

## Block of rapid depolarization induced by *in vitro* energy depletion of rat dorsal vagal motoneurones

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(Received 23 March 1999; accepted after revision 28 April 1999)

1. The ionic mechanisms contributing to the rapid depolarization (RD) induced by *in vitro* ischaemia have been studied in dorsal vagal motoneurones (DVMs) of brainstem slices. Compared with CA1 hippocampal neurones, RD of DVMs was slower, generally occurred from a more depolarized membrane potential and was accompanied by smaller increases in  $[K^+]_o$ .
2. RD was not induced by elevation of  $[K^+]_o$  to values measured around DVMs during *in vitro* ischaemia or by a combination of raised  $[K^+]_o$  and 2–5  $\mu\text{M}$  ouabain.
3. Neither TTX (5–10  $\mu\text{M}$ ) nor TTX combined with bepridil (10–30  $\mu\text{M}$ ), a  $\text{Na}^+$ – $\text{Ca}^{2+}$  exchange inhibitor, slowed RD. Block of voltage-dependent  $\text{Ca}^{2+}$  channels with  $\text{Cd}^{2+}$  (0.2 mM) and  $\text{Ni}^{2+}$  (0.3 mM) led to an earlier onset of RD, possibly because  $[K^+]_o$  was higher than that measured during *in vitro* ischaemia in the absence of divalent ions.
4. When  $[\text{Na}^+]_o$  was reduced to 11.25–25 mM, RD did not occur, although a slow depolarization was observed. RD was slowed (i) by 10 mM  $\text{Mg}^{2+}$  and 0.5 mM  $\text{Ca}^{2+}$ , (ii) by a combination of TTX (1.5–5  $\mu\text{M}$ ), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10  $\mu\text{M}$ ) and D-2-amino-5-phosphonovalerate (AP5, 50  $\mu\text{M}$ ) and (iii) by TTX (1.5–5  $\mu\text{M}$ ) and AP5 (50  $\mu\text{M}$ ).
5.  $\text{Ni}^{2+}$  at concentrations of 0.6 or 1.33 mM blocked RD whereas 0.6 mM  $\text{Cd}^{2+}$  did not. A combination of  $\text{Cd}^{2+}$  (0.2 mM),  $\text{Ni}^{2+}$  (0.3 mM), AP5 (50  $\mu\text{M}$ ) and bepridil (10  $\mu\text{M}$ ) was largely able to mimic the effects of high concentrations of  $\text{Ni}^{2+}$ .
6. It is concluded that RD is due to  $\text{Na}^+$  entry, predominantly through *N*-methyl-D-aspartate receptor ionophores, and to  $\text{Ca}^{2+}$  entry through voltage-dependent  $\text{Ca}^{2+}$  channels. These results are consistent with known changes in the concentrations of extracellular ions when ischaemia-induced rapid depolarization occurs.

When neurones are deprived of oxygen, or oxygen and glucose, changes in membrane potential occur within 30–60 s. Initial changes are relatively small and may include an early short-lasting depolarization due to action potential-dependent neurotransmitter release (Fujiwara *et al.* 1987; Cowan & Martin, 1992). This is followed by either hyperpolarization or depolarization due to modulation of voltage-dependent or ATP-sensitive ion channels, predominantly  $\text{K}^+$  channels (reviewed by Martin *et al.* 1994). As a result there is an early rise in the extracellular  $\text{K}^+$  concentration, to which inhibition of the  $\text{Na}^+$ – $\text{K}^+$ -ATPase may contribute, depending upon the severity of energy substrate deprivation (Martin *et al.* 1994). Ultimately, a rapid depolarization takes place, which is accompanied by an abrupt decrease in extracellular  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{Ca}^{2+}$  concentrations and an increase in extracellular  $\text{K}^+$  concentration (Hansen, 1985).

The mechanisms underlying rapid depolarization (RD) induced by energy deprivation are poorly understood. It occurs *in vivo* and in *in vitro* slice preparations and

superficially resembles the transient, spreading wave of cortical depression which is known as Leão's spreading depression (Leão, 1944). However, RD and Leão's spreading depression can be distinguished from each other by their different sensitivities to *N*-methyl-D-aspartate (NMDA) receptor antagonists. Whereas spreading depression is blocked by NMDA receptor antagonists, RD is not (Hernández-Cáceres *et al.* 1987; Marrannes *et al.* 1988; Haddad & Jiang, 1993).

Various drugs do alter the latency to onset of RD. Application of TTX sufficient to block action potentials delays its onset (Aitken *et al.* 1991; Xie *et al.* 1994). In contrast, blockers of voltage-dependent  $\text{K}^+$  channels bring its onset forward (Aitken *et al.* 1991), as does reduced extracellular  $\text{Ca}^{2+}$  (Young *et al.* 1991; but see Tanaka *et al.* 1997). Antagonists of the NMDA receptor have been reported to accelerate the onset of RD in some studies (Marrannes *et al.* 1988) but not in others (Hernández-Cáceres *et al.* 1987; Aitken *et al.* 1988; Tanaka *et al.* 1997). When AMPA/

kainate receptors are blocked by 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzene(*f*)quinoxaline (NBQX) or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), RD is delayed (Xie *et al.* 1995; Tanaka *et al.* 1997).

Interestingly, RD only occurred in 52% of slices in the presence of  $\text{Ni}^{2+}$  and  $\text{Co}^{2+}$  (2 mM each) and in 3/8 slices when  $\text{Ni}^{2+}$  (2 mM), the NMDA receptor antagonist 3-(( $\pm$ )-2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid (CPP, 10  $\mu\text{M}$ ) and the AMPA/kainate antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX) (10  $\mu\text{M}$ ) were present (Jing *et al.* 1993). A more recent study from the same laboratory reported that a cocktail of  $\text{Ni}^{2+}$ , CPP and DNQX (concentrations as above) and TTX (1  $\mu\text{M}$ ) prevented RD in all CA1 pyramidal cells studied (Müller & Somjen, 1998). Although this result clearly refutes the idea that RD reflects development of a non-specific membrane leakiness, it does not provide full insights into the mechanisms of RD because 2 mM  $\text{Ni}^{2+}$  would be expected to have actions on voltage-dependent  $\text{Ca}^{2+}$  channels (Zamponi *et al.* 1996), the NMDA receptor (Mayer & Westbrook, 1985), the  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger (Kimura *et al.* 1987) and possibly the  $\text{Ca}^{2+}$ -ATPase of the plasma membrane (Enyedi *et al.* 1982).

In this study, the mechanisms underlying RD have been systematically explored in dorsal vagal motoneurons (DVMs) of the brainstem. *In vivo* ischaemia was mimicked *in vitro* by exposing the preparation to a perfusate depleted of oxygen and glucose. Initially it was shown that the characteristics of RD in DVMs are distinctly different from those in CA1 pyramidal neurones. Lowering the extracellular  $\text{Na}^+$  concentration or applying a high concentration of  $\text{Ni}^{2+}$  blocked RD of DVMs, although a slow depolarization still occurred. The reasons for the effectiveness of these treatments were explored using various combinations of drugs. The results demonstrate that ischaemia-induced RD primarily involves entry of  $\text{Ca}^{2+}$  through voltage-dependent  $\text{Ca}^{2+}$  channels and  $\text{Ca}^{2+}$  and  $\text{Na}^+$  through the NMDA receptor ionophore. Parts of the results have been published in abstract form (Martin, 1997, 1998).

## METHODS

### Preparation and solutions

Brainstem or hippocampal slices, 400  $\mu\text{m}$  thick, were prepared from male Wistar rats aged 35–50 days using standard approaches (Cowan & Martin, 1992). Experimental procedures were approved by the Animal Experimentation Ethics Committee at the Australian National University. Rats were initially anaesthetized with halothane (4% in  $\text{O}_2$ ) and when corneal and toe pinch reflexes were absent the skull was rapidly opened and a mid-collicular decerebration performed. For preparation of hippocampal slices the forebrain was removed, a midline transection was made and then both hemispheres glued, midline down, onto a chuck. For preparation of brainstem slices, the bone covering the brainstem was removed, a transection made at the spinomedullary junction, the brainstem turned out of the cranium and glued to a chuck. Throughout the dissection procedures ice-cold artificial cerebrospinal fluid (ACSF) was dripped onto the brain tissue of interest. The chuck and tissue was placed in ice-cold ACSF, whose composition

had been modified from standard (see below) to ensure that its pH at 2 °C was about 7.3 when bubbled with 95%  $\text{O}_2$ -5%  $\text{CO}_2$  (NaCl reduced to 112.5 mM and  $\text{NaHCO}_3$  raised to 32 mM). After cutting (Vibroslice, Campden Instruments Ltd), one slice was immediately placed in a recording chamber at 34 °C. The remainder were placed on a nylon net covering a Petri dish filled with ACSF. This dish was placed in an humidified, carbogenated holding chamber at 30 °C and slices removed as required.

The standard ACSF contained (mM): 124.5 NaCl, 20  $\text{NaHCO}_3$ , 3 KCl, 1.25  $\text{NaH}_2\text{PO}_4$ , 1.25  $\text{MgCl}_2$ , 2  $\text{CaCl}_2$ , together with 9.8 g glucose; when bubbled with 95%  $\text{O}_2$ -5%  $\text{CO}_2$  the pH was about 7.35. ACSF was pumped to the recording chamber via a bubble trap containing a small reservoir of solution (< 0.3 ml), and a solution heater which was placed immediately beside the recording chamber. When *in vitro* ischaemia was induced by switching to standard ACSF bubbled with 95%  $\text{N}_2$ -5%  $\text{CO}_2$  and lacking glucose, the moment at which the first drop of solution entered the bubble trap was marked on chart recordings (see below). The solution took about 20 s to travel from the bubble trap to the recording chamber. Previous measurements in this laboratory have shown that the  $P_{\text{O}_2}$  of the perfusate in the recording chamber is 15–25 mmHg under these experimental conditions (Cowan & Martin, 1995).

In some experiments extracellular  $\text{Na}^+$  ( $[\text{Na}^+]_o$ ) was lowered to 25 mM or less by replacing the NaCl with *N*-methyl-D-glucamine (NMDG) and  $\text{NaHCO}_3$  with HEPES. When the pH was adjusted to 7.35 with 1 M HCl (volume dependent upon  $[\text{Na}^+]_o$  but around 100 ml) the osmolarity was similar to that of normal ACSF. These solutions were bubbled with either 100%  $\text{O}_2$  or 100%  $\text{N}_2$  (ischaemia). In a few cases extracellular  $\text{Cl}^-$  was reduced to 25 mM by reducing NaCl to 15.5 mM and using 104 mM sodium gluconate to maintain osmolarity. When divalent ions were used to block ion channels,  $\text{NaH}_2\text{PO}_4$  was omitted from the ACSF.

Drugs used were tetrodotoxin (TTX), CNQX, 2-amino-5-phosphonopentanoate (D-AP5) (all from Tocris Cookson), bepridil (Research Biochemicals) and ouabain (Sigma).

### Electrophysiological recording and experimental procedures

Intracellular recordings were made from DVMs or CA1 pyramidal cells using 3 M KCl-filled glass electrodes (aluminosilicate glass, SM100F-15, or borosilicate glass, GC100F-15, Clarke Electromedical Instruments). Membrane potential ( $E_m$ ) was measured relative to an agar-KCl electrode placed in the reticular formation of the brainstem or stratum radiatum. DVMs were identified on the basis of their locations, firing patterns and long after-hyperpolarization (see previous work from this laboratory, e.g. Cowan & Martin, 1992, 1995); locations and firing pattern were also used to identify CA1 pyramidal neurones (Costa *et al.* 1991; Andreasen & Lambert, 1995). Membrane potential was recorded in either current-clamp or discontinuous current-clamp mode using an Axoclamp 2A amplifier (Axon Instruments). Input resistance was monitored using small, hyperpolarizing current pulses. Data were digitized (TL-1 DMA Interface, Axon Instruments) at between 4 kHz and 30 kHz, depending upon the speed of the events being monitored, and processed using pCLAMP 5 software (Axon Instruments). Membrane potential was also displayed on a chart recorder (Yew) and on a MacIntosh computer using the MacLab system (digitization rate 4 Hz) with Chart software (ADInstruments, Australia).

When drugs were used for ion substitution experiments, a neurone was exposed to the treatment for a minimum of 10 min before switching to the oxygen- and glucose-depleted perfusate. In some experiments the efficacy of the treatment was tested electrophysiologically, e.g. tests were made for block of action potential by

TTX, reduction in action potential height when  $[Na^+]_o$  was lowered, block of synaptic transmission by CNQX and AP5 or by high  $Mg^{2+}$ -low  $Ca^{2+}$ . To test for block of synaptic transmission, a bipolar stimulating electrode was placed ventral to the dorsal vagal motonucleus (DVMN) and the current was increased until a fast EPSP could be elicited. After application of the test solution, efficacy of treatment was tested at the same stimulus current and at double the stimulus current.

#### Ion-sensitive microelectrodes

On some occasions a  $K^+$ -sensitive microelectrode was placed 200  $\mu m$  into the contralateral DVMN or the CA1 pyramidal cell layer of the hippocampus. These electrodes were pulled from borosilicate glass which had been washed in nitric acid and thoroughly dried. A drop of 2,4 dichloromethylsilane was placed in the tip of each electrode and they were baked overnight at 200 °C. Potassium ionophore I (Cocktail B, Fluka) was introduced into the tip of each silanized electrode using a fine plastic filler and then the electrodes were backfilled with 0.01 M KCl. Electrodes were tested for  $K^+$  sensitivity before and after each experiment using ACSF solutions containing from 1, 3, 10, 25 and 50 mM  $K^+$ , maintained at 34 °C. The relationship between electrode potential and the logarithm of  $K^+$  activity ( $aK$ ) was highly linear ( $r^2 > 0.98$  in all cases), slopes ranged from 48 to 57 mV per log unit and the 10–90% response time ranged from 25 to 30 s. Electrodes were tested for interference from  $Na^+$  and this was found to be negligible in well-silanized electrodes with tips of about 1  $\mu m$ . Data concerning  $aK$  were only analysed in those experiments where electrodes had non-significant differences in  $K^+$  sensitivity before and after each experiment (comparison of slopes using  $F$  test).

#### Data analysis

For data analysis, membrane potential was measured at minute intervals beginning 2 min before the onset of *in vitro* ischaemia. In DVMs the rate of rapid depolarization was estimated as the slope of line fitted, using the least squares linear regression technique, to the membrane potential trajectory between  $-40$  and  $-20$  mV, i.e. the most rapid phase of depolarization. This range was extended if the rapid depolarization clearly began at more negative membrane potentials. In some experiments the membrane potential did not cover the range  $-40$  to  $-20$  mV within 30 min of initiating an ischaemic insult. In these cases the final 40% of the change in membrane potential (the last value being that at 30 min) was fitted with a linear regression to obtain the slope. This portion was used because in most experiments membrane potential changed from about  $-65$  mV to  $-15$  mV and therefore when the slope was fitted over 20 mV this represented 40% of the data range. In hippocampal CA1 neurones, the rate of rapid depolarization was derived from the very obvious region of rapid membrane potential depolarization.

Because the moment at which RD occurred could not be definitively determined, the onset of RD was calculated as one-half of the time taken,  $t_{50}$ , for the membrane potential to reach  $-20$  mV. In some cases the trajectory of the membrane potential was extrapolated from its final point to  $-20$  mV but this was only done if the membrane potential was within 5 mV of  $-20$  mV. This analysis was employed with drug treatments which significantly slowed RD.

The effect of different drug treatments on the rate of ischaemia-induced rapid depolarization was analysed using a one-way analysis of variance (ANOVA) and Dunnett's procedure for multiple comparisons. Differences between DVMs and CA1 neurones, or between *in vitro* ischaemia and ouabain were tested using Student's unpaired  $t$  tests. Differences were considered significant when  $P < 0.05$ .

## RESULTS

### Differences in RD between CA1 neurones and DVMs

The rapid depolarization induced by *in vitro* ischaemia (RD) was significantly slower in DVMs than in CA1 neurones ( $P < 0.0001$ ). In control DVMs, the mean rate of depolarization from digitized records was  $12.3 \pm 2.1$  mV min<sup>-1</sup> ( $n = 15$ , Fig. 1A). Such a rate of depolarization was not usually confused with loss of intracellular penetration. Nevertheless partial or complete recovery of  $E_m$  when the perfusate was replaced with standard ACSF was used as evidence that the depolarization was ischaemia-induced (Fig. 2).

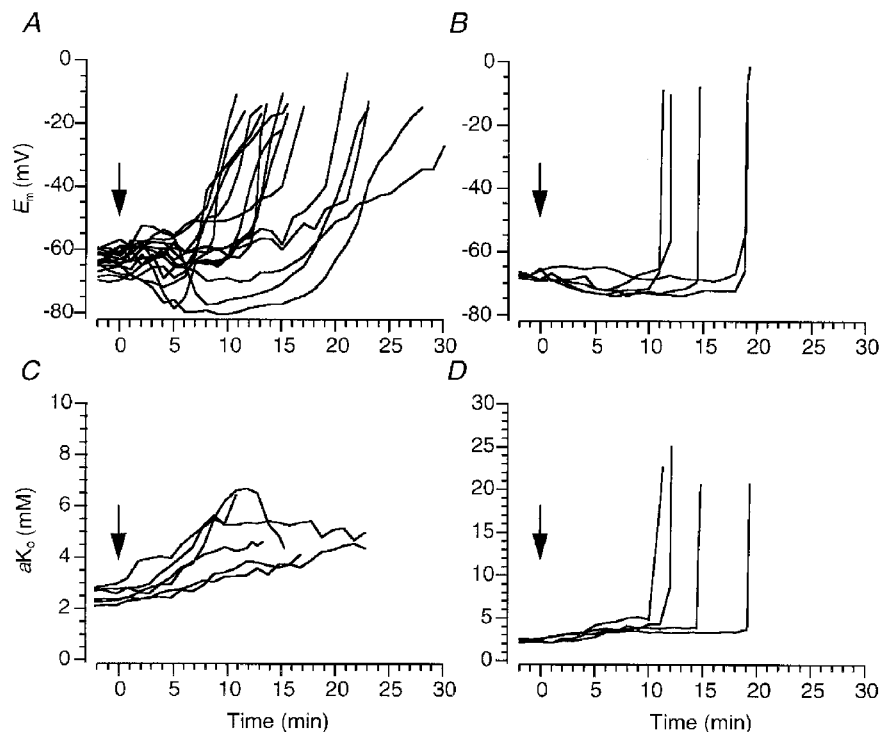
In contrast, RD in CA1 neurones was so fast that it was indistinguishable from the sudden loss of membrane potential that occurs when an electrode 'jumps' out of a neurone (Fig. 1B). Thus, in order to distinguish RD from loss of an intracellular recording it was necessary to show some recovery of  $E_m$  when the perfusate was replaced with standard ACSF (recovery is not shown in Fig. 1B). The mean rate of depolarization was  $6.3 \pm 1.1$  mV s<sup>-1</sup> ( $n = 5$ ), very similar to values reported by Tanaka *et al.* (1997).

In DVMs, changes in  $E_m$  induced by *in vitro* ischaemia were accompanied by small increases in  $aK_o$  from about 3 mM to 5–6 mM. The increase in  $aK_o$  began shortly after the onset of ischaemia and usually rose steadily throughout the period of oxygen and glucose deprivation ( $n = 6$ , Fig. 1C). There was no rapid increase in  $aK_o$  at the time of RD. These data contrast with measurements made in the CA1 cell layer where a dramatic rise in  $aK_o$  to about 25 mM occurred ( $n = 4$ , Fig. 1D).

### Role of $[K^+]_o$ in RD

Experiments were undertaken to ascertain if elevation of  $[K^+]_o$  to values recorded in the DVMN during ischaemia was sufficient to initiate a spiral of rapid depolarization in DVMs. However, even when  $[K^+]_o$  was increased to 10 mM (using KCl or  $K_2SO_4$ ), either as an hyperosmotic solution or with isosmotic replacement of NaCl, DVMs only depolarized by about 10–12 mV and  $E_m$  remained steady thereafter ( $n = 5$ , Fig. 3A). Since the depolarization was substantially less than that predicted by the shift in  $E_K$  this result suggests that the membrane of DVMs has a substantial resting permeability to  $Cl^-$ .

These results also indicate that the  $[K^+]_o$  found in the DVMN during ischaemia does not single-handedly initiate RD. However, elevation of  $[K^+]_o$  in combination with reduced activity of the  $Na^+-K^+-ATPase$  as a result of the reduced availability of ATP during ischaemia could initiate RD. When 2–5  $\mu M$  ouabain was used to block the  $Na^+-K^+-ATPase$  in the presence of 10 mM extracellular  $K^+$ , RD did not occur within 20 min, i.e. within the time frame usually observed for ischaemia-induced RD ( $n = 3$ , Fig. 3B). In 2/3 neurones a relatively slow depolarization started to occur but it was not possible to follow the membrane potential until depolarization occurred because the intracellular recordings

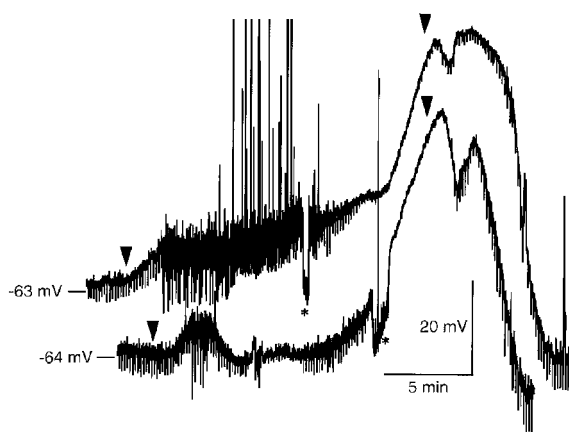


**Figure 1.** Changes in membrane potential and extracellular  $K^+$  activity induced by *in vitro* ischaemia

*A* and *B*, changes in membrane potential of dorsal vagal motoneurons (*A*) and CA1 pyramidal cells (*B*) induced by switching from a standard superfusate to one bubbled with 95%  $O_2$ –5%  $CO_2$  and lacking in glucose. Time zero (arrow) indicates when the new perfusate arrived at the bubble trap in the solution line (see Methods). *C* and *D*, potassium ion activity recorded extracellularly at the same time as some of the recordings in *A* or *B*, respectively. In studies of dorsal vagal motoneurons these measurements were made in the contralateral dorsal vagal motonucleus, and in studies of CA1 pyramidal cells they were made in the pyramidal cell layer.

always became unstable. At 2–5  $\mu M$ , ouabain would be expected to block the  $\alpha_2$  and  $\alpha_3$  isoforms of the  $Na^+$ – $K^+$ –ATPase but probably not the  $\alpha_1$  isoform (for review see Ewart & Klip, 1995). All these isoforms of the  $Na^+$ – $K^+$ –ATPase are present in the brain.

In contrast, 25  $\mu M$  ouabain consistently induced a rapid depolarization within 9–13 min, even when  $[K^+]_o$  was normal (mean rate of depolarization  $12.2 \pm 1.9$  mV min $^{-1}$ ,  $n = 7$ , Fig. 3*C*). During perfusion with ouabain,  $aK_o$  rose from 3 to 5–9 mM ( $n = 3$ , Fig. 3*D*). These results also



**Figure 2.** Original records of rapid depolarization and recovery in dorsal vagal neurones

Raw data, as recorded with the MacLab system, showing response of two DVMs to *in vitro* ischaemia, and their subsequent recovery on return to standard perfusate. Arrowheads indicate when the new perfusate arrived at the bubble trap in the solution line (see Methods). Asterisks mark periods of each trace when hyperpolarizing current was injected into the neurones to return membrane potential to initial values for monitoring of input resistance. Positive-going shifts in membrane potential are action potentials, which were often truncated by the slow digitizing rate (4 samples s $^{-1}$ ).

suggest that the principle reason for ouabain-induced rapid depolarization is not elevation of  $aK_o$ , although this may initiate contributing events.

### Role of $Ca^{2+}$ in RD

Switching to ACSF without added  $Ca^{2+}$  (nominally ' $Ca^{2+}$ -free' perfusate) at the onset of *in vitro* ischaemia did not change the rate of RD but tended to cause an earlier time of onset ( $n=2$ ). At the time of RD there was an unusually large elevation of  $aK_o$  to 10 mM. In control neurones subjected to removal of extracellular  $Ca^{2+}$  only, the membrane depolarized but  $aK_o$  increased by less than 1–2 mM. Thus, in the absence of extracellular  $Ca^{2+}$ , *in vitro* ischaemia induced an unusual rise in  $aK_o$  and for this reason additional experiments were not undertaken.

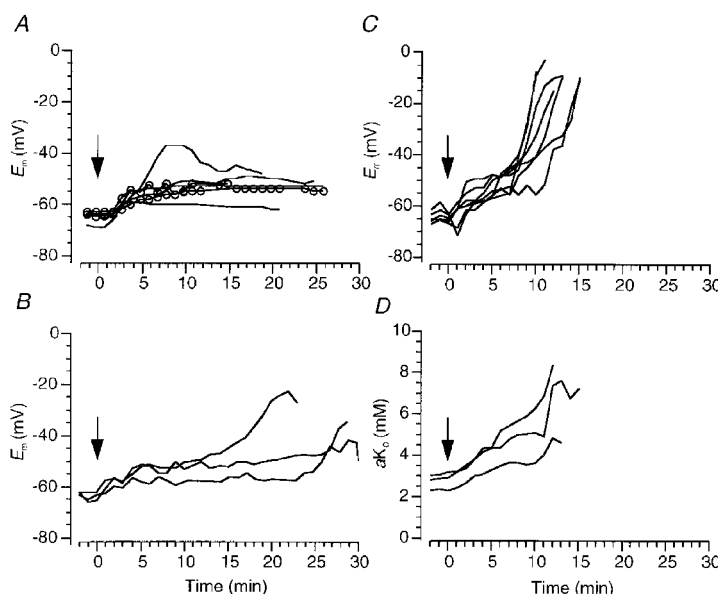
To investigate further the role of  $Ca^{2+}$  in RD a pharmacological approach was used. DVMs possess a variety of voltage-dependent  $Ca^{2+}$  channels (Sah, 1995) which can be blocked by a combination of 0.2 mM  $Cd^{2+}$  and 0.3 mM  $Ni^{2+}$  (A. I. Cowan & R. L. Martin, unpublished observations). While the onset of RD was significantly earlier than in control cells ( $4.4 \pm 0.8$  min ( $n=4$ ), compared with  $8.4 \pm 0.8$  min ( $n=15$ ) in controls; ANOVA followed by Dunnett's test) the rate of depolarization was similar to that in controls (Figs 4A and 6). Again  $aK_o$  tended to rise abruptly and to higher levels ( $n=3$ , Fig. 4B).

These data indicate that when  $Ca^{2+}$  entry into neurones during *in vitro* ischaemia is limited by either lowering extracellular  $Ca^{2+}$  or by blocking voltage-dependent  $Ca^{2+}$  channels with  $Cd^{2+}$  the rise in  $aK_o$  is potentiated. The cause of this was not investigated but the observation may explain why other studies have noted an earlier onset of RD in the presence of  $Cd^{2+}$  (Young *et al.* 1991).

### Role of $Na^+$ in RD

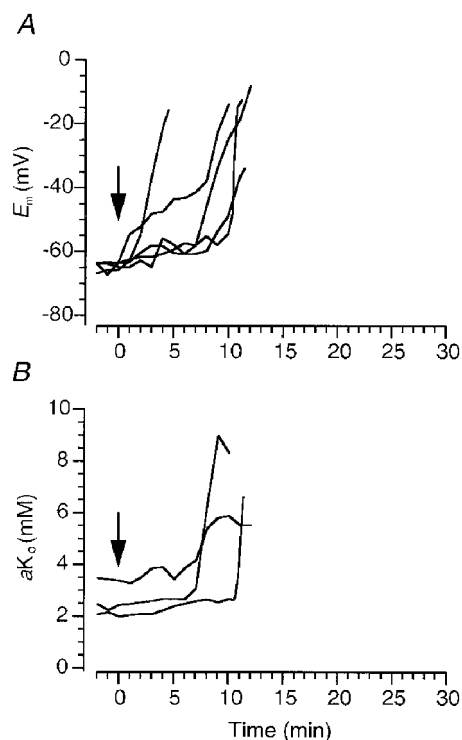
Block of TTX-sensitive voltage-dependent and persistent  $Na^+$  currents (if present in DVMs) with 5–10  $\mu$ M TTX, did not obviously change RD (mean rate of depolarization  $11.6 \pm 3.9$  mV min $^{-1}$ ,  $n=3$ , Figs 5A and 6). This result confirms data reported in numerous previous studies. A combination of 10–30  $\mu$ M bepridil ( $n=3$ ), to block forward  $Na^+$ – $Ca^{2+}$  exchange, and TTX (3–5  $\mu$ M) did not slow RD either (mean rate of depolarization  $21.1 \pm 6.0$  mV min $^{-1}$ ,  $n=3$ , Figs 5A and 6).

However, there are many ways, other than via TTX-sensitive  $Na^+$  channels, that  $Na^+$  can enter the cytosol and effect depolarization. Experiments were attempted in which  $[Na^+]_o$  was reduced to 11.25 mM by isosmotically replacing NaCl with NMDG and replacing  $NaHCO_3$  with Hepes. This treatment alone usually caused depolarization and loss of the intracellular recording. Ultimately, reduction in  $[Na^+]_o$  to 11.25 mM ( $n=1$ ) or 18 mM ( $n=5$ ), or 25 mM ( $n=4$ ) was



**Figure 3.** Effects of raised  $[K^+]_o$  and ouabain on membrane potential and extracellular  $K^+$  activity

A, changes in membrane potential of DVMs induced by switching the superfusate from one containing 3 mM  $K^+$  to one containing 10 mM KCl (—) or 10 mM  $K_2SO_4$  (⊖) at time zero (arrow; see Methods). B, effects on membrane potential of 2–5  $\mu$ M ouabain when  $[K^+]_o$  was raised to 10 mM. C and D, changes in membrane potential (C) and contralaterally measured extracellular  $K^+$  activity (D) induced by 25  $\mu$ M ouabain.

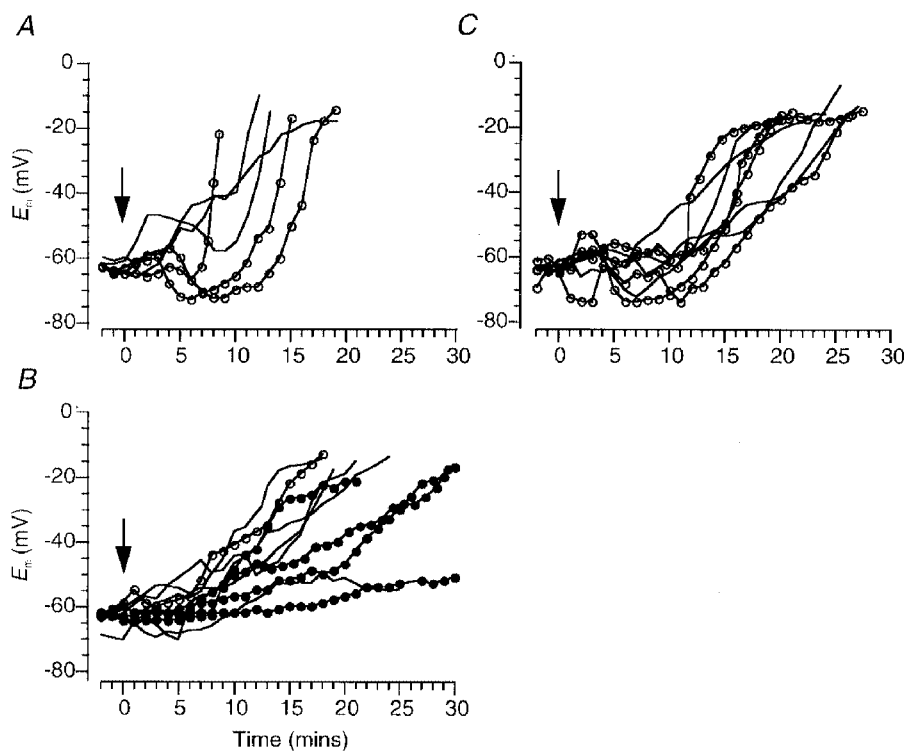


**Figure 4.** Effects of low concentrations of divalent ions on rapid depolarization and extracellular  $K^+$  activity

*A*, changes of membrane potential in DVMs induced by *in vitro* ischaemia in the presence of 0.2 mM  $Cd^{2+}$  and 0.3 mM  $Ni^{2+}$ . Perfusate switched at time zero (arrow; see Methods). *B*, in some experiments extracellular  $K^+$  activity was simultaneously measured in the contralateral dorsal vagal motonucleus.

achieved without loss of recording. With 11.25 and 18 mM  $[Na^+]_o$  the fast action potential was completely abolished whereas in 25 mM  $[Na^+]_o$  the action potential could be elicited, albeit significantly reduced in amplitude.

When *in vitro* ischaemia was initiated in a perfusate with a lowered  $[Na^+]_o$  the response was significantly slower than in controls and at such a rate that RD did not occur (mean rate of depolarization  $3.1 \pm 1.6$  mV  $min^{-1}$ ,  $n = 10$ , Figs 5*B* and 6).



**Figure 5.** Effects of TTX, low  $[Na^+]_o$  and glutamate receptor antagonists on rapid depolarization

Changes in membrane potential of DVMs induced by *in vitro* ischaemia in the presence of: 5–10  $\mu M$  TTX (—) or 3–5  $\mu M$  TTX with 10–30  $\mu M$  bepridil ( $\ominus$ ) (*A*); when the extracellular  $Na^+$  concentration was reduced to 11.25 ( $\ominus$ ), 18 (—) or 25 mM ( $\bullet$ ) (*B*); in the presence of 3–5  $\mu M$  TTX and 50  $\mu M$  AP5, with (—) and without 10  $\mu M$  CNQX ( $\ominus$ ) (*C*). Perfusate switched at time zero (arrow; see Methods).

When  $\text{Na}^+$  was replaced by NMDG the  $[\text{Cl}^-]_o$  was reduced from normal by about 25 mM. To ensure that the elimination of RD was not due to this decrement in  $[\text{Cl}^-]_o$ , two experiments were undertaken in which extracellular  $\text{Cl}^-$  was replaced with gluconate. RD still occurred (data not shown). The ischaemia-induced rise in  $aK_o$  was similar in low  $[\text{Na}^+]_o$  to that observed in the standard ischaemic perfusate.

These results indicate that influx of  $\text{Na}^+$  is an important contributor to RD and therefore blockade of the principal routes of  $\text{Na}^+$  entry should mimic the effects of reducing  $[\text{Na}^+]_o$ . It has been proposed that during ischaemia glutamate release may occur via reverse operation of the glutamate transporter (Madl & Burgesser, 1993) or even as a result of an ischaemia-induced membrane damage (Phillis *et al.* 1994). Such release would not be blocked by TTX but postsynaptic receptors would be activated. DVMs are known to receive a significant glutamatergic synaptic input, which results in activation of both non-NMDA and NMDA receptors (Travagli *et al.* 1991; Willis *et al.* 1996). When AMPA/kainate and NMDA subtypes of glutamate receptors were blocked with a combination of 10  $\mu\text{M}$  CNQX and 50  $\mu\text{M}$  AP5 (in the presence of 3–5  $\mu\text{M}$  TTX,  $n = 4$ ) or with only TTX and AP5 ( $n = 4$ ) the mean rates of depolarization were  $5.5 \pm 1.4$  and  $6.5 \pm 1.1$  mV min $^{-1}$ , respectively (Figs 5C and 6). Although not statistically significant, these rates of depolarization were about half those observed in controls (Fig. 6). EPSPs evoked prior to *in vitro* ischaemia by stimulation ventral to the DVMN were abolished by 10  $\mu\text{M}$  CNQX and 50  $\mu\text{M}$  AP5.

#### RD is slowed by high $\text{Mg}^{2+}$ –low $\text{Ca}^{2+}$

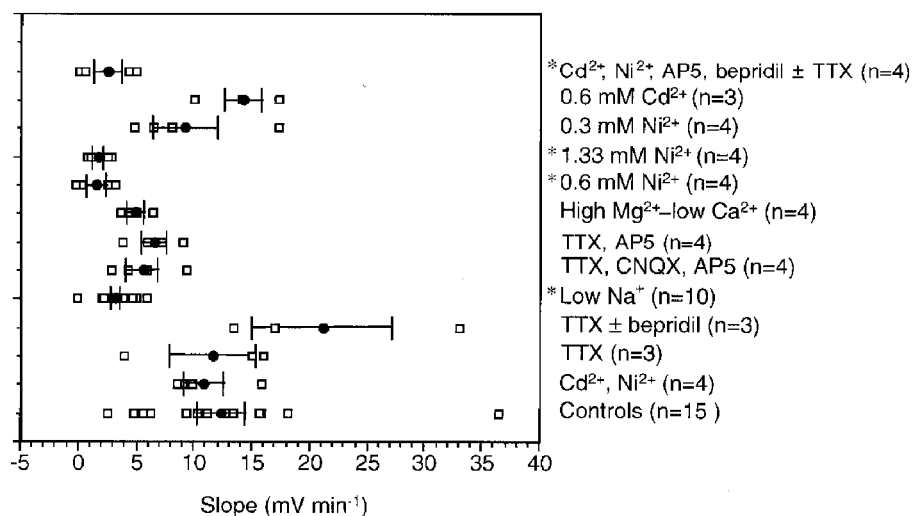
Besides glutamate receptors, DVMs possess postsynaptic nicotinic cholinergic receptors, P2X2 receptors and 5HT3 receptors, all of which may permit  $\text{Na}^+$  entry during *in vitro*

ischaemia if appropriate neurotransmitter release occurs. Thus, the effects of low  $[\text{Na}^+]_o$  on RD may be mimicked by blocking synaptic transmission through simultaneously raising  $[\text{Mg}^{2+}]_o$  to 10 mM and lowering  $[\text{Ca}^{2+}]_o$  to 0.5 mM. This treatment did reduce the rate of ischaemia-induced depolarization, and at  $4.8 \pm 2.5$  mV min $^{-1}$  ( $n = 4$ , Figs 6 and 7A) the mean rate was similar to that observed in the presence of glutamate receptor antagonists.

#### RD is blocked by high concentrations of $\text{Ni}^{2+}$

Studies on hippocampal CA1 neurones have been discordant in relation to the effects of 2 mM  $\text{Ni}^{2+}$  on RD, but in DVMs both 0.6 mM and 1.33 mM  $\text{Ni}^{2+}$  consistently prevented RD. The mean rates of depolarization were  $1.5 \pm 2.5$  and  $1.8 \pm 2.5$  mV min $^{-1}$ , respectively, and these were significantly different from controls (Figs 6 and 7B). In several cells there was an initial early depolarization from rest to about  $-50$  mV, but membrane potential barely changed thereafter. Addition of TTX (5  $\mu\text{M}$ ) and  $\text{Cd}^{2+}$  (0.2 mM), or TTX (5  $\mu\text{M}$ ), CNQX (10  $\mu\text{M}$ ) and AP5 (50  $\mu\text{M}$ ) did not further alter the response ( $n = 2$ , data not shown). In contrast, neither 0.3 mM  $\text{Ni}^{2+}$  ( $n = 4$ ; Fig. 7C) nor 0.6 mM  $\text{Cd}^{2+}$  ( $n = 3$ ; Fig. 7C) slowed RD (mean rates of depolarization  $9.1 \pm 2.5$  and  $14.2 \pm 2.9$  mV min $^{-1}$ , respectively). The effects of 0.6 mM  $\text{Ni}^{2+}$  on the action potential were as would be expected of  $\text{Ca}^{2+}$  channel block (Zamponi *et al.* 1996) and a secondary effect on  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channels, i.e. a slight increase in amplitude, decrease in half-width, and decreases in both the amplitude and duration of the after-hyperpolarization. Furthermore, EPSPs induced by perivagal stimulus were blocked, as they were by 0.6 mM  $\text{Cd}^{2+}$ .

In addition to its action on voltage-dependent  $\text{Ca}^{2+}$  channels, 0.6 mM  $\text{Ni}^{2+}$  would be expected to block NMDA receptors



**Figure 6.** Rates of ischaemia-induced rapid depolarization during various treatments

The size of the symbols prevents all data points from being visible. Means  $\pm$  s.e.m. are indicated by filled circles with bars. A one-way ANOVA demonstrated a highly significant effect of drug treatment on the rate of rapid depolarization ( $P < 0.0001$ ). The asterisks mark those treatments demonstrating significant differences from controls (Dunnett's procedure): low  $[\text{Na}^+]_o$ , 0.6 mM  $\text{Ni}^{2+}$ , 1.33 mM  $\text{Ni}^{2+}$  and combined 0.2 mM  $\text{Cd}^{2+}$ , 0.3 mM  $\text{Ni}^{2+}$ , 50  $\mu\text{M}$  AP5 and 10  $\mu\text{M}$  bepridil  $\pm$  3–5  $\mu\text{M}$  TTX.

(Mayer & Westbrook, 1985) and inhibit the  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger (Kimura *et al.* 1987). Therefore the effects of a combination of 50  $\mu\text{M}$  AP5, 0.2 mM  $\text{Cd}^{2+}$ , 0.3 mM  $\text{Ni}^{2+}$  and 10  $\mu\text{M}$  bepridil on RD were tested (usually in the presence of 3–5  $\mu\text{M}$  TTX). As shown in Fig. 7D, RD did not occur within the 30 min time frame in two cells, and in the other two cells a late, slow depolarization from a relatively positive membrane potential occurred (mean rate of depolarization  $2.4 \pm 2.5$  mV  $\text{min}^{-1}$ ,  $n = 4$ ). Despite the small sample size this result was significantly different from controls (Fig. 6).

## DISCUSSION

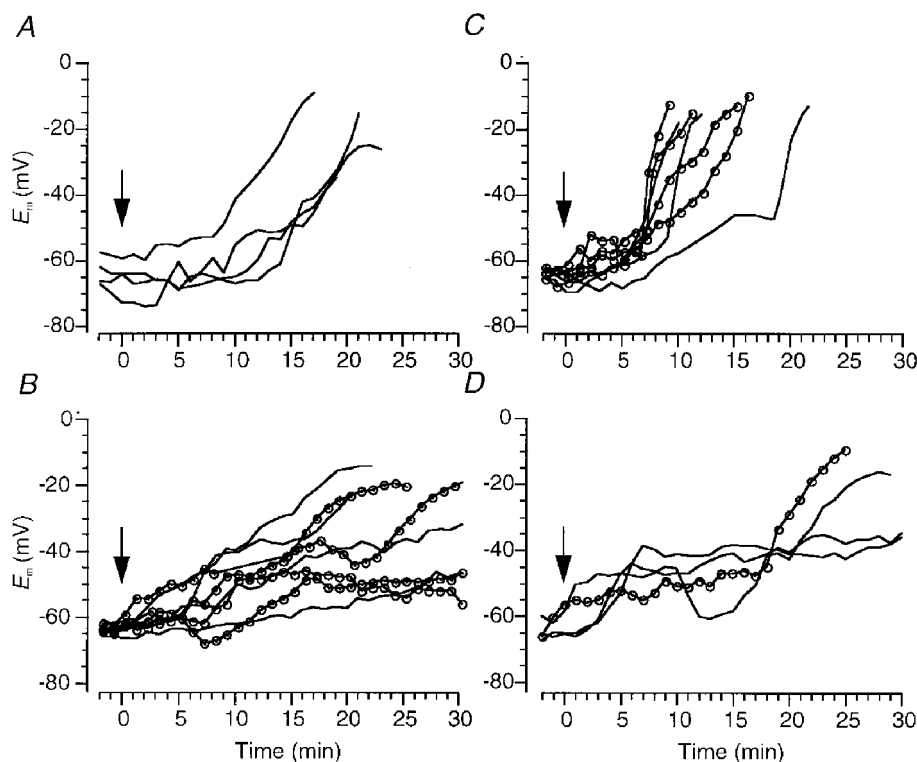
The results presented in this paper clearly indicate that the rapid depolarization induced by *in vitro* ischaemia (RD) in DVMs is not due to development of non-specific membrane leakiness. The idea that RD occurs because the membrane develops a general 'leakiness' has arisen in the literature because many earlier studies failed to find drugs that could block RD. But here application of  $\text{Ni}^{2+}$  in concentrations as low as 0.6 mM, or a combination of TTX, voltage-dependent  $\text{Ca}^{2+}$  channel blockers, an NMDA receptor antagonist and an inhibitor of  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchange eliminated the rapid phase of ischaemia-induced depolarization. Despite the very different rates of ischaemia-induced depolarization of DVMs and CA1 neurones, the mechanisms contributing to RD seem very similar. In CA1 neurones, 2 mM  $\text{Ni}^{2+}$  in combination

with TTX and the glutamate receptor antagonists DNQX and AP5 blocks RD (Müller & Somjen, 1998). Unfortunately, Müller & Somjen did not explore whether the inclusion of the AMPA/kainate receptor antagonist DNQX was absolutely necessary to prevent RD.

Previously, Haddad & Jiang (1993) showed that in brainstem neurones a reduction of  $[\text{Na}^+]_o$  to 5 mM limited the size and speed of RD but the reasons for this were not studied. In CA1 hippocampal neurones, Tanaka *et al.* (1997) and Yamamoto *et al.* (1997) reported that neither a reduction of  $[\text{Na}^+]_o$  to 28.6 mM nor application of 2 mM  $\text{Ni}^{2+}$  prevented RD. However, there was a significant reduction in the rate of depolarization with these treatments. High concentrations (0.25 mM) of AP5 slowed RD but the reduction of  $[\text{Ca}^{2+}]_o$  to 0.25 mM had the most dramatic effects of any treatment (Tanaka *et al.* 1997). However, it is only when drugs are used in combinations, as in the present study, and as reported by Somjen and colleagues (Jing *et al.* 1993; Müller & Somjen, 1998) that RD is prevented.

### Block of action potential and exocytosis

The ability of low  $[\text{Na}^+]_o$  to prevent RD in DVMs suggests that blocking pathways that permit  $\text{Na}^+$  entry should produce a similar result. In keeping with results reported in other studies (see Introduction), TTX did not block RD, which indicates that activation of TTX-sensitive  $\text{Na}^+$  channels or any process which depends upon their action,



**Figure 7.** Treatments which act at multiple targets slow or eliminate rapid depolarization

Ischaemia-induced changes in membrane potential of DVMs in the presence of: 10 mM  $\text{Mg}^{2+}$  and 0.5 mM  $\text{Ca}^{2+}$  (A); 0.6 mM  $\text{Ni}^{2+}$  ( $\ominus$ ) or 1.33 mM  $\text{Ni}^{2+}$  ( $\text{—}$ ) (B); 0.3 mM  $\text{Ni}^{2+}$  ( $\ominus$ ) or 0.6 mM  $\text{Cd}^{2+}$  ( $\text{—}$ ) (C); 0.2 mM  $\text{Cd}^{2+}$ , 0.3 mM  $\text{Ni}^{2+}$ , 50  $\mu\text{M}$  AP5 and 10  $\mu\text{M}$  bepridil, with ( $\text{—}$ ) and without ( $\ominus$ ) 3–5  $\mu\text{M}$  TTX (D). Perfusate switched at time zero (arrow; see Methods).



e.g. physiological exocytotic neurotransmitter release, does not contribute significantly to RD. Nevertheless combined TTX, CNQX and AP5, or TTX and AP5 clearly reduced the rate of depolarization. This suggests that glutamate release by non-exocytotic pathways occurs during ischaemia and is consistent with slice studies showing glutamate release by reversal of the glutamate transporter when ATP is reduced (Madl & Burgesser, 1993) or during oxygen and glucose deprivation (Roettger & Lipton, 1996), and with studies showing that ischaemia-induced glutamate release is largely  $\text{Ca}^{2+}$  independent (Ikeda *et al.* 1989; Rubio *et al.* 1991; Lobner & Lipton, 1993). Activation of AMPA/kainate receptors appears to contribute little to RD in DVMs, possibly because of their rapid desensitization.

When transmission at all synapses was blocked using a high  $\text{Mg}^{2+}$ -low  $\text{Ca}^{2+}$  perfusate, RD was also slowed. This result is inconsistent with the effects of TTX and low concentrations of  $\text{Cd}^{2+}$  and  $\text{Ni}^{2+}$ , which also block synaptic transmission. Thus, high  $\text{Mg}^{2+}$ -low  $\text{Ca}^{2+}$  probably slows RD through inhibition of NMDA receptors (Mayer & Westbrook, 1987), although high concentrations of  $\text{Mg}^{2+}$  have been reported to inhibit the plasmalemmal  $\text{Ca}^{2+}$ -ATPase in red blood cells (Enyedi *et al.* 1982) and the  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchanger in renal epithelial cells (Lyu *et al.* 1991).

#### Actions of $\text{Ni}^{2+}$

Interestingly, concentrations of  $\text{Ni}^{2+}$  as low as 0.6 mM were able to prevent RD. Nickel may protect against RD by four possible modes of action: block of voltage-dependent  $\text{Ca}^{2+}$  channels, block of the NMDA receptor (Mayer & Westbrook, 1985), inhibition of the  $\text{Ca}^{2+}$  pump (Enyedi *et al.* 1982) or block of the  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchanger (Kimura *et al.* 1987). Preliminary observations in this laboratory indicate that the combination of 0.2 mM  $\text{Cd}^{2+}$  and 0.3 mM  $\text{Ni}^{2+}$  provides nearly complete block of all voltage-dependent  $\text{Ca}^{2+}$  channels of DVMs, a result which is consistent with those obtained in CA3 hippocampal neurones (Avery & Johnston, 1996) and neurones from the central amygdala (Yu & Shinnick-Gallagher, 1997). Since neither these concentrations of divalent ions nor 10 mM  $\text{Mg}^{2+}$  and 0.5 mM  $\text{Ca}^{2+}$  were able to block RD as effectively as high  $\text{Ni}^{2+}$ , it is highly unlikely that protection by higher concentrations of  $\text{Ni}^{2+}$  is solely through blockade of voltage-dependent  $\text{Ca}^{2+}$  channels. It is also unlikely that the action of  $\text{Ni}^{2+}$  is mediated solely by block of the NMDA receptor because the effect of  $\text{Ni}^{2+}$  on RD was also more dramatic than that of TTX and AP5.

Typically, 5 mM  $\text{Ni}^{2+}$  is used to block the  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchanger, but in ventricular myocytes 1 mM appears to block its activity by about 60% (Kimura *et al.* 1987). However, results pertaining to the sensitivity of the cardiac splice variant to  $\text{Ni}^{2+}$  cannot necessarily be applied to the neuronal  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchanger because it has been shown that although all three isoforms (NCX1-3) of the  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchanger exist in the brain, only three splice variants of NCX1 (NCX1.4-1.6) are found there and these differ from the cardiac splice variant (NCX1.1) (Quednau *et al.* 1997).

$\text{Ni}^{2+}$  in concentrations greater than 0.2 mM has been shown to inhibit the  $\text{Ca}^{2+}$ -ATPase of red cells by interacting non-specifically with an internal site on the pump (Enyedi *et al.* 1982). Although  $\text{Ni}^{2+}$  may have crossed the plasma membrane it probably did not attain sufficiently high intracellular concentrations to significantly affect the  $\text{Ca}^{2+}$  pump, especially when an effective external concentration was as low as 0.6 mM.

This analysis of the potential actions of  $\text{Ni}^{2+}$  points to the strong likelihood that it prevents RD not by a single action but most probably by partly limiting  $\text{Ca}^{2+}$  influx via voltage-gated  $\text{Ca}^{2+}$  channels and partly limiting both  $\text{Na}^{+}$  and  $\text{Ca}^{2+}$  influx through the NMDA receptor ionophore. Additionally, partial inhibition of the  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchanger could be important. If this is the case then a similar result should be produced by use of 0.2 mM  $\text{Cd}^{2+}$ , 0.3 mM  $\text{Ni}^{2+}$ , AP5 and bepridil. In general this proved to be the case. A residual, late depolarization from very depolarized potentials in some cells treated with these drugs may reflect  $\text{Na}^{+}$  entry via AMPA/kainate receptors.

#### $\text{Na}^{+}$ - $\text{Ca}^{2+}$ exchanger

Because of its electrogenic nature, the  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchanger could contribute to membrane depolarization during ischaemia if it operated in the forward mode. Bepridil was chosen to block this exchanger because it has proved to be neuroprotective in white matter exposed to oxygen and glucose deprivation (reviewed by Stys, 1998). At low concentrations (10  $\mu\text{M}$ ) it targets the  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchanger and the NMDA receptor (Sobolevsky *et al.* 1997) but at higher concentrations (30  $\mu\text{M}$  or more) it has also been shown to depress the sodium current,  $I_{\text{Na}}$ , in ventricular cells. In the presence of TTX no obvious effects of bepridil were observed, suggesting little contribution of electrogenic  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchange to RD. This would be consistent with a report that the NCX1 and 2 isoforms of the exchanger are inhibited by depletion of cellular ATP (Linck *et al.* 1998). Nevertheless, further experiments are required to clearly establish a role, if any, of the exchanger in RD.

#### Residual slow depolarization

In a number of cells a slow depolarization persisted after block of RD. This is consistent with failure of the  $\text{Na}^{+}$ - $\text{K}^{+}$ -ATPase. Perhaps the more intriguing observation is why some cells were able to maintain a membrane potential between -40 and -50 mV for 30 or more minutes. The most likely explanation is that the metabolic demands of these cells have been sufficiently slowed by depletion of  $[\text{Na}^{+}]_o$ , that any residual oxygen available to them is sufficient to fuel the  $\text{Na}^{+}$ - $\text{K}^{+}$ -ATPase.

#### Summary

The results presented in this paper suggest that ischaemia-induced rapid depolarization is due to a combination of  $\text{Na}^{+}$  and  $\text{Ca}^{2+}$  entry, particularly through voltage-dependent  $\text{Ca}^{2+}$  channels and the NMDA receptor ionophore. These results are consistent with studies indicating that at the time of RD

there is an abrupt reduction in  $[Na^+]_o$  and  $[Ca^{2+}]_o$  (Hansen, 1985; Silver & Erecinska, 1990) and with studies showing that in the early phase of ischaemia some of the rise in  $[Ca^{2+}]_i$  can be reduced by blocking NMDA receptors and voltage-dependent  $Ca^{2+}$  channels (Lobner & Lipton, 1993).

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### Acknowledgements

This study was supported by the Australian Research Council. I wish to thank Gerlinde Lenz for technical assistance, especially for her contribution to construction of  $K^{+}$ -selective microelectrodes.

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