J. Physiol. (1982), **332**, *pp.* 459–472
With 5 text-figures
Printed in Great Britain

MECHANISMS INVOLVED IN IRREVERSIBLE ANOXIC DAMAGE TO THE *IN VITRO* RAT HIPPOCAMPAL SLICE

BY IRA S. KASS AND PETER LIPTON

From the Department of Physiology, University of Wisconsin, 1300 University Avenue, Madison, WI 53706, U.S.A.

(*Received 16 March 1982*)

SUMMARY

1. We have studied the effects of anoxia on the recovery of neural transmission between the perforant path and the dentate granule cells in the *in vitro* rat hippocampal slice. There is almost no recovery of the evoked population spike following 10 min of anoxia in slices from adult rats.

2. A 2 h exposure of slices to creatine markedly improves the recovery of the population spike (80 % *vs.* 5 %). The creatine pre-incubation builds up phosphocreatine levels in the slice and prevents the large fall in ATP during anoxia; ATP falls to 7·9 rather than 3·6 nM/mg protein. The intracellular pH of both groups falls to the same level during anoxia.

3. If calcium concentration in the medium is reduced to 0 while magnesium concentration is raised to 10 mM during anoxia the evoked response recovers to about 65 %.

4. The data suggest that an attenuation of the fall in ATP or entry of calcium during anoxia protects the tissue against irreversible transmission damage. Thus both of these factors participate in the generation of this damage. It is not yet clear if they act independently or if one acts by altering the other.

5. In the post-anoxic recovery period the intracellular concentration of potassium is reduced by about 25 %. However, it is still much higher than in slices that show only partial block of the evoked response when treated with ouabain. Therefore a fall in intracellular potassium 1 h after anoxia cannot explain the lack of recovery of the evoked response in adult tissue.

6. ATP levels in the post-anoxic recovery period are reduced from their pre-anoxic levels (9·7 *vs.* 13·9 nM/mg protein). However when azide or antimycin A are used to directly reduce ATP to the level found 1 h after anoxia the evoked response is reduced by only about 45 %. Thus the reduced post-anoxic ATP levels are not sufficient to explain the loss of the evoked response in adult tissue.

7. The data show that the irreversible loss of transmission is not due to decreased cell ATP or to decreased cell K/Na levels 1 h after the anoxic period.

8. Since creatine pre-incubation protects against irreversible transmission loss this compound or one closely related to it may prove useful in attenuating irreversible brain damage *in situ*.

INTRODUCTION

An interruption of oxidative energy metabolism in the brain can lead to the irreversible loss of brain function. The effects of hypoxia and ischaemia, two states that interrupt energy metabolism, have been extensively examined *in vivo* (Siesjo, 1981); however, the cause of irreversible damage remains unclear. Workers have postulated that reduced ATP levels (Ridge, 1971), reduced intracellular pH, (Myers, 1979; Rehncrona, Rosen & Siesjo, 1981) increased intracellular calcium (Hass, 1981), increased free fatty acids (Gardiner, Nilsson, Rehncrona & Siesjo, 1981) and altered free radical concentrations (Chan & Fishman, 1980) during or shortly after anoxia lead to the damage. No electrophysiological basis for the post-anoxic absence of transmission has been determined. One reason that it has been difficult to define the neuronal mechanisms involved in irreversible damage is that studies have generally correlated changes in whole brain metabolism with such global lesions as e.e.g. abnormalities, coma or gross functional deficits. In addition, *in vivo* it is difficult to separate irreversible damage to the circulatory system from damage to the neuronal tissue (Ames, Wright, Kowada, Thurston & Majno, 1968).

In the present study we have established the *in vitro* hippocampal slice as a model system for studying irreversible anoxic damage. We have studied transmission between the perforant bath and the dentate granule cells and have correlated irreversible inhibition of this pathway with metabolic and ionic changes in the tissue.

Our studies suggest that both a decreased ATP level and an increased cytosolic calcium level during anoxia lead to irreversible damage in tissue from adult rats. In addition they suggest that the absence of neural transmission following the anoxic exposure cannot be explained by a simple mechanism in which tissue ATP levels are decreased and the membranes depolarized by a resultant decrease in cell K/Na.

METHODS

Tissue preparation. White male Holtzman rats (110–130 d old) were decapitated and the brain was removed and placed in chilled (3 °C) buffer (for composition, see below). The hippocampi were then dissected free of the brain and sliced free-hand with a razor blade transverse to the longitudinal axis of the hippocampus as described by Skrede & Westgaard (1971). The slice thickness was about 0·5 mm. The tissue was chilled throughout the procedure except for 2 min for removal of the brain and about 2 min for slicing the pre-chilled hippocampi.

Electrophysiology experiments. Slices were placed on a nylon grid and held down by a lucite bar. The slices were totally immersed in buffer within an incubation chamber (5 ml volume) and superfused at a rate of 35 ml/min. The composition of the standard buffer was (mM): NaCl, 126; KCl, 4·0; KH_2PO_4, 1·4; $MgSO_4$, 1·3; $CaCl_2$, 2·4; $NaHCO_3$, 26; and glucose, 4; pH 7·4 at 37 ± 1 °C. The buffer was aerated with 95 % O_2–5 % CO_2 so that the oxygen saturation in the perfusion chamber was about 80 %.

The slices were allowed to recover for at least 1 h prior to placement of the recording and stimulating electrodes. Axons of the perforant path were activated with a tungsten bipolar electrode while extracellular recordings were made from the dentate granule cell layer with a 1–3 MΩ tungsten micro-electrode. The response recorded is called a population spike and represents the summed action potentials of many granule cells (Dudek, Deadwyler, Cotman & Lynch, 1976).

When slices were made 'anoxic' this was done by superfusing with buffer equilibrated with 95 % N_2–5 % CO_2 for 10 min; oxygen saturation in the chamber falls to 5 % in 60 s. These slices were then allowed to recover for 1 h in the normal buffer saturated with 95 % O_2–5 % CO_2. The percentage recovery was calculated as the size of the population spike after 1 h recovery divided by the size

of the population spike before anoxia, all $\times 100$. The size of the population spike was determined to be the mean amplitude of the negative- and positive-going potentials as described by Teyler & Alger (1976).

Biochemical studies: The slices were cut as described in the electrophysiological experiments. However, instead of being placed on a grid and superfused they were placed free floating in a vial with 10 ml of buffer, kept at 37 °C and gased with 95 % O_2–5 % CO_2. Anoxia was induced by bubbling the vial with 95 % N_2–5 % CO_2; the oxygen saturation fell to 4 % in 60 s. At the end of an experiment slices were removed from the vials, placed on a plastic screen and frozen on a metal spatula by placing the spatula in liquid nitrogen. The total time for the freezing process was 15 s. The tissue was stored in liquid nitrogen for less than 1 week. Metabolites were extracted from the slice by homogenizing in 3 N-ice cold perchloric acid as described by Lowry, Passonneau, Hasselberger & Schultz (1964). The pellet was analysed for protein (Lowry, Rosebrough, Farr & Randall, 1952). The metabolites were analysed using standard spectrofluormetric techniques (Lowry & Passonneau, 1972). All enzymes were from Boehringer–Manheim; all other chemicals were from Sigma.

pH measurements. The pH during anoxia was calculated by measuring the concentration of the reactants in the creatine kinase-catalysed reaction. This has been described by Rose (1968) and MacMillan & Siesjo (1972)

$$[H^+] = \frac{[ATP][Cr]}{[PCr][ADP]} K', \tag{1}$$

where K' is the apparent equilibrium constant. Thus pH may be calculated from measurements of ATP, creatine, phosphocreatine and ADP. K' was determined by calculating the steady-state H ion concentration from the distribution of the weak acid DMO (Hinke & Menard, 1976; Roos & Boron, 1981) and substituting this value into eqn. (1).

The value we found for K' in adult rat hippocampal slices was $2{\cdot}8 \times 10^{-8}$; this compares with a value of $7{\cdot}09 \times 10^{-9}$ calculated from *in vivo* data (MacMillan & Siesjo, 1972). It was the same both before and 1 h after the anoxic exposure. We assumed that K' remains unchanged during anoxia and calculated the pH during anoxia from metabolic measurements by substituting into eqn. (1).

In order to measure the pH by the DMO distribution method 0·5 μCi of [^{14}C]DMO and 10 μCi of [^{3}H]inulin (NEN) was added to each vial for 60 min. The slices were then removed and blotted dry. Tissue dry weights and wet weights were determined by drying the tissue for 18 h at 90 °C. The tissue was then extracted in 0·1 N-HNO_3 for 12 h. ^{3}H and ^{14}C were determined in the tissue extract and the bathing medium. Tissue pH (pH_i) was calculated from the following eqn. (Hinke & Menard, 1976):

$$\mathrm{pHi} = \mathrm{pK}' + \log \left[\left(\frac{H_m V_t}{C_t} - 1\right)^{-1} \left(\frac{H_m C_t}{C_m H_t} - 1\right)\left((10^{(\mathrm{pH_e} - \mathrm{pK}'')}) + 1\right) - 1\right] \tag{2}$$

where H_m, d.p.m. inulin/μl media; V_t, total tissue H_2O; C_t, total tissue d.p.m. DMO; C_m, d.p.m. DMO/μl media; H_t, total tissue d.p.m. inulin $\times 0{\cdot}82$; pH_e, pH of incubation medium; pK″, pK of DMO = 6·13.

Extracellular water was calculated as 82 % of the inulin space since it can be shown that 18 % of the inulin is not freely exchangable and is therefore not truly extracellular (Lipton & Heimbach, 1978). The concentrations of sodium and potassium were measured on the same tissue, as described previously (Lipton & Heimbach, 1978). Dry tissue was weighed and extracted as described above. Sodium and potassium were determined in the extract and buffer using flame photometry.

RESULTS

We have investigated the cellular nature of irreversible brain damage using the *in vitro* hippocampal slice preparation. The population spike recorded in the dentate gyrus of adult rats is blocked after 163 ± 10 s of anoxia. 1 h following a 10 min exposure to anoxia the response recovers to only 5 ± 2 % ($n = 26$) of its original amplitude. This is shown in Fig. 1 for a single experiment. We did not test the

response beyond 3 h; however there was no change in the response between 45 min and 3 h after anoxia.

Role of ATP. As shown in Fig. 2 ATP fell dramatically during the anoxic period. To examine whether this fall during anoxia was responsible for irreversible damage we searched for a mechanism to maintain ATP during anoxia.

Previous studies by Whittingham & Lipton (1981) showed that when 25 mM-creatine was included in the perfusate of guinea-pig hippocampal slices the intracellular

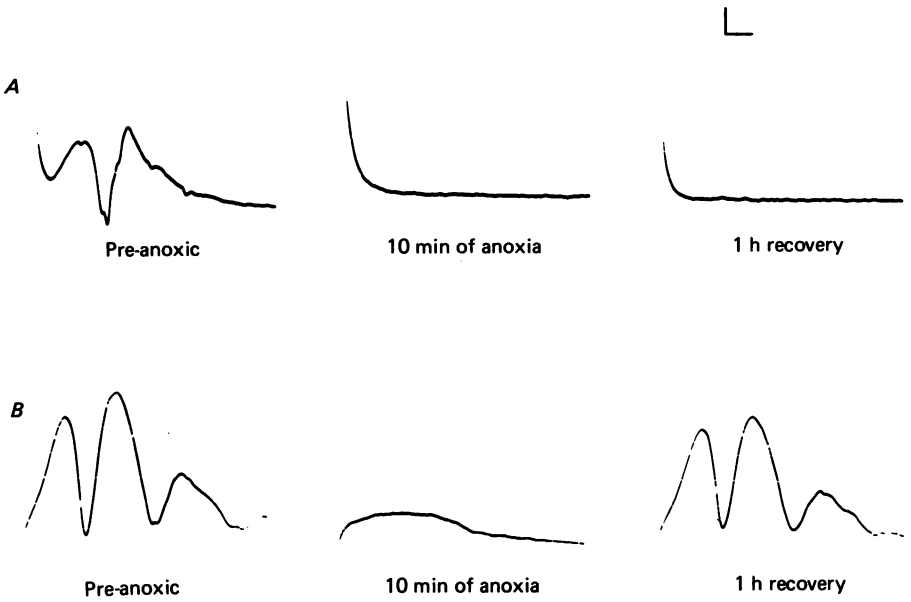

Fig. 1. The effect of anoxia on the evoked response. Responses are recorded in the dentate granule cell layer following stimulation of the perforant path. The responses are shown before, during, and 1 h subsequent to 10 min of anoxia. Scale 200 μV, 1 ms. Positive up in these and all subsequent records. *A*, untreated hippocampal slices. *B*, hippocampal slices were treated for 2 h with 25 mM-creatine and then returned to normal Ringer solution for one half hour before the pre-anoxic response that is shown.

phosphocreatine levels were dramatically increased; this was associated with a prolonged maintenance of tissue ATP concentrations during anoxia (Lipton & Whittingham, 1982). In order to test whether maintenance of ATP levels during anoxia could protect against irreversible damage we pre-incubated the hippocampal slices in 25 mM-creatine for 2 h. Following this, the creatine was removed for 1 h. This did, indeed, lead to a prolonged maintenance of ATP concentrations during anoxia. This is shown in Fig. 2. The ATP concentration during anoxia falls by only 45% compared with a 75% drop in slices which were not exposed to creatine. This protective effect of creatine is correlated with dramatically increased levels of phosphocreatine (PCr) prior to anoxia (124 ± 2 *vs.* 29 ± 1 nM/mg protein). There is a large fall in the PCr to 27 ± 2 nM/mg protein during the anoxic period. Not only does creatine incubation reduce the fall in ATP during anoxia, but it allows a greater

recovery of ATP 1 h following anoxia. This is also shown in Fig. 2. Final levels of ATP are 12 nM/mg protein, compared with 9·6 nM/mg protein in untreated slices.

We next tested the ability of creatine incubation to protect the evoked response against irreversible damage following anoxia. The rate of fall of the evoked response following creatine incubation was far slower than in the control tissue, thus the response is not abolished until more than 6 min after the onset of anoxia (383 ± 62 s). As shown for one experiment in Fig. 1 creatine incubation strongly protects against

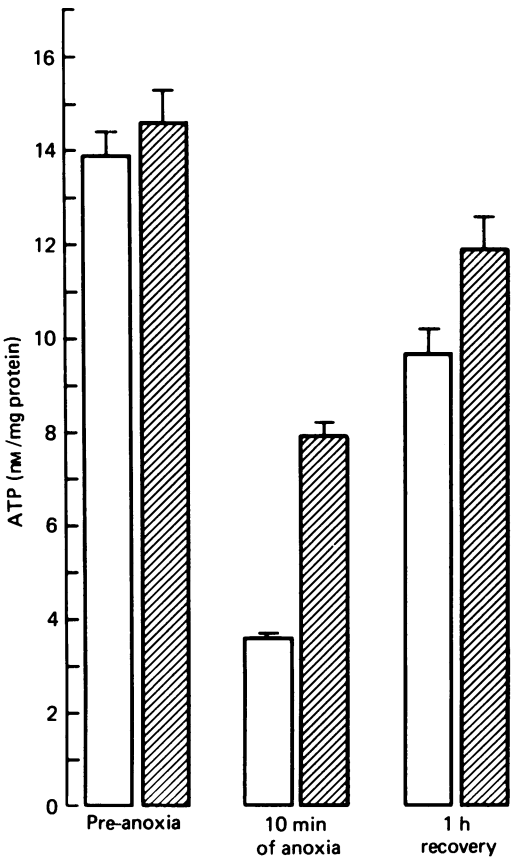

Fig. 2. Concentration of ATP (nM/mg protein) before, at the end of, and 1 h after 10 min of anoxia. Open bars, untreated hippocampal slices; hatched bars, hippocampal slices treated for 2 h with 25 mM-creatine. $n = 6$ for all groups. Bars are ±S.E. of mean.

irreversible damage; the response recovers to 82 ± 4% ($n = 8$) of its original value. These experiments suggest that the fall in tissue ATP during anoxia is associated with the production of irreversible damage.

The 'energy charge' in a cell is thought to have a profound effect on anabolic processes (Atkinson, 1977). Table 1 shows that, although comprised during anoxia, the energy charge returns close to control values for both groups 1 h after anoxia. Thus, irreversible damage does not appear to be due to reduced energy charge levels.

Role of pH and calcium. The change in tissue pH was measured during and after anoxia to see if there was a correlation between changes in intracellular acidity and the irreversible damage to the evoked response. There results are shown in Fig. 3; it is clear that there is no difference between the populations either during anoxia or upon recovery of the response 1 h following anoxia. For both groups, intracellular pH

TABLE 1. Metabolite parameters during and 60 min following anoxia in rat hippocampal slices

Untreated	Pre-anoxia	10 min of anoxia	1 h recovery from anoxia
PCr/ATP	2·12±0·05	0·92±0·06	2·32±0·10
Sum of adenylates	19·0±0·6†	12·8±0·4	14·9±0·7†
Energy charge	0·85±0·01	0·44±0·01	0·80±0·01
Creatine-treated			
PCr/ATP	8·65±0·48	3·34±0·16	9·34±0·60
Sum of adenylates	20·8±0·6*	17·2±0·3	18·0±0·6*
Energy charge	0·83±0·01	0·63±0·02	0·81±0·02

Hippocampal slices from rats were incubated in vials containing oxygenated buffer. Slices from some vials were frozen (Pre-anoxia); the remaining slices were subjected to 10 min of anoxia, then some of these slices were frozen (anoxia); the remaining slices were incubated in oxygenated buffer for 1 h and then frozen (post anoxia). Creatine-treated slices were incubated with creatine for 2 h before the experiment was begun. Sum of adenylates = ATP + ADP + AMP.

$$\text{Energy charge} = \frac{\text{ATP} + 0{\cdot}5\ \text{ADP}}{\text{ATP} + \text{ADP} + \text{AMP}}.$$

Six experiments for each value, values are ±S.E. of mean. Significance of difference determined using Students t test. * $P < 0{\cdot}05$, † $P < 0{\cdot}025$.

returns to its control values during the recovery period. Thus, changes in tissue pH are not correlated with the degree of irreversible damage. We next examined whether calcium entry from the extracellular fluid during anoxia might contribute to irreversible damage in the hippocampal slice. Hippocampal slices were bathed in buffer containing 0 mM-Ca and 10 mM-Mg during the 10 min of anoxia. As shown in Fig. 4 for one experiment these slices exhibited dramatically improved recovery 1 h following anoxia. The mean recovery for six experiments was 66 ± 10 %; this compares with a 5 ± 2 % recovery in control slices.

Site of block of transmission. In one favourable preparation we were able to record both the presynaptic and post-synaptic potentials from the adult hippocampal slice (the presynaptic spike is, in general, difficult to record from the dentate gyrus). This is shown in Fig. 5. Low calcium buffers block synaptic transmission (Katz & Miledi, 1967) and we used a short exposure to 0 Ca/10 mM-Mg buffer to demonstrate that the early potential was, indeed, presynaptic. It remained, while the later response was completely inhibited. We then exposed the slice to 10 min of anoxia in normal buffer; the presynaptic response was completely blocked a few seconds after the post-synaptic population spike. When the slices were allowed to recover in normal oxygenated buffer the post-synaptic response showed no recovery while the presynaptic response recovered rapidly and completely. Thus it appears that impulse propagation towards the nerve terminals is not irreversibly blocked by anoxia.

Reduced ATP levels during recovery. We explored the basis for the decrease in ATP during the recovery period. The PCr/ATP ratio is particularly sensitive to any disturbances in the rate of oxidative energy metabolism: small reductions in the rate of oxidative phosphorylation lead to a fall in this ratio (see Table 3 for example). However, as is shown in Table 1, the PCr/ATP ratios in the post-recovery periods

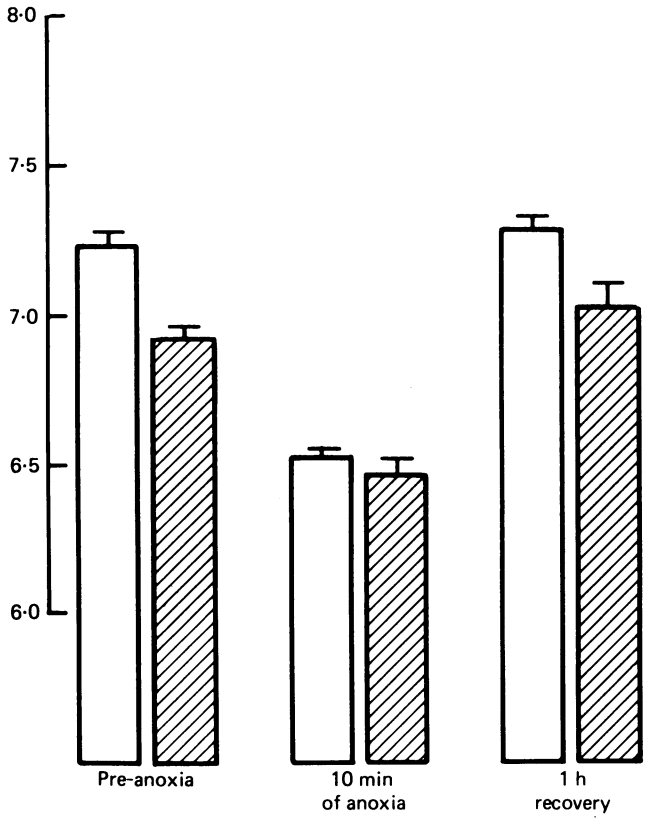

Fig. 3. The effect of anoxia on the intracellular pH of rat hippocampal slices. pH was calculated from measurements of metabolites and substituted into the equation for the creatine kinase equilibrium reaction; the equilibrium constant for this reaction was calculated from the H^+ concentration in control tissue calculated from the distribution of DMO (See Methods). Open bars, pH of untreated tissue; hatched bars, pH from creatine treated tissue, $n = 6$ for all groups. Bars are $\pm$S.E. of mean.

are, in fact, slightly higher than they are in the control periods. This suggests that mitochondrial damage is not the basis for the reduced ATP levels. Also shown in Table 1 is the value for total adenine nucleotides. They are reduced in both groups during the recovery period and the percentage fall is very similar to the percentage fall in ATP. Thus the fall in total nucleotides is reduced in the creatine-treated slices.

Ion changes. The data demonstrate that the irreversible failure of transmission is associated with a decreased tissue ATP with respect to slices in which there is no such

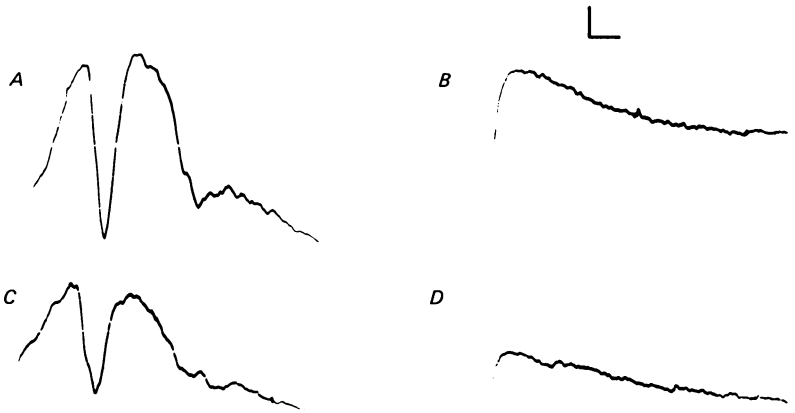

Fig. 4. The effect of removal of extracellular calcium during anoxia on the recovery of the evoked responses. Scale: 100 μV, 1 ms. *A*, the evoked response recorded from the granule cell layer of a hippocampal slice perfused with normal buffer. *B*, the evoked response during anoxia. The slice is perfused with 0 mM-Ca^{2+}–10 mM Mg^{2+} Ringer solution (all other ions as in standard buffer) during the 10 min of anoxia. *C*, the evoked response following 1 h re-perfusion with normal buffer. *D*, the evoked response 1 h following a subsequent 10 min exposure to anoxia in the presence of normal buffer.

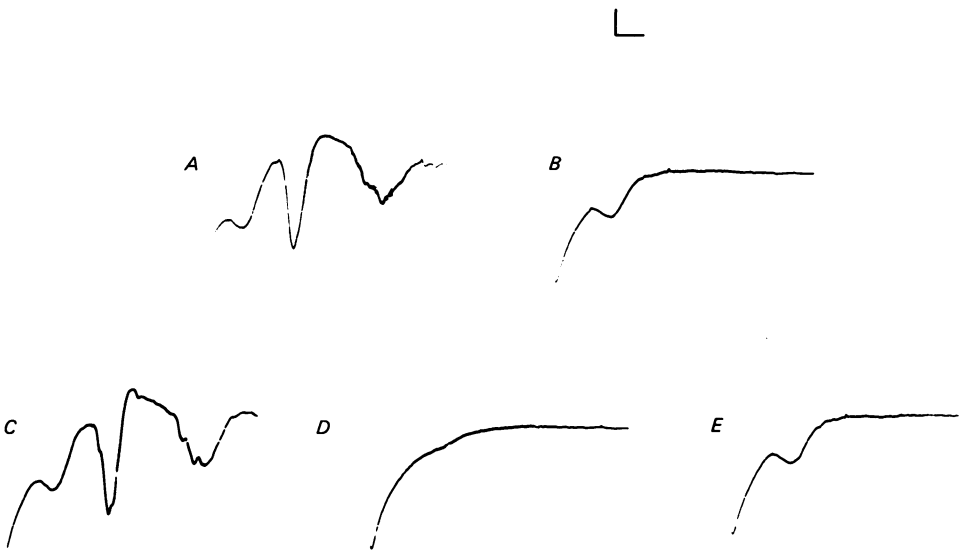

Fig. 5. Effect of anoxia on presynaptic and post-synaptic responses. Scale 200 μV, 1 ms. *A*, the evoked response recorded from the granule cell layer of a hippocampal slice perfused with normal Ringer solution. *B*, the evoked response in the presence of 0 mM-Ca^{2+}–10 mM Mg^{2+} Ringer solution (all other ions as in standard buffer). *C*, the evoked response 30 min after the slice is re-perfused with normal buffer. *D*, the loss of the evoked response during 10 min of anoxia in normal buffer. *E*, the evoked response in normal buffer 1 h after the 10 min of anoxia.

failure of transmission. One basis for the failure of transmission, then, might be that the lowered ATP leads to reduced Na/K ion pumping, subsequent membrane depolarization and conduction block (Lipton & Whittingham, 1979). We explored this hypothesis.

We measured cell sodium and potassium concentrations during anoxia and 1 h following anoxia. The results are shown in Table 2. In all preparations cell potassium

TABLE 2. Ion concentrations during and 60 min following anoxia in rat hippocampal slices

Untreated	Pre-anoxia	10 min of anoxia	1 h recovery from anoxia
Sodium concentration	73±5	132±4	93±9
Potassium concentration	113±6	56±2	82±6
Intracellular water / Dry weight	3·50±0·20	3·80±0·16	4·16±0·24
n	10	7	8
Creatine-treated			
Sodium concentration	74±3	93±2	95±2
Potassium concentration	117±4	85±3	102±5
Intracellular water / Dry weight	4·07±0·09	4·10±0·10	3·97±0·14
n	6	6	6

Procedures as detailed in the legend for Table 1 except the slices were blotted and oven dried instead of frozen. Values are ±S.E. of mean.

is lowered and cell sodium increased 1 h following anoxia. However, comparing creatine-treated with untreated slices it is apparent that creatine treatment greatly attenuates the decrease in cell potassium; the increase in cell sodium is not reduced. Qualitatively, then, these data are consistent with the conclusion that creatine incubation protects against irreversible damage by alleviating membrane depolarization. It is interesting to note how much smaller the decrease in K/Na during anoxia is in the creatine-treated tissue than it is in the untreated slices. This is quite probably related to the much reduced fall in ATP in that tissue and, hence, a smaller inhibition of the Na–K pump. The non-creatine-treated tissue shows a large net gain of ions during and following anoxia, with resultant cell swelling.

Basis for transmission block 1 h after anoxia. The decrease in K/Na and resultant depolarization is a possible basis for the loss of transmission. We wanted to test this possibility more rigorously. To do this, we compared ion changes in the recovery period with ion changes associated with inhibition of transmission by ouabain. Ouabain almost undoubtedly blocks transmission via pump-inhibition and resultant depolarization. The results are shown in Table 3. A concentration of ouabain which blocks transmission by only 40% is associated with a far greater fall in cell K/Na than that which occurs when transmission is completely inhibited 1 h following recovery from anoxia. Complete inhibition by ouabain is associated with an even larger decrease in K/Na. Thus, the decrease in K/Na associated with irreversible transmission failure seems far too small to account for the inhibition of the response.

We addressed the question as to whether the reduction in ATP observed in the

adult slices 1 h after anoxia was adequate to explain the loss of transmission. The tissue was exposed to sodium azide, to inhibit partially mitochondrial oxidative phosphorylation and so reduce ATP levels. We measured the decrease in the evoked response and in cell ATP levels after 30 min; we also measured ion concentrations. The results are shown in Table 3. It is apparent that 1 mM-azide reduces ATP by the same amount as does the 10 min anoxia and 1 h recovery. Yet, the response is

TABLE 3. Effect of sodium azide, antimycin A and ouabain on high energy phosphate ion concentrations in the rat hippocampal slice

	ATP (nM/mg protein)	PCr (nM/mg protein)	K^+ (mM)	Na^+ (mM)	% of control population spike
Control (no anoxia)	13·4±0·4	29·2±2·2	113±6	73±5	—
1 mM-azide	9·4±0·8	11·9±1·3	105±7	72±8	56±13
2 mM-azide	5·5±0·7	2·8±1·0	57±4	114±4	2±2
0·4 μg/ml antimycin A	6·9±0·3	4·7±0·5	—	—	15±6
1×10^{-6}M-ouabain	—	—	54±3	143±5	59±8
3×10^{-6}M-ouabain	—	—	38±2	152±3	0

Experiments in which metabolites and ions were measured were performed in vials as detailed in Tables 1 and 2. Values are for six experiments and each value is ±S.E of mean. The population spike was measured in the superfusion chamber. All measurements were made 60 min after beginning the experimental treatment except in the case of antimycin A. In that case measurements were made 40 min after addition of the drug.

only attenuated by 45% in the presence of azide compared with 95% after the anoxic exposure. Indeed, 2 mM-azide is required to abolish the response and this lowers ATP by twice the amount associated with the irreversible block following 10 min of anoxia. This suggests that reduced ATP during recovery does not completely account for the irreversible transmission block. It is notable that there is little ion change associated with inhibition by 1 mM-azide; this suggests that transmission block due to inhibition of energy metabolism is not simply a result of inhibition of the Na–K pump. In order to confirm that the effect of azide was due to mitochondrial inhibition we tested another well known inhibitor of electron transport. Antimycin A (0·4 μg/ml) was added to the buffer; after 40 min the physiological response declined to about 15% of its control level and ATP concentrations were again far lower than in the tissue irreversibly damaged by anoxia. This is also demonstrated in Table 3.

DISCUSSION

We have established a model for studying the irreversible loss of function in the brain subsequent to anoxia. Others have demonstrated irreversible functional (Glass, Snyder & Webster, 1941), morphological (Kalimo, Rehncrona, Soderfeldt, Olsson & Siesjo, 1981) and biochemical (Dienel, Pulsinelli & Duffy, 1980) changes in the brain *in situ* subsequent to an interruption of brain energy metabolism. We have examined the evoked population spike recorded from granule cells of the hippocampal slice and

used this as an indication of brain function. This population spike is eliminated for at least 3 h following a 10 min exposure of the slice to anoxia; we consider this an irreversible functional loss although we have not examined events at times greater than 3 h subsequent to anoxia.

The population spike can be almost completely protected against irreversible damage by pre-exposure of the tissue to 25 mM-creatine. Acute effects of anoxia have similarly been attenuated by creatine incubation; Whittingham & Lipton (1981) showed prolonged maintenance of the evoked response during anoxia with pre-exposure to creatine. These results along with our own suggest that creatine might be useful for protecting brain function *in situ*; however, it is not clear whether it can be accumulated sufficiently by the brain of intact organisms. The creatine analogue, cyclocreatine, can readily accumulate in brain tissue *in situ* and it does slow the fall in high energy metabolites during ischaemia (Woznicki & Walker, 1980); however, no functional tests have been reported. The basis of the protection afforded by creatine is probably its conversion to phosphocreatine and subsequent maintenance of ATP levels during the anoxic period; it more than doubled the level of ATP observed at the end of a 10 min anoxic period. Indeed, one of the most dramatic effects of anoxia is the rapid fall in ATP and phosphocreatine (Ridge, 1971). The fact that pre-incubation in creatine was able to maintain ATP levels during anoxia and also protected the slices against irreversible loss of the evoked response strongly suggests that this loss of ATP is a factor leading to irreversible damage.

A fall in pH is associated with an interruption of oxidative metabolism; however we have been unable to show an independent correlation between the intracellular pH during anoxia and irreversible damage. Thus, pH falls to the same level in control tissue and tissue which has been exposed to creatine while the degree of damage is almost nil in the latter. Recent studies have shown a correlation *in situ* between severe lactic acidosis and irreversible damage during ischaemia or oligaemia (Myers, 1979; Rehncrona *et al.* 1981). Lactate accumulation in these studies is between 20 and 30 mM, this will produce a fall in tissue pH to values as low as 6·4 to 6·0 (Siesjo, 1978). Irreversible damage occurs in the present studies at higher levels of pH. Another possible explanation for the difference between our conclusions and those cited above is that the large acidosis may well be acting on the vasculature to cause localized oedema. Also, localized lactate build-up may be leading to damage in the *in situ* studies; this will not occur in our study because of the high rate of tissue superfusion. Siesjo (1981) has pointed out that there is, in general, no good correlation between intracellular pH changes and irreversible brain damage in different conditions of metabolic stress.

Harris, Symon, Branston & Bayhan (1981) demonstrated massive Ca influx into brain tissue during *in vivo* ischaemia. High cytosolic Ca levels recently have been postulated to trigger ischaemic damage in both brain (Hass, 1981; Siesjo, 1981) and heart tissue (Hearse, 1977). Our results support this hypothesis. When the influx of Ca during anoxia is prevented by incubating in 0 mM-Ca/10 mM-Mg buffer irreversible damage is markedly reduced. Calcium is normally removed from the cell by a Na–Ca exchange that depends on the sodium gradient (Baker, 1969) and by a calcium pump that depends on ATP (Sorenson & Mahler, 1981). The reduced ATP levels during anoxia will thus impair Ca removal and the increased Na may actually lead to a net

Ca influx via the reversible Na–Ca exchange mechanism (Mullins & Requena, 1981). Additionally, the depolarization of the cell during anoxia might lead to the opening of Ca channels (Fishman & Spector, 1981) allowing further influx of calcium. Indeed, the maintenance of high K–Na during anoxia may well be the mechanism by which creatine incubation protects against irreversible damage. Increased Ca levels have been shown to activate proteases (Pant & Gainer, 1980) and phospholipases (Derksen & Cohen, 1975) which could lead to irreversible cell damage.

We examined cell metabolites and ion levels 1 h after anoxia to see if they could explain the absence of the evoked potential in the recovery period. Both ATP levels and the K–Na ratio show incomplete recovery from anoxia; with creatine pre-treatment these values return more closely to control levels. The reduced levels of ATP correlate with a loss of total adenine nucleotides; this is very probably due to a loss of adenosine from the tissue during anoxia; a similar phenomenon has been observed *in vivo* (Ridge, 1971). Thus, the increase in AMP during anoxia could lead to the production of adenosine which would leak from the cell.

The decreased ATP 1 h after anoxia could affect the K–Na ratio by reducing the activity of the Na–K pump. The reduced K–Na would lead to a depolarization which might be sufficient to block transmission. However, it appears that this mechanism is not, in fact, responsible for the irreversible signal loss. We blocked the pump directly with ouabain; the minimal concentration of ouabain that completely blocks the evoked response causes a far more profound reduction in K–Na than is found 1 h after anoxia. We made an estimate of the membrane potential associated with the different treatments. Using a P_{Na}/P_K of 0·02, a ratio determined for olfactory cortex cells *in vitro* (Scholfield, 1978), and ignoring chloride, the control membrane diffusion potential (Goldman, 1943) is 70 mV. The calculated depolarization with ouabain is 25 mV while the depolarization 1 h after anoxia is only 7 mV. Using 1 mM-azide we reduced ATP to the level found 1 h after anoxia; this fall in ATP only reduced the evoked response by 50 % compared with the 95 % inhibition seen during the recovery period. Thus, the reduced ATP levels during this period do not appear sufficient to explain the irreversible blockage of transmission. This inference was confirmed by our studies with antimycin A. The dependence of the evoked response on ATP levels in our studies is similar to that seen by Yamamoto & Kurokawa (1970) in the guinea-pig olfactory cortex.

In addition to these conclusions the studies with azide demonstrate that reduced oxidative metabolism can affect transmission independently of an effect on potassium and sodium balance. Thus, K–Na ratios are virtually unchanged when the tissue is exposed to 1 mM-azide in spite of the 50 % fall in the evoked potential.

Our results, then, suggest that during anoxia both an influx of calcium and a decrease in cell ATP contribute to irreversible damage. It is possible that the lowered ATP is important because it allows an increased influx of calcium. We postulate that the high calcium may lead to irreversible damage by activating the catabolism of macromolecules. The end result of this process is to inhibit transmission in a manner other than via depolarization-induced conduction block.

The research was supported by NIH grant NS-15878 and fellowship 5F32 NS06316 from NIH to Ira S. Kass. We acknowledge with pleasure the technical assistance of Ms Karen Robacker and the secretarial assistance of Ms Carrie Poole.

REFERENCES

AMES, A., WRIGHT, R. L., KOWADA, M., THURSTON, J. M. & MAJNO, G. (1968). Cerebral ischemia. II. The no-reflow phenomenon. *Am. J. Pathol.* **53**, 437–453.

ATKINSON, D. E. (1977). *Cellular Energy Metabolism and its Regulation*. New York: Academic Press, Inc.

BAKER, P. F. (1969). Sodium-calcium exchange across the nerve cell membrane. In *Calcium and Cell Function*, ed. Cuthbert, A. W., New York: St. Martin's Press.

CHAN, P. H. & FISHMAN, R. A. (1980). Transient formation of superoxide radicals in polyunsaturated fatty acid-induced brain swelling. *J. Neurochem.* **35**, 1004–1007.

DERKSEN, A. & COHEN, P. (1975). Pattern of fatty acid release from endogenous substrates by human platelet homogenates and membranes. *J. biol. Chem.* **250**, 9342–9347.

DIENEL, G. A., PULSINELLI, W. A. & DUFFY, T. E. (1980). Regional protein synthesis in rat brain following acute hemispheric ischemia. *J. Neurochem.* **35**, 1216–1226.

DUDEK, F. E., DEADWYLER, S. A., COTMAN, C. W. & LYNCH, G. (1976). Intracellular responses from granule cell layer in slices of rat hippocampus: perforant path synapse. *J. Neurophysiol.* **39**, 384–393.

FISHMAN, M. C. & SPECTOR, I. (1981). Potassium current suppression by quinidine reveals additional calcium currents in neuroblastoma cells. *Proc. natn. Acad. Sci. U.S.A.* **78**, 5245–5249.

GARDINER, M., NILSSON, B., REHNCRONA, S. & SIESJO, B. K. (1981). Free fatty acids in the rat brain in moderate and severe hypoxia. *J. Neurochem.* **36**, 1500–1505.

GLASS, H. G., SNYDER, F. F. & WEBSTER, E. (1941). The rate of decline and resistance to anoxia of rabbits, dogs and guinea pigs from the onset of viability to adult life. *Am. J. Physiol.* **140**, 609–615.

GOLDMAN, D. E. (1943). Potential, impedance and rectification in membranes. *J. gen. Physiol.* **27**, 37–60.

HARRIS, R. J., SYMON, L., BRANSTON, N. M. & BAYHAN, M. (1981). Changes in extracellular calcium activity in cerebral ischemia. *J. Cereb. Blood Flow Metabol.* **1**, 203–209.

HASS, W. K. (1981). Beyond cerebral blood flow, metabolism and ischemic thresholds: Examination of the role of calcium in the initiation of cerebral infarction. In *Cerebral Vascular Disease*, vol. 3, Proceedings of the 10th Salzburg Conference on Cerebral Vascular Disease, eds. MEYER, J. S., LECHNER, H., REIVICH, M., OTT, E. O. & ARABINAR, A., pp. 3–17. Amsterdam: Excerpta Medica.

HEARSE, D. J. (1977). Reperfusion of the ischemic myocardium. *J. mol. cell. Cardiol.* **9**, 605–616.

HINKE, J. A. M. & MENARD, M. R. (1976). Intracellular pH of single crustacean muscle fibres by the DMO and electrode methods during acid and alkaline conditions. *J. Physiol.* **262**, 533–552.

KALIMO, H., REHNCRONA, S., SODERFELT, B., OLSSON, Y. & SIESJO, R. K. (1981). Brain lactic acidosis and ischemic cell damage: 2. histopathology. *J. Cereb. Blood Flow Metab.* **1**, 313–327.

KATZ, B. & MILEDI, R. (1967). The timing of calcium action during neuromuscular transmission. *J. Physiol.* **189**, 535–544.

LIPTON, P. & HEIMBACH, C. J. (1978). Mechanism of extracellular potassium stimulation of protein synthesis in the *in vitro* hippocampus. *J. Neurochem.* **31**, 1299–1307.

LIPTON, P. & WHITTINGHAM, T. S. (1979). The effect of hypoxia on evoked potentials in the *in vitro* hippocampus. *J. Physiol.* **287**, 427–438.

LIPTON, P. & WHITTINGHAM, T. S. (1982). Reduced ATP concentration as a basis for synaptic transmission failure during hypoxia in the *in vitro* guinea-pig hippocampus. *J. Physiol.* **325**, 51–65.

LJUNGGREN, B., NORBERG, K. & SIESJO, B. K. (1974). Influence of tissue acidosis upon restitution of brain energy metabolism following total ischemia. *Brain Res.* **77**, 173–186.

LOWRY, O. H. & PASSONNEAU, J. V. (1972). *A Flexible System of Enzymatic Analysis*. New York: Academic.

LOWRY, O. H., PASSONNEAU, J. V., HASSELBERGER, F. X. & SCHULTZ, D. W. (1964). Effect of ischemia on known substrates and cofactors of and glycolytic pathway in the brain. *J. biol. Chem.* **239**, 18–30.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1952). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

MACMILLAN, V. & SIESJO, B. K. (1972). Intracellular pH of the brain in arterial hypoxemia evaluated with the CO_2 method and from the creatine phosphokinase equilibrium. *Scand. J. clin. Lab. Invest.* **30**, 117–125.

MULLINS, L. J. & REQUENA, J. (1981). The late calcium channel in squid axon. *J. gen. Physiol.* **78**, 683–700.

MYERS, R. E. (1979). A unitary theory of causation of anoxic and hypoxic brain pathology. In *Advances in Neurology*, vol. 26, ed. FAHN, S., DAVIS, J. N. & ROWLAND, L. P., pp. 195–215. New York: Raven Press.

PANT, H. C. & GAINER, H. (1980). Properties of calcium-activated protease in squid axoplasm which selectively degrades neurofilament proteins. *J. Neurobiol.* **11**, 1–12.

REHNCRONA, S., ROSEN, I. & SIESJO, B. K. (1981). Brain lactic acidosis and ischemic cell damage. 1. Biochemistry and Neurophysiology. *J. Cereb. Blood Flow Metabol.* **1**, 297–311.

RIDGE, J. W. (1971). Hypoxia and the energy charge of the cerebral adenylate pool. *Biochem. J.* **127**, 351–355.

ROOS, A. & BROWN, W. F. (1981). Intracellular pH. *Physiol. Rev.* **61**, 296–434.

ROSE, I. A. (1968). The state of magnesium in cells as estimated from the adenylate kinase equilibrium. *Proc. natn. Acad. Sci. U.S.A.* **61**, 1079–1086.

SCHOLFIELD, C. N. (1978). Electrical properties of neurones in the olfactory cortex slice *in vitro*. *J. Physiol.* **275**, 535–546.

SIESJO, B. K. (1978). *Brain Energy Metabolism*. New York: Wiley.

SIESJO, B. K. (1981). Cell damage in the brain: a speculative synthesis. *J. Cereb. Blood Flow Metab.* **1**, 155–185.

SKREDE, K. K. & WESTGAARD, R. H. (1971). The transverse hippocampal slice: a well-defined cortical structure maintained *in vitro*. *Brain Res.* **35**, 589–593.

SORENSEN, R. G., & MAHLER, H. R. (1981). Calcium-stimulated adenosine triphosphatases in synaptic membranes. *J. Neurochem.* **37**, 1407–1418.

TEYLER, T. J. & ALGER, B. E. (1976). Monosynaptic habituation in the vertebrate forebrain: the dentate gyrus examined *in vitro*. *Brain Res.* **115**, 413–425.

WHITTINGHAM, T. S. & LIPTON, P. (1981). Cerebral synaptic transmission during anoxia is protected by creatine. *J. Neurochem.* **37**, 1618–1621.

WOZNICKI, D. T. & WALKER, J. B. (1980). Utilization of cyclo-creatine phosphate, an analogue of creatine phosphate, by mouse brain during ischemia and its sparing action on brain energy reserves. *J. Neurochem.* **34**, 1247–1253.

YAMAMOTO, C. & KUROKAWA, M. (1970). Synaptic potentials recorded in brain slices and their modification by changes in the level of tissue ATP. *Exp. Brain Res.* **10**, 159–170.