Oxygen deprivation inhibits Na^+ current in rat hippocampal neurones via protein kinase C

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1. Hippocampal neurones respond to acute oxygen deprivation (hypoxia) with an inhibition of whole-cell Na^+ current (I_{Na}), although the mechanism of the inhibition is unknown. Kinases can modulate I_{Na} and kinases are activated during hypoxia. We hypothesized that kinase activation may play a role in the hypoxia-induced inhibition of I_{Na}.

2. Single electrode patch clamp techniques were used in dissociated hippocampal CA1 neurones from the rat. I_{Na} was recorded at baseline, during exposure to kinase activators (with and without kinase inhibitors), and during acute hypoxia (with and without kinase inhibitors).

3. Hypoxia (3 min) reduced I_{Na} to 38·1 ± 4·5% of initial values, and shifted steady-state inactivation in the negative direction. Hypoxia produced no effect on activation or fast inactivation.

4. Protein kinase A (PKA) activation with 2·5 mM adenosine 3′,5′-cyclic adenosine monophosphate, N^6,O^2-dibutyryl, sodium salt (db-cAMP) resulted in reduction of I_{Na} to 62·8 ± 5·5% without an effect on activation or steady-state inactivation. I_{Na} was also reduced by activation of protein kinase C (PKC) with 5 nM phorbol 12-myristate 13-acetate (PMA; to 40·0 ± 3·7%) or 50 μM 1-oleoyl-2-acetyl-*sn*-glycerol (OAG; to 46·1 ± 2·8%). In addition, steady-state inactivation was shifted in the negative direction by PKC activation. Neither the activation curve nor the kinetics of fast inactivation was altered by PKC activation.

5. The response to PKA activation was blocked by the PKA inhibitor *N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinolinesulphonamide (H-89; 30 μM) and by 30 μM of PKA inhibitory peptide PKA_{5-24} (PKAi). PKC activation was blocked by the kinase inhibitor 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7; 100 μM), by the PKC inhibitor calphostin C (10 μM), and by 20 μM of the inhibitory peptide PKC_{19-31} (PKCi).

6. The hypoxia-induced inhibition of I_{Na} and shift in steady-state inactivation were greatly attenuated with H-7, calphostin C, or PKCi, but not with H-89 or PKAi.

7. We conclude that hypoxia activates PKC in rat CA1 neurones, and that PKC activation leads to the hypoxia-induced inhibition of I_{Na}.

Voltage-gated Na^+ channels play a crucial role in neuronal function in the central nervous system (CNS). The action potentials that are initiated and propagated by these channels are essential for communication between neurones and are necessary for many physiological functions. In addition to involvement in normal physiological events, there is mounting evidence that Na^+ channels in the CNS play an important role in pathophysiological events such as hypoxia (Urenjak & Obrenovitch, 1996). Studies have shown that reducing Na^+ channel activity (e.g. by application of tetrodotoxin) attenuates neuronal hypoxic responses and reduces hypoxia-induced neuronal injury and death *in vitro* (Weber & Taylor, 1994) and *in vivo* (Prenen, Go, Postema, Zuiderveen & Korf, 1988).

Recent reports from our laboratory are of particular interest with respect to the relation between Na^+ channels and hypoxia (Cummins, Donnelly & Haddad, 1991; Cummins, Jiang & Haddad, 1993). These studies have demonstrated that neurones in the adult neocortex and hippocampus respond to acute hypoxia with a rapid reduction in whole-cell Na^+ current (I_{Na}). This inhibition of I_{Na} is due, at least

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in part, to a negative shift in steady-state inactivation, reducing the availability of Na^+ channels for activation at the same membrane potential. We have previously suggested that this inhibition of Na^+ channels during hypoxia is an adaptive response that enhances neuronal tolerance of a low-oxygen environment and may delay hypoxia-induced neuronal injury and death (Cummins *et al.* 1991, 1993). Inhibition of Na^+ channel activation would result in reduced neuronal activity and Na^+ influx across the neuronal membrane, thereby reducing the metabolic demand on neurones during a time when energy production is reduced (Astrup, Sorensen & Sorensen, 1981).

The cellular mechanism(s) underlying the hypoxia-induced inhibition of I_{Na} is (are) not known. One mechanism found in excitable cells, including neurones, that can result in Na^+ current inhibition is protein phosphorylation (Cukierman, 1996). Several studies have shown that both protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) are capable of phosphorylating the brain Na^+ channel (Costa & Catterall, 1984; Rossie & Catterall, 1987), and kinase activation can result in a reduction of I_{Na} and changes in the voltage dependence of activation and inactivation (Dascal & Lotan, 1991; Numann, Catterall & Scheuer, 1991; Gershon, Weigl, Lotan, Schreibmayer & Dascal, 1992; Li, West, Lai, Scheuer & Catterall, 1992; Godoy & Cukierman, 1994). However, the physiological significance of Na^+ current modulation is unknown.

We hypothesized that exposure to hypoxia activates a kinase that results in inhibition of I_{Na} in adult CNS neurones. To address this question, we used acutely dissociated neurones from the CA1 region of the hippocampus from rats at postnatal days P17–25. We recorded the whole-cell Na^+ current (I_{Na}) in these neurones with single electrode patch clamp techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981), and studied baseline recordings of I_{Na} during normoxia, the response of I_{Na} to kinase activation (with and without kinase inhibitors), and the response of I_{Na} to hypoxia (with and without kinase inhibitors).

METHODS

Tissue preparation and neurone isolation

Neurones were dissociated with a modification of the method of Kay & Wong (1986). We used Sprague–Dawley rats at postnatal day P17–25. At this age, hippocampal neurones respond rapidly with a reduction in Na^+ current when exposed to hypoxia while neurons from younger animals (P2–7) do not (Cummins *et al.* 1991). In addition, electrophysiological properties of cortical neurones at this age are similar to those in older animals (McCormick & Prince, 1987) and the yield of viable mature neurones with our dissociation technique is higher at this age than at later times in development. The rats were deeply anaesthetized with methoxyflurane and decapitated. The cortex was removed and submerged in iced Ringer solution (mM: 125 NaCl, 3 KCl, 1·3 $MgSO_4$, 2·5 $CaCl_2$, 2·5 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose; pH 7·4; equilibrated with 95% O_2–5% CO_2). After 1 min, the cortex was removed from the Ringer solution and the hippocampus was isolated. Slices (300–400 μM) were taken with a tissue chopper and transferred to a chamber containing Hepes-buffered saline (mM: 140 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 25 Glucose; pH 7·4; equilibrated with 100% O_2). After 10–30 min, trypsin (0·02–0·04%, Type XI, Sigma) was added to the incubation chamber. Following 30 min of exposure to trypsin, the slices were washed and incubated for 15 min with protease (0·01–0·02%, Type XIV, Sigma). The tissue was then washed (2 × 30 min) before being prepared for recording. Immediately before recording, a hippocampal slice was removed from the chamber and the CA1 region was cut away from the remaining tissue. CA1 neurones were acutely isolated by gentle trituration with fire-polished Pasteur pipettes. The cell suspension was plated on coverslips, transferred to the recording chamber (room temperature, 22–24 °C), perfused with a low-Na^+ solution (see below) at 1–2 ml min^{-1}, and viewed on an inverted microscope with Hoffman optics.

Morphological and electrophysiological criteria for recording

As in our previous publications (Cummins *et al.* 1991, 1993) we considered cells to be neurones and to be viable when they had a smooth surface, a three-dimensional contour, a pyramidal shape with an apical dendrite, and no signs of swelling. Cells that exhibited a flat contour, a grainy surface, a round or swollen shape, or no processes were considered to be of non-neuronal origin, severely injured, or dead, and no attempt was made to record from them. Electrophysiological criteria for acceptable recordings were as follows. (a) Seal resistance: neurones were considered for recording when the seal resistance after patching the cell was $\geqslant 5$ GΩ. In preliminary experiments, recordings from cells with a seal resistance of < 5 GΩ were often found to be unstable or exhibited a rapid run-down of the Na^+ current after initiation of whole-cell recording. (b) Whole cell recording: only neurones with a holding current of $\leqslant 0{\cdot}1$ nA (holding potential, −100 mV) upon initiation of whole-cell recording were used for data collection. Cells with holding currents greater than 0·1 nA often exhibited rapid deterioration and run-down of the Na^+ current within minutes of whole-cell recording. These cells were considered injured or leaky and were not used. (c) Voltage error: recordings from some cells exhibited voltage clamp problems due in part to voltage errors arising from large Na^+ currents or large series resistance. These problems were exaggerated when recordings were performed in an extracellular Na^+ concentration of 140 mM. Reducing the extracellular Na^+ concentration to 20 mM decreased the peak Na^+ current by ~30–60%, and reduced the potential voltage error by a similar amount. Adequate voltage control was assessed by comparing I–V curves from two holding potentials, −100 and −70 mV. Recordings were considered acceptable when both scaled I–V curves were identical and smoothly graded over the voltage range of activation (~−50 to −10 mV). In addition, low-resistance electrodes (2–4 MΩ) and $\geqslant 80$% series resistance compensation were used to reduce voltage errors due to series resistance. Neurones were accepted for analysis when the compensated voltage error was $\leqslant 5$ mV. Cells with larger voltage errors (> 5 mV) often exhibited voltage clamp problems and were not used in the data analysis. Therefore, only neurones that met the following electrophysiological criteria were used for data analysis in this study: (a) seal resistance of $\geqslant 5$ GΩ, (b) holding current of $\leqslant 0{\cdot}1$ nA (holding potential, −100 mV), (c) peak current $\leqslant 4$ nA (holding potential, −70 mV; step to −10 mV), (d) uncompensated series resistance of $\leqslant 10$ MΩ, (e) peak compensated voltage error of $\leqslant 5$ mV, (f) identical scaled activation curves at −100 and −70 mV, and (g) stable Na^+ current (holding potential, −70 mV; step to −10 mV) for $\geqslant 1$ min.

Electrophysiological recordings

Whole cell recordings with single electrode patch clamp techniques were used to study the Na^+ current (Hamill *et al.* 1981). All recordings were performed at room temperature (22–24 °C) in a low-Na^+ saline solution (mM: 20 NaCl, 120 choline chloride, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 25 Glucose, 0·5 $CdCl_2$; pH 7·4; 305 mosmol l^{-1}). Electrodes were pulled on a Flaming–Brown micropipette puller (Model P-87, Sutter Instrument Co., Novato, CA, USA) from borosilicate capillary glass (1·2 mm o.d., 0·69 mm i.d.). The electrodes were fire polished, and resistances were 2–4 MΩ. For most recordings of Na^+ currents, the patch pipette contained (mM): 130 CsF, 10 Na-Hepes, 1 $CaCl_2$, 10 EGTA, 1 $MgCl_2$, 10 TEA-Cl, 10 Glucose; pH 7·4; 300 mosmol l^{-1}. Some experiments were performed with caesium aspartate in the pipette in place of CsF since it has been suggested that fluoride may affect some cellular processes (Kay, 1992). K^+ currents were blocked with TEA and Cs^+ in the pipette, and Ca^{2+} currents were blocked with Cd^{2+} in the bath solution. The remaining inward current was considered to be Na^+ current because it was reduced with the reduction in extracellular Na^+ and blocked by 1 μM tetrodotoxin (data not shown). Linear leak subtraction was performed on-line based on five hyperpolarizing pulses. Currents were low-passed filtered (Bessel 4-pole filter, −3 dB at 5 kHz), digitized at 20 kHz, and stored on computer disk for later analysis (pCLAMP 6, Axon Instruments). Junction potentials were nulled before seal formation, and capacitance and resistance artifacts were compensated using the circuitry of the amplifier (Axopatch-1C amplifier, Axon Instruments). Steady-state inactivation curves were obtained using the peak current measured with a step depolarization to −10 mV from each prepulse (200 ms, −130 to −20 mV in 10 mV increments) from a holding potential of −100 mV. Voltage dependence of activation was determined from the conductance G, where $G = I_{peak}/V_{test} - V_{rev}$) at each depolarization voltage (20 ms) over the range of −65 to +30 mV (+5 mV increments) from a holding potential of −70 mV. Whole cell Na^+ current (I_{Na}) was defined as the peak Na^+ current measured with a depolarizing step (8 ms) to −10 mV from a holding potential of −70 mV.

Application of hypoxia, kinase activators and kinase inhibitors

Hypoxia was induced by switching from control perfusate (low Na^+; see above) equilibrated with room air to the same solution equilibrated with 100% N_2 for > 2 h. The bath solution was exchanged within ~20 s. An oxygen scavenger ($Na_2S_2O_4$; 1 mM) was added to the hypoxic perfusate immediately prior to the initiation of the hypoxic exposure, reducing the P_{O_2} to < 1 Torr (Jiang & Haddad, 1994). Exposure to hypoxia without $Na_2S_2O_4$ produced similar results for I_{Na} (data not shown). The protein kinase C activators 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) and phorbol 12-myristate 13-acetate (PMA) were obtained from LC Laboratories, Woburn, MA, USA. The inactive phorbol ester 4-α-phorbol 12-myristate 13-acetate (4-α-PMA) was from Research Biochemicals International (RBI). These compounds are often dissolved in dimethyl sulphoxide (DMSO). However, preliminary experiments with DMSO (0·1% v/v) in the bath produced a dramatic decrease of I_{Na} after 3 min in four of the six cells examined (to 47·4 ± 3·9%, $n = 4$). Therefore, these compounds (OAG, PMA and 4-α-PMA) were dissolved in ethanol (EtOH) before dilution to working concentrations; the final working concentration of EtOH was ≤ 0·1% (v/v). Exposure to the PKC activators was initiated by switching from control perfusate to the same perfusate containing the particular activator. OAG was used at a concentration of 50 μM (Marchetti & Brown, 1988). Preliminary experiments with lower concentrations of OAG (1–10 μM) produced inconsistent results for the response of I_{Na}, and a higher concentration (100 μM) produced a similar inhibition of I_{Na} (data not shown). PMA and 4-α-PMA were used at 5 nM (Dascal & Lotan, 1991). The protein kinase A activator adenosine 3′,5′-cyclic adenosine monophosphate, N^6,O^2-dibutyryl, sodium salt (db-cAMP; Calbiochem, La Jolla, CA, USA) was applied in the perfusate at 2·5 mM (Ono, Kiyosue & Arita, 1989). Lower concentrations of db-cAMP (1 mM) failed to produce a significant inhibition of I_{Na}, and higher concentrations (5 mM) produced similar results (data not shown). Kinase inhibitors were added to the pipette solution. 1-(5-Isoquinolinesulphonyl)-2-methylpiperazine (H-7; LC Laboratories) was used at 100 μM (Kostyuk, Lukyanetz & Ter-Markosyan, 1992). The PKC inhibitor calphostin C was dissolved in EtOH (final working EtOH concentration ≤ 0·1% v/v) and stored in the dark at −80 °C. Calphostin C was used at 10 μM (Budworth & Gescher, 1995). The PKC inhibitory peptide PKC_{19-31} (PKCi) was obtained from Alexis Corporation, San Diego, CA, USA and was used at 20 μM (Takano, Stanfield, Nakajima & Nakajima, 1995). The PKA inhibitory peptide PKA_{5-24} (PKAi) was obtained from Alexis Corporation and was used at 30 μM (Kapur & MacDonald, 1996). The protease inhibitor leupeptin (100 μM; Sigma) was included in the pipette when PKCi or PKAi was used (Cantrell, Ma, Scheuer & Catterall, 1996). The PKA inhibitor *N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinolinesulphonamide (H-89; Calbiochem) was used at 30 μM (Chijiwa *et al.* 1990). Lower concentrations of the kinase inhibitors were inconsistent in blocking the response to kinase activation (50 μM OAG or 2·5 mM db-cAMP), and higher concentrations showed similar results (data not shown). All other compounds were from Sigma. Appropriate control experiments were done for all vehicles and compounds (see Table 1).

Data analysis

Steady-state inactivation curves were obtained with a Boltzmann fit of the data using

$$I/I_{max} = 1/(1 + \exp((V - V_{½})/k)),$$

where I_{max} is the peak current, I is the peak current (step depolarization to −10 mV) measured from each prepulse, V is the prepulse (holding potentials, −130 to −20 mV; 10 mV increments), $V_{½}$ is the half-inactivation, and k is the slope factor. Activation curves were obtained with a Boltzmann fit of the data using

$$G/G_{max} = 1/(1 + \exp((V_{½} - V)/k)),$$

where G_{max} is the peak conductance, G is the conductance at each test voltage, V is the test voltage (−65 to +30 mV; 5 mV steps; holding potential, −70 mV), $V_{½}$ is the half-activation, and k is the slope factor. Changes in Na^+ current are expressed as the percentage of the initial current recorded at ~2–3 min of whole-cell recording. Changes in activation and steady-state inactivation were compared using the $V_{½}$ from the Boltzmann fit. Grouped data are presented as means ± S.E.M. Differences were considered significant when $P < 0·05$ (ANOVA, Bonferroni–Dunn test, Student's t test).

RESULTS

Baseline recordings of whole-cell Na^+ current (I_{Na}) in dissociated CA1 neurones

The whole-cell Na^+ current (I_{Na}) recorded with a step depolarization to −10 mV from a holding potential of −70 mV stabilized by 1–2 min after rupture of the membrane. Experiments were performed between 2 and 10 min after the start of whole-cell recording. Control studies showed a slow run-down of peak I_{Na} over 3 min of

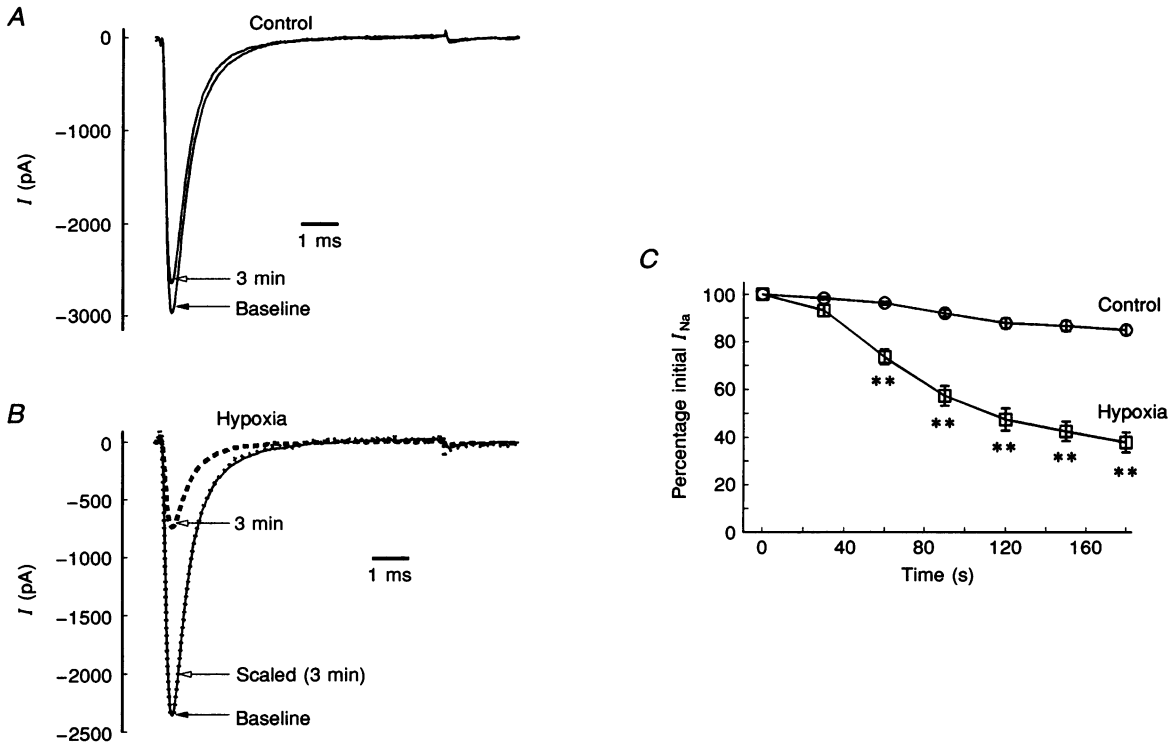

Figure 1. Hypoxia inhibits whole-cell Na^+ current (I_{Na})

A, representative control traces of I_{Na} ($V_h = -70$ mV; step to -10 mV) show a slight run-down of I_{Na} after 3 min. *B*, hypoxic exposure dramatically reduces I_{Na} after 3 min. The scaled trace shows that hypoxia inhibits I_{Na} without an effect on activation or inactivation kinetics. *C*, grouped data (means ± S.E.M.) comparing control ($n = 10$) and hypoxia ($n = 10$). Hypoxia was initiated at time = 0. ** $P < 0{\cdot}001$.

recording to 84·9 ± 2·1% of the initial current ($n = 10$; mean ± S.E.M.; Fig. 1*A* and *C*). In addition, both the steady-state inactivation curve (initial $V_{½} = -59{\cdot}6 \pm 1{\cdot}2$ mV; $\Delta V_{½} = -4{\cdot}7 \pm 0{\cdot}6$ mV; Fig. 2*A*) and the activation curve (initial $V_{½} = -29{\cdot}5 \pm 0{\cdot}9$ mV; $\Delta V_{½} = -3{\cdot}6 \pm 1{\cdot}0$ mV, Fig. 2*B*) showed a drift over time of $\sim -1{\cdot}0$ mV min^{-1}.

Hypoxia inhibits I_{Na}

When the extracellular bath was changed from control perfusate to the hypoxic solution, there was a rapid inhibition of peak I_{Na} with no effect on the kinetics of activation or inactivation (Fig. 1*B* and *C*). After 3 min of hypoxic exposure, I_{Na} was reduced to 38·1 ± 4·5% of

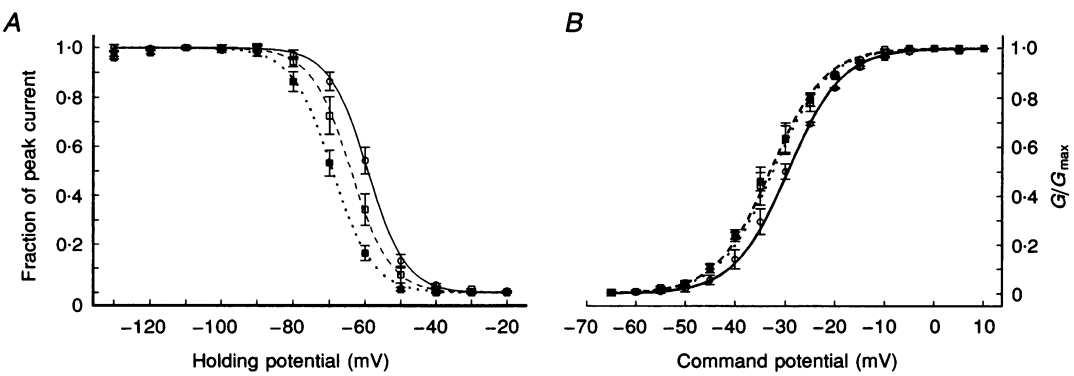

Figure 2. Hypoxia (3 min) shifts steady-state inactivation in the negative direction

A, control recordings at 3 min ($n = 10$) show a slow drift in steady-state inactivation. Hypoxia ($n = 10$) results in a shift of steady-state inactivation twice that of control ($V_{½} = -10{\cdot}0 \pm 1{\cdot}0$ *vs.* $-4{\cdot}7 \pm 0{\cdot}6$ mV; $P < 0{\cdot}001$). *B*, hypoxia does not affect activation. ○, baseline; □, control; ■, hypoxia. Means ± S.E.M.; curves from Boltzmann fit.

Table 1. Vehicle and inhibitor controls

	I_{Na} (% initial I_{Na})	Activation $\Delta V_{½}$ (mV)	Inactivation $\Delta V_{½}$ (mV)	n
Control	84·9 ± 2·1	−3·6 ± 1·0	−4·7 ± 0·6	10
H-89 (in), 30 μM	80·4 ± 5·1	−3·1 ± 0·2	−4·8 ± 0·7	5
EtOH (ex), 0·1–1·0% v/v	82·5 ± 4·5	−4·0 ± 0·7	−4·6 ± 0·6	8
EtOH (in), 0·1% v/v	82·5 ± 2·9	−2·8 ± 0·3	−3·8 ± 0·3	6
H-7 (in), 100 μM	92·0 ± 5·0	−3·2 ± 2·0	−3·9 ± 0·5	3
Calphostin C (in), 10 μM	80·4 ± 1·8	−3·3 ± 1·0	−4·2 ± 0·6	5
Leupeptin (in), 100 μM	82·4 ± 9·0	−4·4 ± 0·5	−4·7 ± 0·8	3
PKCi (in), 20 μM	85·3 ± 1·9	−2·2 ± 0·9	−4·8 ± 0·5	3
PKAi (in), 30 μM	83·0 ± 1·2	−3·0 ± 0·7	−3·5 ± 1·1	3

Recordings of peak whole-cell Na^+ current (I_{Na}), activation, and steady-state inactivation after 3 min exposure to vehicle or kinase inhibitors were not different from control. I_{Na} is presented as the percentage of initial current. Activation and inactivation are presented as the change in the $V_{½}$ from a Boltzmann fit of the data. (in) intracellular; (ex) extracellular.

baseline ($n = 10$; Fig. 1*C*; $P < 0·001$). In addition, steady-state inactivation was shifted by −10·0 ± 1·0 mV *vs.* a shift of −4·7 ± 0·6 mV during control recordings (Fig. 2*A*; $P < 0·001$), while the change in activation was not different from control ($\Delta V_{½} = -4·1 \pm 0·9$ mV; Fig. 2*B*). Similar results were obtained with caesium aspartate in place of CsF in the pipette (40·1 ± 5·4%; $\Delta V_{½} = -10·0 \pm 1·2$ mV; $n = 6$; $P < 0·001$).

Kinase inhibition attenuates the hypoxia-induced inhibition of I_{Na}

Several kinases, including protein kinase A (PKA) and protein kinase C (PKC), have been implicated in the modulation of neuronal Na^+ channels and Na^+ current (Dascal & Lotan, 1991; Numann *et al.* 1991; Gershon *et al.* 1992; Li *et al.* 1992; Godoy & Cukierman, 1994). To determine if kinase activation was involved in the hypoxia-induced inhibition of I_{Na}, we used the non-specific kinase inhibitor H-7. Initial experiments showed that the response of I_{Na} to 50 μM of the protein kinase C activator OAG was blocked with 100 μM H-7 (82·8 ± 2·4% of initial I_{Na}; $n = 6$). When neurones were exposed to hypoxia for 3 min with 100 μM H-7 in the pipette, the hypoxia-induced inhibition of I_{Na} was greatly attenuated (71·3 ± 6·9% of initial I_{Na}; $P < 0·001$; $n = 5$; Fig. 3) compared with hypoxia (38·1 ± 4·5% of initial I_{Na}; Fig. 3). H-7 also attenuated the hypoxia-induced shift in steady-state inactivation ($\Delta V_{½} = -5·9 \pm 0·6$ *vs.* −10·0 ± 1·0 mV; $P < 0·01$). A higher concentration of H-7 (400 μM) showed no further attenuation of the hypoxia-induced inhibition of I_{Na} (69·2 ± 3·5% of initial I_{Na}; $n = 2$). Recordings with intracellular H-7 alone were not different from control (Table 1).

The results with H-7 suggested that kinase activation was playing a role in the hypoxia-induced inhibition of I_{Na}. However, H-7 is a relatively non-specific kinase inhibitor (Kawamoto & Hidaka, 1984). To determine which kinase(s) may be involved in the hypoxia-induced inhibition of I_{Na}, experiments were performed with the PKA-specific blockers H-89 and PKA_{5-24} (PKAi), and also the PKC-specific blockers calphostin C and PKC_{19-31} (PKCi).

PKA inhibition does not alter the hypoxia-induced inhibition of I_{Na}

To investigate a possible role of protein kinase A (PKA) in modulation of I_{Na}, neurones were exposed for 3 min to 2·5 mM adenosine 3′,5′-cyclic monophosphate, N^6,O^2-di-

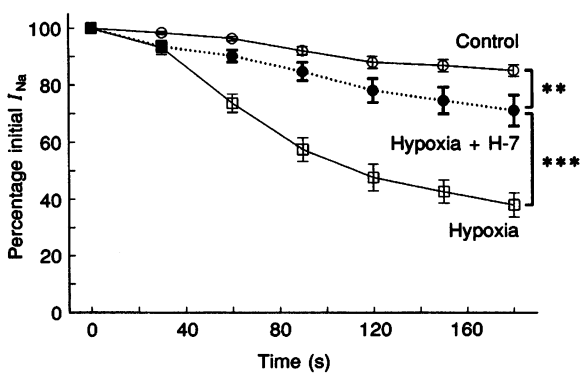

Figure 3. Kinase inhibition with H-7 attenuates the hypoxia-induced inhibition of I_{Na}

The hypoxia-induced inhibition of I_{Na} is greatly attenuated when neurones are exposed to hypoxia with 100 μM of the kinase inhibitor H-7 in the pipette ($n = 5$). Control, $n = 10$; hypoxia, $n = 10$; means ± S.E.M. ** $P < 0·01$; *** $P < 0·001$.

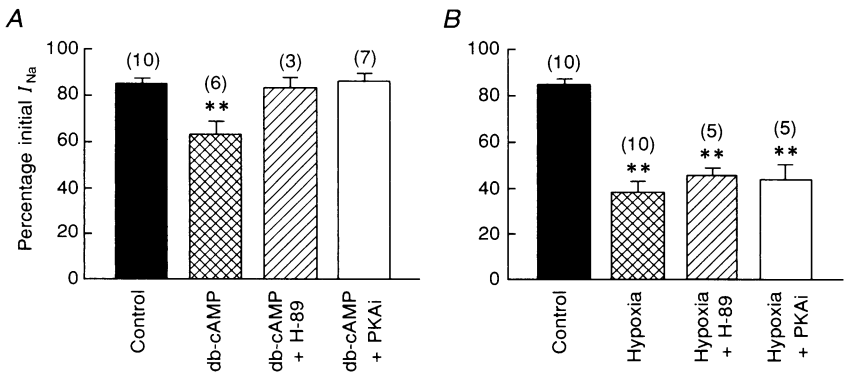

Figure 4. The protein kinase A (PKA) blockers H-89 and PKAi do not alter the hypoxia-induced inhibition of I_{Na}

A, activation of PKA with 2·5 mM db-cAMP inhibits I_{Na}. The inhibition is blocked with H-89 (30 μM) or PKAi (30 μM). *B*, H-89 and PKAi have no effect on the response of I_{Na} to hypoxia. I_{Na} after 3 min; means ± S.E.M.; ** $P < 0{\cdot}001$ *vs.* control; values of n shown in parentheses.

butyryl, sodium salt (db-cAMP) to determine if PKA activation altered I_{Na} in dissociated CA1 neurones. Activation of PKA reduced I_{Na} to 62·8 ± 5·5% of the initial value ($n = 6$; $P < 0{\cdot}001$; Fig. 4*A*) without an effect on inactivation ($\Delta V_{½} = -4{\cdot}6 \pm 0{\cdot}5$) or activation ($\Delta V_{½} = -3{\cdot}7 \pm 0{\cdot}7$ mV). The response of I_{Na} to 2·5 mM db-cAMP was blocked with 30 μM of the PKA inhibitor H-89 (83·0 ± 4·3% of initial I_{Na}; $n = 3$; Fig. 4*A*) or 30 μM PKAi (86·0 ± 3·1% of initial I_{Na}; $n = 7$; Fig. 4*A*), while recordings with H-89 or PKAi alone were not different from control (Table 1).

After determining an effective concentration of PKA inhibitors, neurones were exposed to hypoxia (3 min) with 30 μM H-89 or 30 μM PKAi in the pipette. The hypoxia-

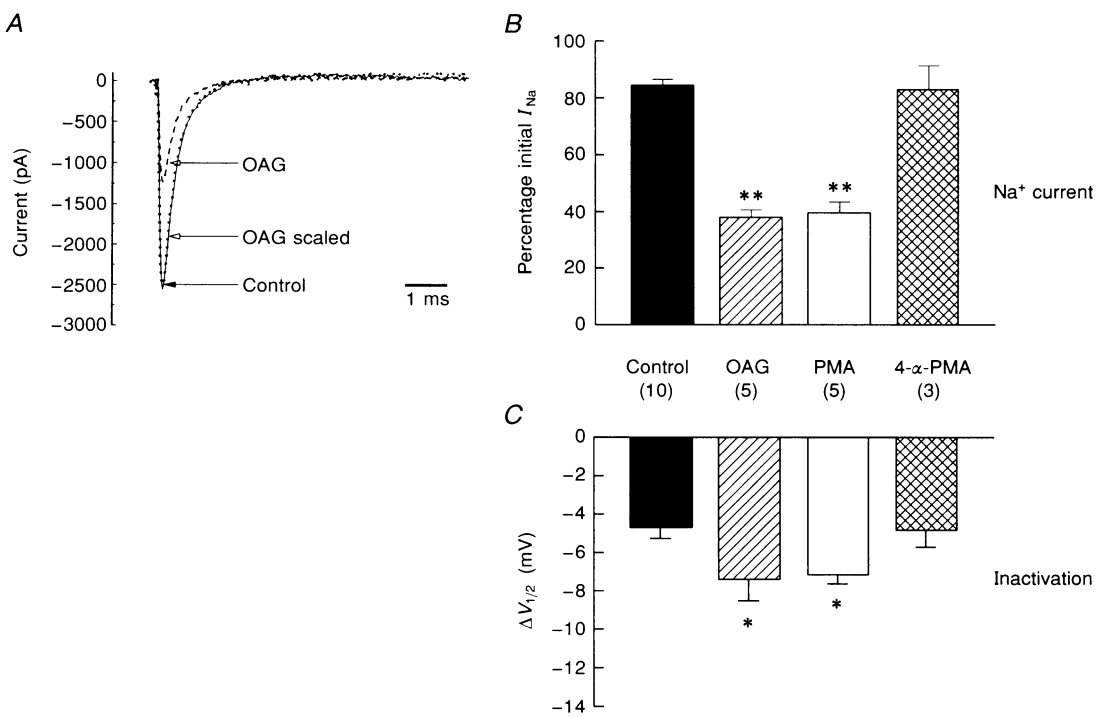

Figure 5. PKC activation (3 min) reduces I_{Na} and shifts steady-state inactivation in the negative direction

A, 50 μM OAG reduces I_{Na} without an effect on activation or inactivation kinetics. *B*, activation of PKC with 50 μM OAG or 5 nM PMA reduces I_{Na} after 3 min, while the inactive phorbol ester 4-α-PMA (5 nM) has no effect. *C*, OAG and PMA shift steady-state inactivation in the negative direction, while 4-α-PMA has no effect. Means ± S.E.M.; * $P < 0{\cdot}01$; ** $P < 0{\cdot}001$ *vs.* control. Values of n are shown in parentheses.

induced inhibition of I_{Na} was not altered by inclusion in the pipette of H-89 (45·2 ± 3·6% of initial I_{Na}; $n = 5$; Fig. 4*B*) or PKAi (44·0 ± 6·0% of initial I_{Na}; $n = 5$; Fig. 4*B*). Also, the hypoxia-induced shift in inactivation was not attenuated with H-89 ($\Delta V_{½} = -9·2 \pm 0·6$ mV) nor PKAi ($\Delta V_{½} = -11·0 \pm 2·1$ mV).

PKC inhibition attenuates the hypoxia-induced inhibition of I_{Na}

Extracellular application of 50 μM OAG or 5 nM PMA to activate protein kinase C (PKC) was used to study the effect of PKC on I_{Na}. Neurones exposed to 50 μM OAG or 5 nM PMA exhibited a reduction in I_{Na} without an effect on activation or inactivation kinetics (Fig. 5*A*). Application of OAG ($n = 5$) or 5 nM PMA ($n = 5$) resulted in a reduction of I_{Na} over 3 min to 46·1 ± 2·8% and to 40·0 ± 3·7% of baseline, respectively (Fig. 5*B*; $P < 0·001$), and a shift in steady-state inactivation ($\Delta V_{½} = -7·4 \pm 1·1$ and $-7·2 \pm 0·4$ mV, respectively; Fig. 5*C*; $P < 0·01$). Activation was not affected by OAG ($\Delta V_{½} = -3·9 \pm 0·7$ mV) nor PMA ($\Delta V_{½} = -2·7 \pm 1·2$ mV). The inactive phorbol ester 4-α-phorbol 12-myristate 13-acetate (5 nM) had no effect on I_{Na} (82·9 ± 8·5% of initial I_{Na}; $\Delta V_{½}$ activation = −4·4 ± 0·7 mV; $\Delta V_{½}$ inactivation = −4·8 ± 0·9 mV; Fig. 5*B* and *C*; $n = 3$). In addition, neurones exposed to OAG and hypoxia simultaneously showed a response that was similar to hypoxia or OAG alone (I_{Na} reduced to 38·8 ± 5·9% of initial I_{Na}; $\Delta V_{½}$ inactivation = −9·5 ± 1·1 mV; $n = 3$). Vehicle controls with extracellular application of EtOH were not different from control (Table 1).

The response of I_{Na} to 50 μM OAG (3 min) was blocked with the PKC-specific inhibitors calphostin C (10 μM; 83·0 ± 4·0% of initial I_{Na}; $P < 0·001$; inactivation $\Delta V_{½} = -3·9 \pm 0·6$ mV; $P < 0·05$; $n = 5$) or PKCi (20 μM; 85·9 ± 4·0% of initial I_{Na}; $P < 0·001$; inactivation $\Delta V_{½} = -4·4 \pm 0·8$ mV; $P < 0·05$; $n = 6$). Recordings with intracellular EtOH, intracellular calphostin C, or intracellular PKCi were not different from control (Table 1).

Concentrations of PKC inhibitors that were effective in blocking the I_{Na} response to PKC activation were used during exposure to hypoxia to determine the role of PKC in the hypoxia-induced inhibition of I_{Na}. When the PKC-specific inhibitors calphostin C (10 μM; $n = 12$) or PKCi (20 μM; $n = 5$) were included in the pipette during hypoxic exposure, the hypoxia-induced inhibition of I_{Na} was greatly attenuated (calphostin C: 62·6 ± 4·1% of initial I_{Na}; $P < 0·001$; PKCi: 63·5 ± 1·4% of initial I_{Na}; $P < 0·001$; Fig. 6*B*). The PKC inhibitors also attenuated the hypoxia-induced shift in steady-state inactivation (calphostin C: $\Delta V_{½} = -6·1 \pm 0·6$ mV; $P < 0·01$; PKCi: $\Delta V_{½} = -4·6 \pm 0·8$ mV; $P < 0·01$). Higher concentrations of PKCi produced no further attenuation of the hypoxia-induced inhibition of I_{Na} (50 μM PKCi: 62·1 ± 3·1% of initial I_{Na}; $n = 4$; 100 μM PKCi: 61·3 ± 8·2% of initial I_{Na}; $n = 3$). A similar attenuation was seen with PKCi during hypoxia when caesium aspartate was used in the pipette (68·0 ± 3·9% of initial I_{Na}; $\Delta V_{½} = -5·1 \pm 2·2$ mV; $n = 5$; $P < 0·01$). Recordings with intracellular EtOH (38·9 ± 7·5% of initial I_{Na}; inactivation $\Delta V_{½} = -9·2 \pm 0·9$ mV; $n = 6$) or intracellular leupeptin (41·3 ± 5·5% of initial I_{Na}; inactivation $\Delta V_{½} = -8·8 \pm 1·1$ mV; $n = 3$) did not alter the I_{Na} response to hypoxia.

DISCUSSION

The principal finding of this study is that hippocampal CA1 neurones respond to a physiological stimulus (i.e. hypoxia) with an inhibition of whole-cell Na^+ current (I_{Na}), and that this hypoxia-induced inhibition of I_{Na} is mediated largely by PKC activation. This conclusion is based on the results that demonstrate that (a) hypoxia inhibits I_{Na} and shifts steady-state inactivation, (b) activation of PKC inhibits I_{Na} and shifts steady-state inactivation, and (c) blockers of PKC greatly attenuate the hypoxia-induced inhibition of I_{Na} and shift in steady-state inactivation. The results demonstrate an important link between physiological events and Na^+ current modulation.

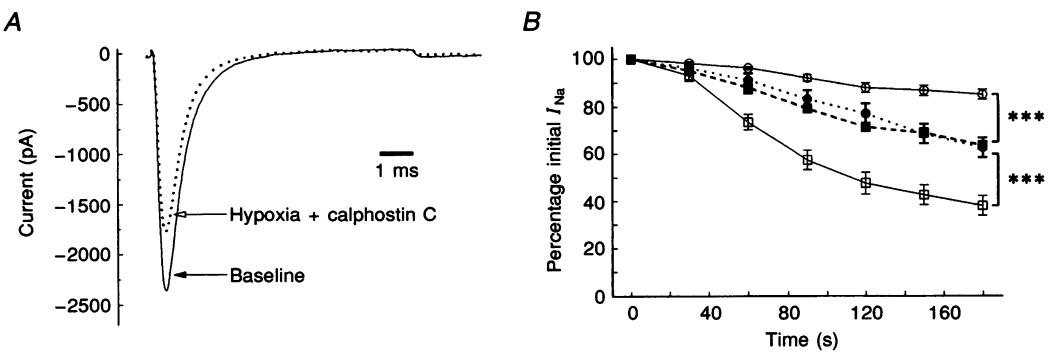

Figure 6. PKC blockers attenuate the hypoxia-induced inhibition of I_{Na}

A, the PKC-specific inhibitor calphostin C (10 μM) attenuated the I_{Na} response to 3 min of hypoxia. *B*, grouped data for control ($n = 10$), hypoxia ($n = 10$), and hypoxia with 10 μM calphostin C ($n = 12$) or 20 μM PKCi ($n = 5$) in the pipette. ○, control; □, hypoxia; ●, hypoxia + calphostin C; ■, hypoxia + PKCi. Means ± S.E.M.; *** $P < 0·001$.

Hypoxia inhibits I_{Na}

The data presented in this study confirm and expand on our previous work (Cummins *et al.* 1991, 1993) that showed that metabolic inhibition with cyanide or exposure to hypoxia produces a rapid and dramatic decrease in I_{Na} in mature CA1 and neocortical neurones. This inhibition is due, at least in part, to a negative shift in steady-state inactivation. The experiments with aspartate in place of fluoride in the pipette demonstrate that fluoride ions do not alter the hypoxic response. Also, the hypoxia-induced inhibition of I_{Na} is not affected by possible washout effects of whole-cell recording since the hypoxic response is also seen in perforated patch recordings (Cummins *et al.* 1991). The hypoxia-induced inhibition of I_{Na} may be a general response of cortical neurons to hypoxia and could underlie the rapid disappearance of the EEG during hypoxic events (Rossen, Kabat & Anderson, 1943).

We believe that this inhibition of I_{Na} may be an adaptive cellular mechanism that increases neuronal tolerance to a low-oxygen environment. A large proportion of the energy produced in the CNS (~50%) is used to maintain ionic gradients across the neuronal membrane, and a major consumer of ATP is the Na^+–K^+-ATPase (Erecinska & Silver, 1989). Inhibition of Na^+ channel activation would reduce neuronal activity and reduce Na^+ ion influx across the neuronal membrane. This would result in a reduction in energy demand at a time when energy production is severely compromised. Reducing energy demand when energy supply is diminished during hypoxia is a prominent survival mechanism in many hypoxia-tolerant species (Hochachka, Lutz, Sick, Rosenthal & van den Thillart, 1993). For example, there is a major decrease in expression of Na^+ channels in turtle brain during exposure to hypoxia (Perez-Pinzon, Rosenthal, Sick, Lutz, Pablo & Mash, 1992), which may play a role in the remarkable tolerance to hypoxic stress of turtle brain compared with mammalian brain (Sick, Rosenthal, LaManna & Lutz, 1982).

PKA and PKC inhibit I_{Na}

Several reports (Gershon *et al.* 1992; Li *et al.* 1992) have shown that PKA reduces I_{Na} without an effect on activation or steady-state inactivation. In our study, application of db-cAMP to activate PKA produced an inhibition of I_{Na} without an effect on activation or steady-state inactivation in agreement with these reports. We believe that this response was mediated by PKA because the response was blocked by two PKA-specific inhibitors, H-89 and PKAi.

Previous investigations also have demonstrated that I_{Na} can be modulated by PKC (Cukierman, 1996). In the present study, activation of PKC with OAG or PMA resulted in a decrease in I_{Na} and a shift of steady-state inactivation in the negative direction. We believe that the reduction in I_{Na} was mediated by PKC and not the result of a non-specific effect because the inactive phorbol ester 4-α-PMA had no effect on I_{Na}, and the response to OAG was blocked by the kinase inhibitor H-7 and two PKC-specific inhibitors, calphostin C and PKCi. Non-specific effects of peptide dialysis should not play a role in the attenuation since the PKAi peptide did not alter the response of I_{Na} to hypoxia.

The response of neuronal I_{Na} to PKC activation has been studied in several different experimental systems (Dascal & Lotan, 1991; Numann *et al.* 1991; Godoy & Cukierman, 1994). Although these studies agree that PKC activation reduces I_{Na}, there is not a general agreement on the effect of PKC on the voltage dependence of activation or inactivation. Numann *et al.* (1991) found a slowing of fast inactivation but no change in steady-state inactivation in primary cultured rat brain cells and in Chinese hamster ovary (CHO) cells expressing type IIA α subunits. Godoy & Cukierman (1994) reported a shift of steady-state inactivation in the negative direction in N1E-115 mouse neuroblastoma cells, and Dascal & Lotan (1991) described a shift in activation in the positive direction using an oocyte expression system. Another recent study (Cantrell *et al.* 1996) in dissociated neurones reported a decrease in I_{Na} with PKC activation, a slowing of fast inactivation, and no change in activation or steady-state inactivation. The different results between these studies concerning the voltage-dependent properties of I_{Na} could be due to different models (e.g. dissociated neurons *vs.* expression systems), different cell types (e.g. CA1 *vs.* CHO cells), different PKC activators (e.g. OAG *vs.* PMA; see Godoy & Cukierman, 1994), or different solvents (e.g. DMSO *vs.* EtOH; see Methods).

Inhibition of PKC, but not PKA, attenuates the hypoxia-induced inhibition of I_{Na}

A role for PKC in the hypoxia-induced inhibition of I_{Na} was suggested by our results demonstrating that PKC activation and hypoxia produced similar effects on I_{Na}. Initial experiments with the kinase inhibitor H-7, which inhibits PKC activation at the catalytic site of the enzyme, showed an attenuation of the hypoxia-induced inhibition of I_{Na}. However, H-7 is a relatively non-specific kinase inhibitor (Kawamoto & Hidaka, 1984). Therefore, to determine if the kinase involved in the hypoxic response was PKC, we used the PKC-specific inhibitors calphostin C and PKCi, which inhibit PKC at the activation site of the enzyme (House & Kemp, 1987; Kobayashi, Nakano, Morimoto & Tamaoki, 1989). Calphostin C and PKCi also attenuated the I_{Na} inhibition and the negative shift in steady-state inactivation normally seen during hypoxic exposure. In addition, exposure to hypoxia and PKC activation simultaneously produced no additive effects on I_{Na}. These data support the conclusion that the hypoxia-induced inhibition of I_{Na} is mediated by PKC activation.

In contrast to the attenuation of the hypoxia-induced inhibition of I_{Na} seen with PKC inhibition, exposure to hypoxia in the presence of the PKA-specific blockers H-89 and PKAi failed to alter the hypoxia-induced inhibition of I_{Na} or the hypoxia-induced shift in steady-state inactivation, suggesting that PKA activation is not involved in the inhibition of I_{Na} during acute oxygen deprivation.

PKC activation during hypoxia

Activation of kinases, in particular PKC, during hypoxia has been described in several reports. For example, PKC is rapidly translocated from the cytosol to the neuronal membrane in response to hypoxia (Wieloch, Bergstedt & Hu, 1993), an event associated with activation of PKC (Bell, 1986). Activation of PKC can be one of the key events that occurs in the CNS in response to oxygen deprivation, although reports differ on whether PKC activation in response to hypoxia is neurotoxic (Cardell & Wieloch, 1993) or neuroprotective (Madden, Clark, Kochhar & Zivin, 1991). The data in this study suggest a neuroprotective role for PKC activation during the initial response to acute hypoxia.

Although PKC is implicated in the inhibition of I_{Na} during hypoxia, the substrate for PKC phosphorylation that leads to this inhibition is unknown. Studies have shown that the Na^+ channel is a substrate for PKC-mediated phosphorylation (Costa & Catterall, 1984) and that phosphorylation of the Na^+ channel by PKC is sufficient for the inhibition of I_{Na} (West, Numann, Murphy, Scheuer & Catterall, 1991). Therefore, the direct phosphorylation of Na^+ channels by PKC may produce the hypoxia-induced inhibition of I_{Na}.

In this report, PKC inhibitors blocked the hypoxia-induced inhibition of I_{Na} at 1 min of hypoxic exposure. However, after 3 min of hypoxic exposure, the blockage was incomplete, suggesting that there may be other mechanisms involved in the hypoxia-induced inhibition of I_{Na}. One compound that could play an additional role in the inhibition of I_{Na} during hypoxia is arachidonic acid (AA). AA is known to be produced rapidly during hypoxia (Bazan, 1970), and AA has been shown to modulate ion channels (Meves, 1994). In particular, AA has been shown to be involved in I_{Na} inhibition (Fraser, Hoehn, Weiss & MacVicar, 1993). Therefore, the hypoxia-induced inhibition of I_{Na} may be the result of parallel and related cellular pathways which lead to a reduction in I_{Na}.

The results demonstrate that the hypoxia-induced inhibition of I_{Na} is greatly attenuated with blockers of PKC leading to the conclusion that PKC is activated by hypoxia, and that PKC activation mediates the hypoxia-induced inhibition of I_{Na}. This study describes a novel finding that demonstrates a unique and interesting coupling between extracellular physiological and/or pathophysiological events and Na^+ current modulation in CA1 neurones. We propose that the PKC-mediated inhibition of I_{Na} during hypoxia is a protective cellular response that may promote neuronal tolerance to acute oxygen deprivation and delay hypoxia-induced neuronal injury or death.

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