

Inhibition of oxidative metabolism increases persistent sodium current in rat CA1 hippocampal neurons

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(Received 22 December 1997; accepted after revision 22 April 1998)

1. Whole-cell patch-clamp recordings from freshly dissociated rat CA1 neurons revealed a large transient Na^+ current ($I_{\text{Na,T}}$) and a smaller, inactivation-resistant persistent Na^+ current ($I_{\text{Na,P}}$). Both currents could be blocked with TTX.
2. The average current densities of $I_{\text{Na,T}}$ and $I_{\text{Na,P}}$ in thirty cells were 111.0 ± 9.62 and 0.87 ± 0.13 pA pF⁻¹, respectively.
3. Inhibiting oxidative phosphorylation by adding 5 mM sodium cyanide to the pipette solution significantly increased the amplitude of $I_{\text{Na,P}}$ but had no significant effect on the amplitude of $I_{\text{Na,T}}$.
4. Exposing CA1 neurons to hypoxia for more than 7 min caused an increase in the amplitude of $I_{\text{Na,P}}$. There was also a delayed decrease in the amplitude of $I_{\text{Na,T}}$.
5. $I_{\text{Na,P}}$ was more sensitive to the Na^+ channel blockers TTX and lidocaine than $I_{\text{Na,T}}$. The IC_{50} for the effect of TTX on $I_{\text{Na,P}}$ was 9.1 ± 1.2 nM whereas the IC_{50} for $I_{\text{Na,T}}$ was 37.1 ± 1.2 nM, approximately 4-fold higher. Lidocaine (lignocaine; 1 μM) reduced $I_{\text{Na,P}}$ to 0.24 ± 0.15 of control ($n = 4$) whereas $I_{\text{Na,T}}$ was essentially unaffected (0.99 ± 0.11 , $n = 4$).
6. These results show that $I_{\text{Na,P}}$ is increased when oxidative metabolism is blocked in CA1 neurons. The persistent influx of Na^+ through non-inactivating Na^+ channels can be blocked by concentrations of Na^+ channel blockers that do not affect $I_{\text{Na,T}}$.

Reduced oxygen supply to the brain results in a loss of ion homeostasis and energy depletion that leads to cell death. An early effect of hypoxia in neurons is an increase in intracellular sodium concentration ($[\text{Na}^+]_i$) which is followed by an elevation in intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) (Friedman & Haddad, 1994*a,b*). In animal models of ischaemia, a low concentration of extracellular sodium ($[\text{Na}^+]_o$) and low concentrations of Na^+ channel blockers (which do not affect normal action potential firing) have been shown to prevent the rise in $[\text{Ca}^{2+}]_i$ and extracellular glutamate concentration, the decrease in $[\text{ATP}]_i$ and neuronal damage (Weber & Taylor, 1994; Fried, Amorim, Chambers, Cottrell & Kass, 1995; Taylor, Burke & Weber, 1995). It has been suggested, therefore, that an early increase in $[\text{Na}^+]_i$ activates Na^+ - Ca^{2+} and glutamate exchangers in neurons in reverse mode leading to a rise in $[\text{Ca}^{2+}]_i$ and glutamate release from cells (Stys, Waxman & Ransom, 1991; Taylor *et al.* 1995). The reason for the increase in $[\text{Na}^+]_i$ has not been explained. Because the Na^+ influx and depolarization recorded during hypoxia are sustained (Taylor, 1995), it has been thought that the classical transient Na^+ channel current ($I_{\text{Na,T}}$) could not be responsible. However, a persistent, slowly inactivating, TTX-sensitive Na^+ current has been recorded in neurons (Gilly & Armstrong, 1984; French &

Gage, 1985; French, Sah, Buckett & Gage, 1990; Taylor, 1993; Crill, 1996) and it has been shown that hypoxia can cause an increase in a persistent sodium current ($I_{\text{Na,P}}$) in cardiac muscle (Ju, Saint & Gage, 1996). In normal healthy cells $I_{\text{Na,P}}$ has been thought to function as a pacemaker current, contributing to synaptic potentials, enhancing rhythmicity and enabling repetitive firing of action potentials (Taylor, 1993). The aim of this study was to investigate the effect of inhibiting oxidative metabolism on $I_{\text{Na,P}}$ in CA1 neurons and to assess the sensitivity of the current potentiated by hypoxia to TTX and lidocaine. We report here that inhibition of oxidative metabolism increases the persistent Na^+ current in hippocampal neurons and propose that this is an early event during hypoxia that leads to Ca^{2+} accumulation and consequent damage to neurons. Some of these results have been presented previously in abstract form (Hammarström, Lim & Gage, 1996).

METHODS

Dissociation of CA1 neurons

Young adult rats (14–21 days old) were decapitated with a small animal guillotine (anaesthesia under CO_2) and the brain quickly transferred to ice-cold artificial cerebrospinal fluid (ACSF) (124 mM

NaCl, 26 mM NaH₂CO₃, 3 mM KCl, 1.3 mM MgSO₄, 2.5 mM NaH₂PO₄ and 20 mM glucose) gassed with 95% CO₂-5% O₂ (BOC gases, Australia). Brain slices 500 μ m thick were cut on a vibratome, in the presence of ice-cold ACSF equilibrated with 95% CO₂-5% O₂. The brain slices were enzymatically treated for 30 min with 200 units papain (Worthington Biochemicals), 1.1 mM cysteine (Sigma), 0.2 mM EDTA and 13.4 μ M mercaptoethanol at 35 °C, following the adaptation (French *et al.* 1990) of the procedure originally described by Kay & Wong, 1987. The CA1 region was subsequently removed and individual neurons obtained by careful mechanical trituration with a glass pipette. These methods have been approved by the Animal Experimentation Ethics Committee at the Australian National University (Ethics Approval No. J.NS.82.98).

Electrophysiological recording and data analysis

Tight gigaohm seal patch-clamp techniques using standard glass pipettes (GC150F-15, Clarke Electromedical Instruments) were used to record currents in whole-cell configuration from CA1 neurons. Neurons that were flat, swollen or grainy in appearance were not used. Pipettes were pulled on a List Medical vertical pipette puller (L/M-3P-A) and had resistances of 6–10 M Ω when filled with pipette solution. In order to obtain reproducible results, currents were not recorded for the first 10–15 min after achieving the whole-cell configuration.

Tetrodotoxin (TTX) (Boehringer Mannheim) and lidocaine (lignocaine; Sigma) were applied to whole cells through fine (200 μ m i.d.) tubes carefully positioned to cause a rapid change in concentration of drugs close to the patched cell. Hypoxia was achieved by perfusing the patched cell through a fine stainless steel tube with a solution vigorously bubbled with 100% N₂ (BOC gases). This technique has already been used in our laboratory and produces a rapid decrease in oxygen tension around the cell (Ju *et al.* 1996).

The bath solution contained (mM): 135 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 5 CoCl₂, 5 CsCl and 10 Tes adjusted to a pH of 7.4 with NaOH. The pipette solution contained (mM): 125 CsF, 5 NaF, 10 KCl, 10 Tes and 10 EGTA, pH 7.4. In experiments in which

5 mM sodium cyanide (BDH Chemicals, Australia) was added to the pipette solution the concentration of CsF in the pipette solution was reduced to 120 mM. In most instances 10 mM glucose was also included in the bath solution but its absence or presence did not appear to affect the Na⁺ currents (data not shown). Stocks of TTX and lidocaine were diluted in bath solution to the required concentration immediately prior to the experiment. All solutions were of similar osmolality (290 \pm 10 mosmol kg⁻¹).

Whole-cell currents were recorded with an Axopatch-1D amplifier (Axon Instruments) in response to voltage protocols imposed using an IBM-compatible PC and digital-to-analog interface. Currents were analysed using computer techniques and 'in-house' software (Ju, Saint & Gage, 1992; Ju *et al.* 1996). Subtraction of traces recorded before and after exposure to a high concentration of TTX (0.5 μ M) was used throughout to isolate TTX-sensitive Na⁺ currents (Fig. 1).

The amplitude of $I_{Na,T}$ was measured at its peak and the amplitude of $I_{Na,P}$ was measured at the end of a 400 ms voltage pulse. These techniques have been described in more detail elsewhere (Ju *et al.* 1992, 1996).

All values are expressed as means \pm 1 s.e.m. and the number of cells (*n*) in each group is given. Statistical analysis was performed using Student's two-tailed unpaired *t* test.

RESULTS

Control $I_{Na,P}$ and $I_{Na,T}$

Sodium currents were recorded first in the absence and then in the presence of 0.5 μ M TTX (Fig. 1A).

Subtraction of the traces revealed TTX-sensitive currents. An example of this procedure is shown in Fig. 1B. In this instance, the pipette contained 5 mM sodium cyanide. With this protocol, a transient and a persistent Na⁺ current could be seen free from contamination by other currents.

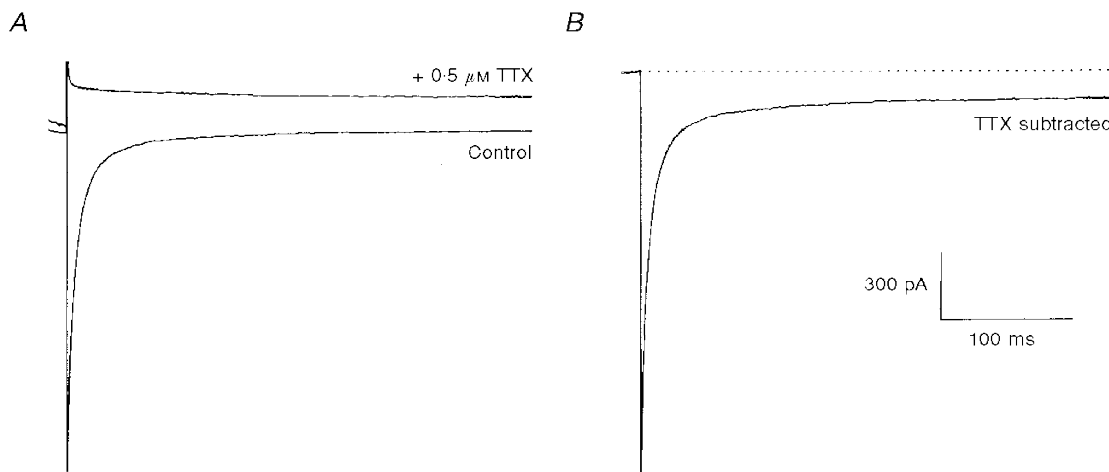


Figure 1. Recording TTX-sensitive current

Currents shown were recorded with an electrode containing 5 mM sodium cyanide 15 min after the whole-cell seal was obtained. *A*, the two traces show currents recorded before (lower trace) and after exposure of the neuron to 0.5 μ M TTX. The currents were generated by a voltage step to -30 mV from a holding potential of -100 mV. *B*, the TTX-sensitive current obtained by subtracting the two traces in *A*. The transient current is truncated to show the persistent current in more detail. The scale bars apply for both *A* and *B*. The dashed line denotes the subtracted current level before the depolarizing pulse (zero current).

The current densities of $I_{\text{Na,T}}$ and $I_{\text{Na,P}}$ in control solution measured in thirty cells in this way were: peak $I_{\text{Na,T}}$, 111.0 ± 9.62 pA pF⁻¹; $I_{\text{Na,P}}$, 0.87 ± 0.13 pA pF⁻¹. That is, the persistent Na⁺ current was 0.7–1% of the peak transient current amplitude. As has been reported previously (French *et al.* 1990; Alzheimer, Schwandt & Crill, 1993; Crill, 1996), $I_{\text{Na,P}}$ activated at more negative potentials ($V_{1/2}$ about -50 mV) than $I_{\text{Na,T}}$ and did not show the same inactivation properties as $I_{\text{Na,T}}$. When the pre-pulse potential was altered between -150 and 0 mV, $I_{\text{Na,P}}$ persisted at pre-pulse potentials that inactivated $I_{\text{Na,T}}$.

Effect of inhibiting oxidative metabolism with sodium cyanide

In order to block oxidative metabolism, neurons were exposed to 5 mM sodium cyanide through the pipette solution (Elliott, Smith & Allen, 1989; Ju *et al.* 1996). Solutions contained 10 mM glucose so that glycolysis was not impaired. With this treatment, the amplitudes of $I_{\text{Na,P}}$ were much larger than the control amplitudes, as illustrated in Fig. 2A.

In ten out of fifteen cells in which the effect of cyanide was measured after the cell interior had been perfused with the drug for 10–15 min, the current density of $I_{\text{Na,P}}$ recorded at the end of a 400 ms depolarizing pulse to -30 mV was 3.10 ± 0.71 pA pF⁻¹ ($n = 10$), significantly greater than in the absence of cyanide ($P < 0.0001$). The current density of $I_{\text{Na,T}}$ during exposure to cyanide was 119.70 ± 18.72 pA pF⁻¹ ($n = 10$), not significantly different from control values ($P > 0.05$). Hence, cyanide caused an increase in the ratio of $I_{\text{Na,P}}$ to $I_{\text{Na,T}}$ to more than 2% (0.022 ± 0.003 , $n = 10$). In three out of the fifteen cells, a progressive time-dependent increase in the amplitude of $I_{\text{Na,P}}$ was observed (in the other

twelve cells the increase appeared to have stabilized 15–20 min after the whole-cell seal was obtained). One such cell is shown in Fig. 2A where the $I_{\text{Na,P}}:I_{\text{Na,T}}$ ratio increased 3-fold (from 0.004 to 0.012) over a time period of 1 h.

Effects of hypoxia

In order to establish that the increase in $I_{\text{Na,P}}$ caused by cyanide was due to inhibition of oxidative metabolism rather than a non-specific effect of the drug, the effect of reducing the O₂ tension by perfusing a cell with solution that had been bubbled with 100% N₂ was examined in ten neurons. Exposure of a cell to a hypoxic solution clearly increased the amplitude of $I_{\text{Na,P}}$ (Fig. 2B). In some neurons, a progressive increase in the amplitude of $I_{\text{Na,P}}$ over time could be observed and currents recorded in one such neuron are shown in Fig. 2B. $I_{\text{Na,P}}$ significantly increased from 1.21 ± 0.24 to 2.12 ± 0.34 pA pF⁻¹ ($n = 10$, $P < 0.05$) during hypoxia. In contrast, there was generally a decrease in the amplitude of $I_{\text{Na,T}}$ in neurons exposed to hypoxia in this way (0.87 ± 0.10 , $n = 9$). The ratio of $I_{\text{Na,P}}$ to $I_{\text{Na,T}}$ increased 3-fold during hypoxia (from 0.014 ± 0.04 to 0.042 ± 0.018 of $I_{\text{Na,T}}$, $n = 9$).

An increase in the amplitude of $I_{\text{Na,P}}$ during hypoxia was seen at all potentials, as can be seen in the current–voltage curves obtained from one neuron in Fig. 3A. Currents were recorded over a range of potentials before and after exposure to TTX and the amplitude of $I_{\text{Na,P}}$ was measured from the subtracted current at the end of a 400 ms depolarizing pulse. Results shown in Fig. 3A were obtained before (●) and after 12–15 min of hypoxia (○). The lines through the data points were obtained from best fits to the

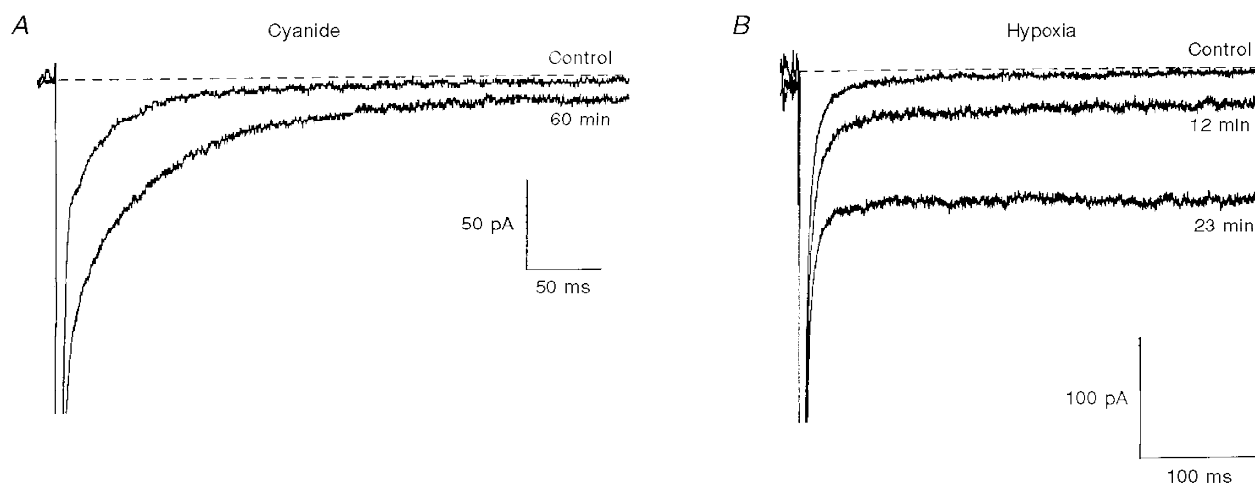


Figure 2. Inhibition of oxidative metabolism by hypoxia or cyanide increases persistent Na⁺ current

A, TTX-sensitive currents recorded from a neuron after 10 and 60 min of internal sodium cyanide exposure. Normally (in 12 out of 15 cells) the increase was large and stabilized within 10–15 min after the whole-cell seal was obtained. Currents shown were generated by a depolarizing step to -30 mV from a holding potential of -100 mV. B, TTX-sensitive currents recorded from a neuron before (top trace) and after exposure to hypoxic solution for 12 min (middle trace) and 23 min (lower trace). Currents were generated by a voltage step to -20 mV from a holding potential of -100 mV. The dashed line denotes the subtracted current level before the depolarizing pulse (zero current).

conductance–voltage plots from the same cell (Fig. 3B) of the Boltzmann-type equation:

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

where G_{\max} is the maximum conductance, V the test potential, V' the potential at which G was 50% of G_{\max} and k a slope factor.

Before exposure to hypoxia, G_{\max} was 0.18 nS, V' was -49 mV and k was 8.8. Following 12–15 min hypoxia, G_{\max} had increased to 0.33 nS, V' was -60 mV and k was 11.8. It can be seen that $I_{\text{Na,P}}$ is present at potentials near the resting membrane potential (around -70 mV) and reaches a peak at about -40 mV, a range that would be important in modulating the rate of action potential firing, or rhythmicity, in these cells.

Effects of Na⁺ channel blockers

TTX. The effects of a range of concentrations of TTX (0.5–500 nM) on Na⁺ current recorded with pipettes containing 5 mM sodium cyanide were examined. The persistent and transient sodium currents were evoked by a test pulse to -30 mV from a holding potential of -100 mV. Control responses were always established before and after exposure to solutions containing TTX. A solution containing $0.5 \mu\text{M}$ TTX was applied after each lower concentration of TTX so that the TTX-sensitive Na⁺ currents could be isolated by subtraction of the currents recorded in the presence of the high concentration of TTX. Currents analysed were obtained by subtraction of the currents remaining after exposure of the neuron to the supramaximal TTX concentration.

There was a noticeable difference in the sensitivity of $I_{\text{Na,P}}$ and $I_{\text{Na,T}}$ to TTX. At a concentration of 5 nM, TTX caused an obvious reduction in the amplitude of $I_{\text{Na,P}}$ (Fig. 4A) whereas the peak amplitude of $I_{\text{Na,T}}$ was not significantly affected.

In five cells exposed to 5 nM TTX, $I_{\text{Na,P}}$ was reduced to 0.72 ± 0.03 of the control value (Fig. 4C). In the same cells, $I_{\text{Na,T}}$ was 0.91 ± 0.03 of control which was significantly different from the effect of this concentration of TTX on $I_{\text{Na,P}}$ ($P < 0.005$). In the presence of 50 nM TTX, $I_{\text{Na,P}}$ was almost completely blocked while $I_{\text{Na,T}}$ was reduced by about 50% (Fig. 4A). In four cells exposed to 50 nM TTX, $I_{\text{Na,P}}$ was 0.03 ± 0.03 of control whereas the peak $I_{\text{Na,T}}$ was 0.40 ± 0.12 of control (Fig. 4C). In the presence of $0.5 \mu\text{M}$ TTX, both $I_{\text{Na,P}}$ and $I_{\text{Na,T}}$ were completely abolished (Fig. 1A). A least-squares fit of the Hill equation to the data gave an IC_{50} of 9.1 ± 1.2 nM for $I_{\text{Na,P}}$, which was significantly lower than the IC_{50} for $I_{\text{Na,T}}$, which was 37.1 ± 1.2 nM, approximately 4-fold higher ($P < 0.001$). A similar difference in the sensitivity of $I_{\text{Na,T}}$ and $I_{\text{Na,P}}$ to TTX was seen in neurons not exposed to cyanide (data not shown).

Lidocaine. The effects of lidocaine on the Na⁺ currents were examined in a similar manner. In three experiments in which a neuron was exposed to 3 nM lidocaine, the effects on both $I_{\text{Na,P}}$ and $I_{\text{Na,T}}$ were small (0.67 ± 0.33 and 0.91 ± 0.03 of control, respectively; Fig. 4D). At 30 nM, lidocaine caused a larger decrease in $I_{\text{Na,P}}$ (Fig. 4B) while having little effect on $I_{\text{Na,T}}$. In four cells, 30 nM lidocaine reduced $I_{\text{Na,P}}$ to 0.47 ± 0.01 of control; $I_{\text{Na,T}}$ was 0.97 ± 0.04 of control,

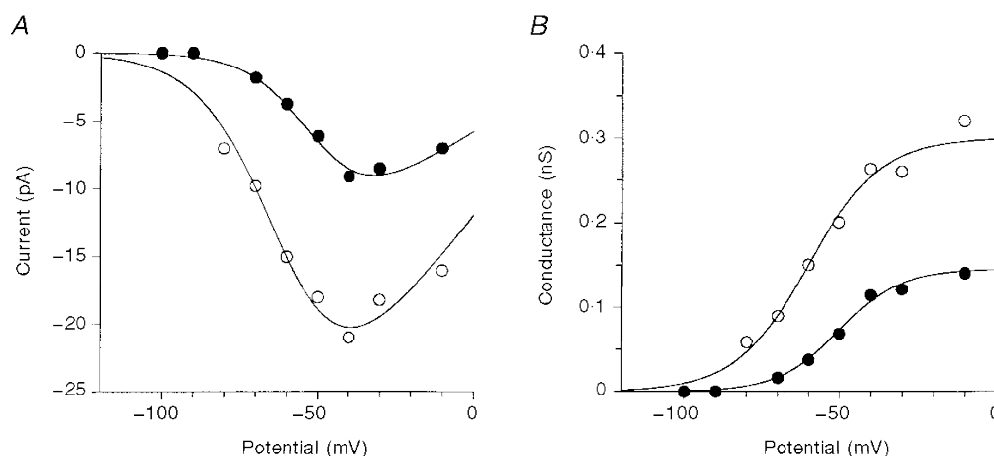


Figure 3. Current–voltage and conductance–voltage relationships before and after hypoxia

A, current–voltage relationship of the TTX-sensitive persistent Na⁺ current during normoxia (●) and after 12–15 min of hypoxia (○). The lines through the data points were obtained as described in the text. B, conductance–voltage relationship of the TTX-sensitive persistent Na⁺ current during normoxia (●) and after 12–15 min of hypoxia (○). The amplitude of $I_{\text{Na,P}}$ was measured at the end of a 400 ms depolarizing pulse and divided by $V - E_0$ to obtain the conductance (E_0 is the potential at which $I = 0$). The lines through the data points were the best fits of the equation: $G(V) = G_{\max}/(1 + \exp((V' - V)/k))$. Further details are given in the text.

which was significantly different from the effect of this concentration on $I_{\text{Na,P}}$ ($P < 0.001$). At $1 \mu\text{M}$, lidocaine reduced $I_{\text{Na,P}}$ further to 0.24 ± 0.15 whereas $I_{\text{Na,T}}$ was 0.99 ± 0.11 of control (Fig. 4D, $n = 4$). Hence, $I_{\text{Na,P}}$ responded to inhibition by lidocaine at concentrations that had little, if any, effect on $I_{\text{Na,T}}$.

DISCUSSION

These results show that inhibition of oxidative metabolism by sodium cyanide or hypoxia increases $I_{\text{Na,P}}$ while having little effect on $I_{\text{Na,T}}$. It should be noted that, in these experiments, the intracellular $[\text{Na}^+]_i$ was kept constant so that any change in I_{Na} must have been due to a change in conductance rather than a change in driving force on sodium ions. The situation in cells *in vivo* would be different in that $[\text{Na}^+]_i$ may change as extrusion mechanisms fail to cope with Na^+ influx. Cyanide and hypoxia had similar effects, as has been described previously (Bickler & Hansen, 1994; Ju *et al.* 1996), indicating that they had a common mechanism of action.

Our results demonstrate the existence of a larger than normal persistent I_{Na} during hypoxia. An increase in $I_{\text{Na,P}}$ would be expected to have a significant effect on neuronal function. Firstly, it would increase the excitability of neurons and disrupt the normal oscillations in membrane potential and rhythmic firing of action potentials (Alonzo & Llinás, 1989; Amitai, 1994). Similar effects have been observed when $I_{\text{Na,P}}$ is increased with anemone toxin which significantly enhances bursting behaviour in rat neocortical pyramidal neurons (Mantegazza, Franceschetti & Avanzini, 1998). Secondly, an increase in a non-inactivating Na^+ current is probably the primary cause of the increase in $[\text{Na}^+]_i$ observed during hypoxia (Friedman & Haddad, 1994a) and may be involved both directly and indirectly in the sequence of events, including an increase in $[\text{Ca}^{2+}]_i$, that culminate in cell death caused by hypoxia. It has been shown that removing Na^+ channel inactivation with agents such as veratridine during normoxia increases $[\text{Ca}^{2+}]_i$ (Haigney, Lakatta, Stern & Silverman, 1994). Furthermore, neuronal damage caused by hypoxia or ischaemia can be

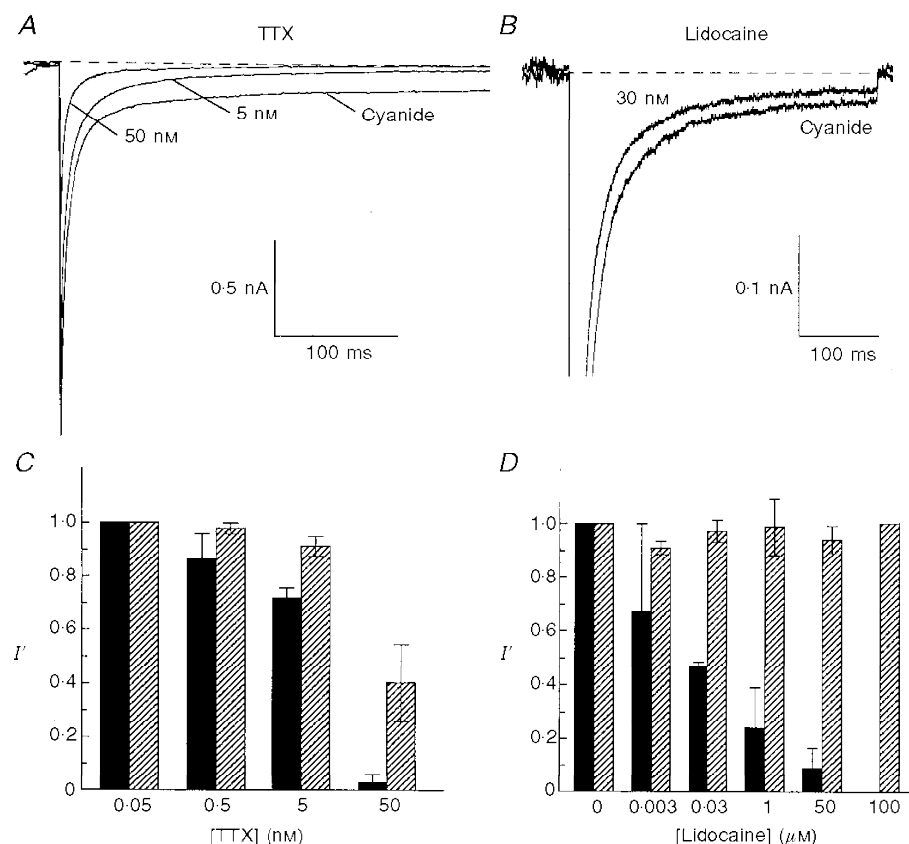


Figure 4. Low concentrations of TTX and lidocaine block the persistent Na^+ current more than the transient Na^+ current

A, effects of 5 and 50 nM TTX on the persistent Na^+ current generated by a voltage step from -100 to -30 mV 15–25 min after whole-cell seal was obtained. The pipette contained 5 mM cyanide. B, the persistent Na^+ current after approximately 25 min of sodium cyanide exposure is depressed by 30 nM lidocaine. Currents were generated by a voltage pulse to -30 mV from a holding potential of -100 mV. C and D, the relationship between the concentration of TTX (C) or lidocaine (D) and depression of the normalized amplitudes ($I = I/I_{\text{control}}$) of the transient (▨) and persistent (■) Na^+ current. The bars show the average normalized amplitude of the current and the vertical lines denote 1 s.e.m.

alleviated by Na^+ channel blockers such as TTX and lidocaine at concentrations so low that action potentials are not blocked. The rise in $[\text{Na}^+]_i$ caused by an uncompensated increase in $I_{\text{Na,P}}$ would reverse the $\text{Na}^+-\text{Ca}^{2+}$ exchanger and lead to an increase in $[\text{Ca}^{2+}]_i$ and consequent cell damage. The depolarization caused by an increase in Na^+ permeability could activate voltage-sensitive Ca^{2+} channels and this would tend to load cells with Ca^{2+} also. Thus, an increase in $I_{\text{Na,P}}$ may be the primary change induced by hypoxia that eventually leads to cell death. Selective inhibition of the hypoxia-induced increase in neuronal $I_{\text{Na,P}}$ by low concentrations of TTX and lidocaine provides an explanation for earlier reports that low concentrations of Na^+ channel inhibitors prevent the $[\text{Ca}^{2+}]_i$ increase and cell damage caused by hypoxia, while leaving normal action potential firing unchanged (Haigney *et al.* 1994; Fried *et al.* 1995; Taylor *et al.* 1995). Finally, the increase in $[\text{Na}^+]_i$ would depress re-uptake of glutamate that is coupled to the $[\text{Na}^+]$ gradient. This, together with increased secretion of glutamate caused by the increase in $[\text{Ca}^{2+}]_i$, could explain the increase in extracellular glutamate concentration that is observed during hypoxia (Sanchez-Prieto & Gonzalez, 1988; Nicholls & Attwell, 1990).

The reason for the selective increase in $I_{\text{Na,P}}$ during hypoxia is not clear. It has been postulated that the channels responsible for $I_{\text{Na,P}}$ are the same as those that generate $I_{\text{Na,T}}$ and that a small fraction of Na^+ channels entering a non-inactivating mode generates $I_{\text{Na,P}}$ (Alzheimer *et al.* 1993; Crill, 1996). Alternatively, the $I_{\text{Na,P}}$ channels may be different from $I_{\text{Na,T}}$ channels. For example, cloned Na^+ type III channels have slower activation and particularly slower inactivation than type I and II Na^+ channels but type III channels are expressed only at very early stages of development (Taylor, 1995). Furthermore, mutations in Na^+ channel protein have been shown to cause a larger than normal $I_{\text{Na,P}}$ (Rojas, 1996). Hence, the increase in the amplitude of $I_{\text{Na,P}}$ may be due to increased probability of opening of 'persistent Na^+ channels' or to an increased proportion of 'normal' Na^+ channels (most probably of type I or maybe type II) switching to a non-inactivating state, possibly because of a change in phosphorylation mediated by protein kinase C.

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Acknowledgements

A. K. M. Hammarström was a Postdoctoral Fellow supported by the National Heart Foundation of Australia and the Bruce Foundation (Anutech Pty Ltd). We are grateful to Michelle Lim for her help with dissociating cells.

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