activities during hypoxia/ischemia are poorly known with no information available for PKC θ and mixed/conflict results for PKC βII (increased, decreased or no change) (Krupinski et al., 1998; Li et al., 2006; Selvatici et al., 2003, 2006; Sieber et al., 1998); and (4) more importantly, their inhibitors are relatively specific and accountable based on published literature (Kaminski et al., 2007; Karagiannides et al., 2011; Omiyi et al., 2005). Many traditional inhibitors of PKC isozymes influence cellular functions with cross reaction, while the inhibitors we used in the present study are those that specifically block the interactions between isozymes and the specific isozyme PKC-binding proteins called the receptors for activated C kinase. This in turn blocks the translocation of activated PKC isozymes to specific subcellular compartments that are unique for each isozyme, thus specifically blocking their effects (Bright and Mochly-Rosen, 2005; Chou and Messing, 2005; Kaminski et al., 2007; Karagiannides et al., 2011; Omiyi et al., 2005). Though individual PKC isozymes may mediate different and sometimes opposing functions after activation by the same stimulus (Chen et al., 2001), and play unique roles in mediating both survival and cell death pathways in the brain (Wang et al., 2004), our results suggest that both of these isozymes play a similar role in DOR signaling in inhibiting the functions of Na^+ channels. PKC βII belongs to conventional PKCs that are activated by DAG and Ca^{2+} , while PKC θ is activated by DAG but not Ca^{2+} (Bright and Mochly-Rosen, 2005; Chou and Messing, 2005). Therefore, it seems that DAG is critically required for transduction of DOR signal to Na^+ channels. Indeed, DAG may be one of the most important downstream signals of DOR (Law et al., 2000; Yang et al., 2010).

Voltage-gated Na^+ channels are primary mediators of the collapse of ion homeostasis during hypoxia/ischemia (Chao and Xia, 2010). Blockade of Na^+ channels and Na^+ influx through them has been shown to attenuate/postpone anoxic depolarization, and prevent hypoxia/

ischemia-induced neuronal injury and death (Banasiak et al., 2004; Müller and Somjen, 2000). PKC θ has been recently reported to be deleterious in the brain because mice deficient in PKC θ demonstrate normal brain morphology without any signs of edema and swelling due to cerebral ischemia caused by microvascular disease, unlike wild-type mice exhibiting distinct signs of ischemic brain damage in experimental cerebral malaria (Fauconnier et al., 2011). In contrast to this result, our data showed PKC θ may mediate DOR neuroprotection against anoxic Na⁺ influx via Na⁺ channels though no information is available regarding the functional linking with Na⁺ channels. The discrepancy regarding the role of PKC θ in cerebral ischemia may be attributed to the differences in the models and insults. In contrast to PKC θ , conventional PKC β II damages cardiac function (Omiyi et al., 2005) but protects the brain in hypoxic/ischemic and toxic stress (Cordey and Pike, 2006; Li et al., 2005). Consistent with the notion of its protection in the brain, our results suggest that PKC β II signals the DOR neuroprotection by stabilizing anoxia-induced Na⁺ derangement via Na⁺ channels.

In conclusion, we found that DOR activation inhibits both anoxia and ischemia-induced Na⁺ influx through TTX-sensitive Na⁺ channels; Na⁺ channel opener can enhance anoxic Na⁺ influx or induce excessive Na⁺ influx in normoxia, which can also be reduced by DOR activation. The DOR action against the disruption of Na⁺ homeostasis is dependent on PKC, especially PKC β II and PKC θ isozymes. To our knowledge, this is the first demonstration of PKC-mediated DOR attenuation of anoxic/ischemic Na⁺ influx through Na⁺ channels in the cortex.

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Abbreviations

[Na ⁺] _o	extracellular Na ⁺ concentrations
ACSF	artificial cerebro-spinal fluid
CHEL	chelerythrine
DAG	diacylglycerol
DOR	delta-opioid receptors
MOR	μ -opioid receptors
NTI	naltrindole
OGD	oxygen-glucose deprivation
PKC	protein kinase C
PKC β II PI	protein kinase β II peptide inhibitor
PKC θ PI	protein kinase C θ pseudosubstrate inhibitor
TTX	tetrodotoxin

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Fig. 1.

Effect of Na^+ channel blockade on anoxia-induced Na^+ influx in mouse cortical slices.

Anoxia induced a sudden large drop in $[Na^+]_o$ (A), which could be completely abolished in most slices (7 out of 11) by voltage-gated Na^+ channel blocker, TTX (B). OGD test showed these slices in B had good viability (C). Of the remaining 4 (36%) TTX-treated slices that still responded to anoxia, two showed a reduced drop in anoxia-induced $[Na^+]_o$ (D) and two had no appreciable changes in anoxia-induced $[Na^+]_o$ drop. These results suggest that the blockade of voltage-gated Na^+ channels in the cortex largely blocked the anoxia-induced Na^+ influx in the cortex.

Fig. 2.

Effect of DOR activation with UFP 512 (1, 5 μM) on anoxia-induced Na^+ influx in mouse cortical slices. Trace recordings of A: control (Cont), B: UFP 512 (1 μM), C: UFP 512 (5 μM). D–F are statistical results of each recording parameter. * $p < 0.05$, ** $p < 0.01$ as compared with the controls. Note that UFP 512 at 1 and 5 μM significantly attenuated anoxic decrease in $[\text{Na}^+]_o$, suggesting that DOR activation attenuates anoxic Na^+ influx.

Fig. 3. Effect of DOR activation with UFP 512 on ischemia-induced Na^+ influx in mouse cortical slices. Trace recordings of A: anoxia; B: oxygen-glucose deprivation (OGD); C: OGD+UFP 512 (5 μM). D–F are statistical results of each recording parameter. **p<0.01, ***p<0.001 as compared with anoxia; ##p<0.01, ###p<0.001 as compared with OGD. Note that OGD induced a sudden drop in $[\text{Na}^+]_o$ to a greater extent than anoxia in all the slices investigated, which could be attenuated by UFP 512 (5 μM).

Fig. 4. DOR antagonist blocked the DOR attenuation of anoxic Na^+ influx. Trace recordings of A: control (Cont), B: UFP 512 (5 μM), C: Naltrindole (NTI) (1 μM) and D: NTI+UFP 512. E–G are statistical results of each recording parameter. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared with the controls; ### $p < 0.001$ vs. UFP 512. Note that NTI (1 μM) alone significantly increased anoxia-induced drop of $[\text{Na}^+]_o$, with a significant shortened T_{max} and prolonged recovery from anoxic Na^+ drop, suggesting a tonic activity of endogenous DOR. In the presence of NTI (1 μM), UFP 512 (5 μM) could not inhibit anoxic Na^+ influx anymore.

Fig. 5.

Effect of DOR activation on veratridine-enhanced anoxic drop in $[Na^+]_o$. Trace recordings of A: control (Cont), B: UFP 512 (5 μ M), C: veratridine (1 μ M) and D: veratridine+UFP 512. E–G are statistical results of each recording parameter. * $p < 0.05$, ** $p < 0.01$ as compared with the controls; ### $p < 0.001$ vs. UFP 512; && $p < 0.01$, &&& $p < 0.001$ as compared with veratridine. Note that perfusion of 1 μ M veratridine itself for 20 min could not produce any obvious changes in $[Na^+]_o$ in normoxia, but greatly enhanced anoxia-induced Na^+ influx, which could be greatly attenuated by DOR activation with 5 μ M UFP 512.

Fig. 6.

Effect of DOR activation on Na^+ influx induced by Na^+ channel opener veratridine in normoxia. Trace recordings of A: veratridine (5 μM) (ver-5), B: UFP 512 (5 μM), C: veratridine (10 μM) (ver-10) and D: UFP 512 (5 μM). E–G are statistical results of each recording parameter. * $p < 0.05$, ** $p < 0.01$ as compared with the controls; ### $p < 0.001$ vs. ver-5. Perfusion of 5 and 10 μM of veratridine induced a drop in $[\text{Na}^+]_o$ to the same degree under normoxia; but a very short period of perfusion with 10 μM veratridine was sufficient to induce a major decrease in $[\text{Na}^+]_o$. Activation of DOR with UFP 512 (5 μM) significantly attenuated Na^+ influx induced by 5 but not 10 of veratridine under normoxia.

Fig. 7.

Involvement of PKC in DOR-mediated inhibition of anoxia-induced Na^+ influx. Trace recordings of A: control (Cont), B: UFP 512 (5 μ M), C: chelerythrine (CHEL) (10 μ M) and D: CHEL+UFP 512. E–G are statistical results of each recording parameter. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared with the controls; ## $p < 0.01$, ### $p < 0.001$ vs. UFP 512. Note that DOR-mediated inhibition of anoxia-induced Na^+ influx was blocked by broad-spectrum PKC blocker, chelerythrine.

Fig. 8.

Involvement of PKCβII in DOR-mediated inhibition of anoxia-induced Na⁺ influx. Trace recordings of A: control (Cont), B: UFP 512 (5 μM), C: PKCβII peptide inhibitor (PKCβII PI) (0.1 μM) and D: PKCβII PI+UFP 512. E–G are statistical results of each recording parameter. **p*<0.05, ***p*<0.01 as compared with the controls; #*p*<0.05, ##*p*<0.01 vs. UFP 512. Note that DOR-mediated inhibition of anoxia-induced Na⁺ influx was blocked by PKCβII peptide inhibitor.

Fig. 9.

Involvement of PKC θ in DOR-mediated inhibition of anoxia-induced Na^+ influx. Trace recordings of A: control (Cont), B: UFP 512 (5 μ M), C: PKC θ pseudosubstrate inhibitor (PKCq PI) (0.1 μ M) and D: PKCq PI+UFP 512. E–G are statistical results of each recording parameter. * p <0.05, ** p <0.01, *** p <0.001 as compared with the controls; # p <0.05, ### p <0.001 vs. UFP 512; & p <0.05 as compared with PKCq PI. Note that DOR-mediated inhibition of anoxia-induced Na^+ influx was blocked by PKC θ pseudosubstrate inhibitor.