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Neither Moderate Hypoxia nor Mild Hypoglycaemia Alone Causes Any Significant Increase in Cerebral $[Ca^{2+}]_i$: Only a Combination of the Two Insults Has This Effect. A ^{31}P and ^{19}F NMR Study

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Abstract: (1) The energy state and free intracellular calcium concentration ($[Ca^{2+}]_i$) of superfused cortical slices were measured in moderate hypoxia (~65 μM O_2), in mild hypoglycaemia (0.5 m*M* glucose), and in combinations of the two insults using ^{19}F and ^{31}P NMR spectroscopy. (2) Neither hypoxia nor hypoglycaemia alone caused any significant change in $[Ca^{2+}]_i$. Hypoxia caused a 40% fall in phosphocreatine (PCr) content but not in ATP level, and hypoglycaemia produced a slight fall in both (as expected from previous studies). These changes in the energy state recovered on return to control conditions. (3) A combined sequential insult (hypoxia, followed by hypoxia plus hypoglycaemia) produced a 100% increase in $[Ca^{2+}]_i$ and a decrease in PCr level to ~25% of control. The reverse combined sequential insult (hypoglycaemia, followed by hypoglycaemia plus hypoxia) had the same effect. On return to control conditions there was some decrease in $[Ca^{2+}]_i$ and a small increase in PCr content, but neither recovered to control levels. (4) Exposure of the tissue to the combined simultaneous insult (hypoxia plus hypoglycaemia) immediately after the control spectra had been recorded resulted in a fivefold increase in $[Ca^{2+}]_i$ and a similar decrease in PCr level to 20–25% of control. There was little if any change of $[Ca^{2+}]_i$ or PCr level on return to control conditions. (5) These results are discussed in terms of metabolic adaptation of some but not all of the cortical cells to the single type of insult, which renders the tissues less vulnerable to the combined insult. **Key Words:** Hypoxia—Hypoglycaemia—Free intracellular Ca^{2+} concentration—^{31}P NMR spectroscopy—^{19}F NMR spectroscopy.
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The dependence of the brain on its blood supply of oxygen and glucose, coupled with its short supplies of endogenous substrates, renders it particularly vulnerable to deprivation of these nutrients in hypoxia, hypoglycaemia, and ischaemia, leading ultimately to cell death. Ischaemia, whether "global," as in arterial occlusion cardiac arrest, or "focal," caused by a local blood clot or haemorrhage (as in stroke), represents a mixed biochemical insult. Not only is there deprivation of glucose and oxygen and of other essential nutrients, such as vitamins, essential amino acids, and essential fatty acids, but the cells are also unable to excrete potentially toxic metabolites such as lactate, glutamine, and ammonia. A useful strategy to examine the underlying effects on cerebral metabolism is to concentrate on individual aspects of the combined insult, such as studying hypoxia and hypoglycaemia separately. These metabolic insults are also important in the effects on cerebral function of, e.g., hypoxia resulting from lung dysfunction and the hypoglycaemia that can occur in diabetes.

Current hypotheses on the events leading to cell death in ischaemia focus on the excitotoxic effect of the neurotransmitter glutamate and, more specifically, the role of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor, which regulates a magnesium-gated calcium channel. It is suggested that the excess glutamate released on ischaemia reacts with the NMDA receptor, thus allowing the entry of calcium, which causes a cascade of metabolic events leading to cell death (see Choi, 1988; Siesjö and Bengtsson, 1989). There is good evidence for the pivotal role of an increased free intracellular calcium concentration ($[Ca^{2+}]_i$) in ischaemia, and it is often assumed that the damage caused by hypoxia and hypoglycaemia may also be due to increased $[Ca^{2+}]_i$ (see, e.g., Gibson et al., 1989; Siesjö and Bengtsson, 1989; Duchen et al., 1990).

Lee et al. (1990) observed a slow, steady increase in

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Abbreviations used: $[Ca^{2+}]_i$, free intracellular calcium concentration; 5FBAPTA, 5,5′-F_2-1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; NMDA, *N*-methyl-D-aspartate; PCr, phosphocreatine.

$[Ca^{2+}]_i$ with no detectable effect on ATP in chromaffin cells cultured for 10 min in media pregassed with 100% N_2. However, the O_2 concentration of their media (12 μM) was much lower than that in the present study (see Discussion). Most of the evidence supporting the view that hypoxic cell death is caused by an increased calcium concentration is based on the use of "chemical hypoxia," i.e., the use of metabolic poisons such as barbiturates, cyanide, iodoacetate, and rotenone. Thus, considerable increases in $[Ca^{2+}]_i$ with cyanide have been observed in synaptosomes (Gibson et al., 1989), in cultured hippocampal neurones (Dubinsky and Rothman, 1991), and transiently in chromaffin cells (Carroll et al., 1992). However, Dubinsky and Rothman (1991) could find no neurotoxicity and commented that "under our experimental conditions we found no correlation between Ca^{2+} and neuronal degeneration." Carroll et al. (1992) found no correlation between ATP depletion and $[Ca^{2+}]_i$—they observed a temporary two- to fivefold increase in $[Ca^{2+}]_i$ with little effect on viability and that massive depletion of ATP occurred at normal levels of $[Ca^{2+}]_i$. Iodoacetate also causes large increases in $[Ca^{2+}]_i$: with amobarbital in ROC-1 glial cells (Jurkowitz-Alexander et al., 1992) and with rotenone in synaptosomes (Kauppinen et al., 1988). The latter investigators commented that they found cell damage and ATP depletion to be caused by the poisons independent of changes in $[Ca^{2+}]_i$. Other workers have also reported that anoxia or metabolic poisons may cause cell death without an increase in $[Ca^{2+}]_i$ (Cheung et al., 1986; Lemasters et al., 1987; see Siesjö and Bengtsson, 1989). The use of metabolic poisons that interfere with glycolysis (barbiturates or iodoacetate) or the electron transport system (rotenone or cyanide) are often referred to as "chemical hypoxia," which is to be regretted—their effects are not analogous to true hypoxia, because they may interfere with enzymes of diverse metabolic pathways.

Scrutiny of the literature reveals that there have been very few studies on the effects of hypoglycaemia directly on $[Ca^{2+}]_i$: Uematsu et al. (1989) reported a large increase in $[Ca^{2+}]_i$ when the EEG was isoelectric from insulin-induced severe hypoglycaemia in cat brain in vivo, but Siesjö and Deshpande (1987) found no change in total tissue calcium content in analogous studies on the rat. This is not surprising in view of the very low $[Ca^{2+}]_i$ compared with the total tissue calcium level. Simon et al. (1986), using microscopy of rat hippocampus, found that severe hypoglycaemia did not produce the mitochondrial accumulation of Ca^{2+} seen in ischaemia. Therefore, although it is frequently stated that the cell damage caused by hypoxia and hypoglycaemia would be expected to involve increased $[Ca^{2+}]_i$, there is little direct evidence in support.

Although there are uncertainties in the quantitative degree of true hypoxia produced by the use of gas mixtures, this can be monitored by well-documented effects on the energy state. ATP tends to be preserved at the expense of phosphocreatine (PCr) in hypoxia, whereas levels of both fall together in hypoglycaemia (Bachelard et al., 1985). In this study we have examined the effects of hypoxia and hypoglycaemia on $[Ca^{2+}]_i$ and the energy state in intact metabolising cerebral tissue by the NMR techniques previously reported (Bachelard et al., 1988; Badar-Goffer et al., 1990*a,b*; Ben-Yoseph et al., 1990).

The results show (a) that moderate hypoxia produces an initial fall in $[Ca^{2+}]_i$, followed by a return to the normal level; (b) that mild hypoglycaemia (0.5 mM glucose) produces an "unstable" $[Ca^{2+}]_i$, in that the level oscillated about the mean control level but did not change significantly; (c) that only the combined insult of hypoxia plus hypoglycaemia causes a significant rise in $[Ca^{2+}]_i$; and (d) that sequential insults, i.e., hypoxia followed by hypoxia plus hypoglycaemia, or vice versa, were less injurious than the immediate combined insult, which has intriguing and novel implications in our understanding of metabolic adaptation in the brain.

MATERIALS AND METHODS

Treatment of tissues

Tissue slices from the guinea-pig cerebral cortex were prepared as previously described (Badar-Goffer et al., 1990*a*). After a preincubation period of 30 min in control media to restore normal levels of metabolites such as lactate, ATP, and PCr, the slices were transferred to the superfusion apparatus and placed within the NMR magnet (Bachelard et al., 1985). The control superfusion medium contained 124 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.4 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose, gassed with O_2/CO_2 (95:5) at 37°C. After control ^{19}F and ^{31}P NMR spectra had been acquired, the superfusion medium was replaced by media containing 0.5 mM glucose, by media gassed with N_2/CO_2 [95%/5%; moderate hypoxia, see Cox et al., 1983)], or by a combination of both, as indicated in the text. Further NMR spectra were then acquired.

Measurement of $[Ca^{2+}]_i$

This was performed as previously described (Bachelard et al., 1988) except that we had to modify the amount of the calcium indicator [5,5′-F_2-1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (5FBAPTA)] we preloaded into the tissues. On moving to Nottingham we built a new superfusion apparatus enabling NMR observation of ~0.8–1 g of tissue, in contrast to the earlier apparatus (described in detail in Bachelard et al., 1985), which required 3.5–4 g of tissue. When we applied the earlier loading conditions for our current experiments, the sensitivity of the ^{19}F NMR spectra was enhanced, but some impairment of the energy state (ATP and PCr) was observed in the ^{31}P NMR spectra, in comparison with spectra recorded in the absence of the indicator. We concluded that this reflected a higher concentration of indicator loaded, due to the partition coefficient between the slices and media. We therefore halved the concentration of 5FBAPTA in the loading medium, and no

deleterious effect on the energy state of the slices was then observed. Each set of experiments was performed in the absence and presence of 5FBAPTA, to ensure that the presence of the indicator was not having an adverse effect on the energy state of the tissue. The disadvantage of the lower amount of 5FBAPTA present initially in the tissues was a lower signal/noise ratio in the ^{19}F NMR spectra so that the total time available for acquisition of data was shortened from the previous time of 6 h to ~4 h [owing to slow washout of the indicator from the tissue (see Badar-Goffer et al., 1990*b*)].

For calculation of $[Ca^{2+}]_i$ we had previously used a K_D value of 708 n*M* for the dissociation constant of Ca^{2+}-5FBAPTA (Bachelard et al., 1988). However, we have recently been advised by Dr. G. A. Smith (Department of Biochemistry, Cambridge), who donated the indicator, that he has since made a more accurate measurement (Kirschenlohr et al., 1988) for the K_D (540 n*M* at 30°C). Allowing for the slight temperature dependence of the K_D (Harrison and Bers, 1987), we have used a value of 600 n*M* in these studies at 37°C.

NMR spectroscopy

NMR data were obtained using an AMX-500 Bruker wide-bore spectrometer operating at 202.46 MHz for ^{31}P and 470.51 MHz for ^{19}F. The magnetic field was shimmed using the ^1H resonance of the water of the superfusing medium to achieve a line-width usually of <5 Hz, which increased to <12 Hz in the presence of the tissues. ^{31}P spectra were accumulated as 360 transients using 60° radiofrequency pulses repeated every 2 s. ^{19}F data were accumulated in blocks of 5,000 transients using 45° radiofrequency pulses, a sweep width of 10 kHz, and an interpulse interval of 0.2 s. ^{19}F spectra were obtained by Fourier transformation using a line-broadening (exponential weighting) of 70 Hz. Values for $[Ca^{2+}]_i$ were calculated from the ratios of the areas of bound and free resonances in the ^{19}F spectra, multiplied by the dissociation constant of the 5FBAPTA (600 n*M*; see above).

RESULTS

The value for $[Ca^{2+}]_i$ of 282 ± 47 n*M* (mean ± SD) obtained from 12 separate experiments in this study (Table 1) is lower than our previously reported results (Badar-Goffer et al., 1990*b*; Ben-Yoseph et al., 1990). The ratio of Ca^{2+}-bound to free 5FBAPTA is similar to that observed previously, and the lower calculated value for $[Ca^{2+}]_i$ results from the lower value for K_D used (see Materials and Methods). It falls within the range (100–500 n*M*) reported from other studies on neuronal preparations, as previously discussed (Bachelard et al., 1988). Brooks and Aaronson (1991) subsequently reported a value of 245 n*M* from the use of fura 2 on cortical slices.

Hypoxia

Subjecting the slices to moderate hypoxic conditions resulted in an initial significant decrease in the measured $[Ca^{2+}]_i$ from 282 ± 47 to 167 ± 29 n*M* (Table 1). This can be seen in Fig. 1, where the peak of Ca^{2+}-bound 5FBAPTA is greatly reduced (c) compared with control (a) after 20–30 min of hypoxia. In some experiments when spectra were observed at earlier time points (within 10 min of hypoxia), often the bound peak of 5FBAPTA could not be detected above the noise. Subsequent spectra, taken under continued hypoxic conditions, showed an increase in $[Ca^{2+}]_i$ to values of 271 ± 49 n*M*, similar to control, and remained so for >3 h of hypoxia. This implies that a rapid fall in $[Ca^{2+}]_i$ is followed by a slow rise, which finally achieves a steady state with values similar to control.

TABLE 1. *Measurements of $[Ca^{2+}]_i$ and PCr content in guinea-pig cerebral cortical slices under conditions of low oxygen and/or low glucose*

Conditions	n	5FBAPTA	$[Ca^{2+}]_i$	PCr
Control	12	+	282 ± 47	100
Hypoxia	2	−	ND	60 ± 12[a]
Hypoxia (20 min)	5	+	167 ± 29[a]	59 ± 6[a]
Hypoxia (50 min)	4	+	271 ± 49	59 ± 6[a]
Low glucose	1	−	ND	61
Low glucose[b]	5	+	392 ± 128	73 ± 19
Combined sequential[c]				
A	1	−	ND	10
A	4	+	596 ± 164[a]	28 ± 11[a]
B	1	−	ND	24
B	3	+	541 ± 108[a]	26 ± 19[a]
Combined immediate				
C	2	+	1,340, 1,225	20, 25

Data for $[Ca^{2+}]_i$ (n*M*) and PCr (% of control) are mean ± SD values, where n is the number of individual experiments. ND, not detectable owing to the absence of indicator. For $[Ca^{2+}]_i$, the total combined sequential level (A + B, n = 7) was 572 ± 131 n*M* ($p < 0.01$).

[a] $p < 0.01$.

[b] Values fluctuated with time above and below the mean, which was not significantly different from control; hence, the SD value is larger than control or hypoxia (see text).

[c] Experiment A, hypoxia, then followed by hypoxia plus 0.5 m*M* glucose; experiment B, 0.5 m*M* glucose, then followed by 0.5 m*M* glucose plus hypoxia; and experiment C, hypoxia with 0.5 m*M* glucose directly after acquisition of control spectra.

The ^{31}P spectra showed no significant effect of hypoxia on the ATP level (Fig. 1b and d), whereas the PCr resonance was reduced to 59 ± 6% of control, almost identical to its reduction in the absence of 5FBAPTA (Table 1). This decrease in PCr content remained constant over times of up to 3 h of hypoxia, suggesting a new steady state in energy metabolism, which is in line with our previous studies. On returning the tissue to control conditions (data not shown), the PCr level recovered to 100% of control in both the presence and absence of the indicator.

Low glucose

We chose 0.5 m*M* glucose as a "threshold" for hypoglycaemia, as our previous studies had shown that this concentration produced little decrease in ATP or PCr content but had a marked effect on synaptic activ-

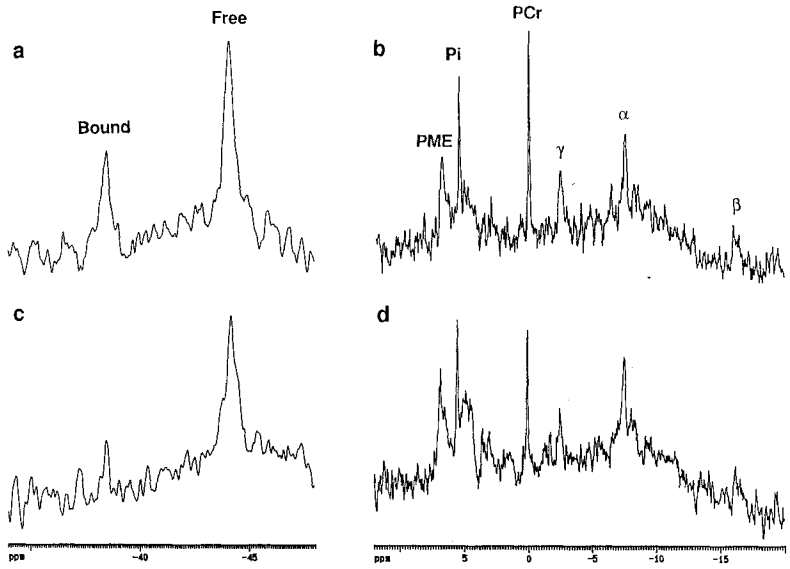

FIG. 1. ^{19}F (**a** and **c**) and ^{31}P NMR (**b** and **d**) spectra of guinea-pig cerebral cortical slices superfused in control (a and b) and hypoxic (c and d) media. The experimental conditions are described in the text. The ^{19}F NMR spectra show a decrease in $[Ca^{2+}]_i$ after 15 min of hypoxia, and the ^{31}P NMR spectra show the decrease in PCr but not ATP content after 1 h of hypoxia. PME, phosphomonoester.

ity (Cox et al., 1983; Bachelard et al., 1984, 1985). Changing from 10 (control) to 0.5 m*M* glucose resulted in a mean ± SD value for $[Ca^{2+}]_i$ of 392 ± 128 n*M* (Table 1). The large increase in the SD (compared with the control SD value of 47) reflects the scatter of results that we observed throughout the low glucose experiments, suggesting some metabolic or membrane instability. Longer times of exposure to the low glucose revealed a similar variation in the $[Ca^{2+}]_i$. The spectra of Fig. 2 show no obvious change in $[Ca^{2+}]_i$ (a and c) with a slight but inconsistent fall (b and d) in PCr content (Table 1) and in ATP level to ~75% of control. On returning the tissues to control conditions the ATP and PCr values recovered to >90% (data not shown).

Combined hypoxia and hypoglycaemia

We decided to study the combined insult, i.e., the combination of moderate hypoxia and low glucose, in three ways: experiment A, low oxygen, followed by low glucose with low oxygen (Fig. 3); experiment B, low glucose, followed by low oxygen with low glucose (Fig. 4); and experiment C, introduction of low oxygen and low glucose simultaneously immediately after control spectra had been recorded (Fig. 5). As can be seen in the spectra of Fig. 3, there was no obvious increase in $[Ca^{2+}]_i$ when the tissues were subjected to some 40 min of hypoxia alone (a and c), but when 0.5 m*M* glucose was present in the hypoxic medium (e), there was a clear increase in $[Ca^{2+}]_i$. The values of Table 1 show that this combined sequential insult

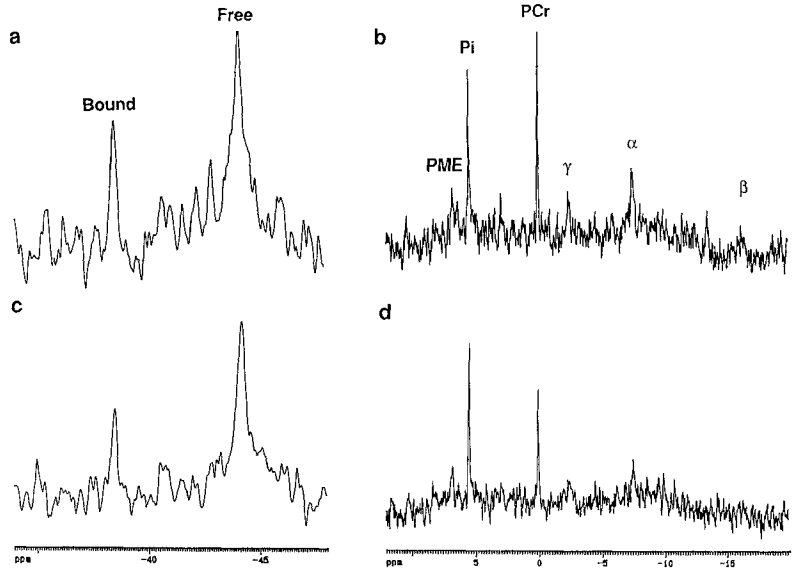

FIG. 2. ^{19}F (**a** and **c**) and ^{31}P NMR (**b** and **d**) spectra of guinea-pig cerebral cortical slices superfused in control medium (a and b) and in media containing 0.5 m*M* glucose (c and d). The experimental conditions are described in the text. The ^{19}F NMR spectra show no change in $[Ca^{2+}]_i$, and the ^{31}P NMR spectra show decreases in both PCr and ATP content in hypoglycaemia. PME, phosphomonoester.

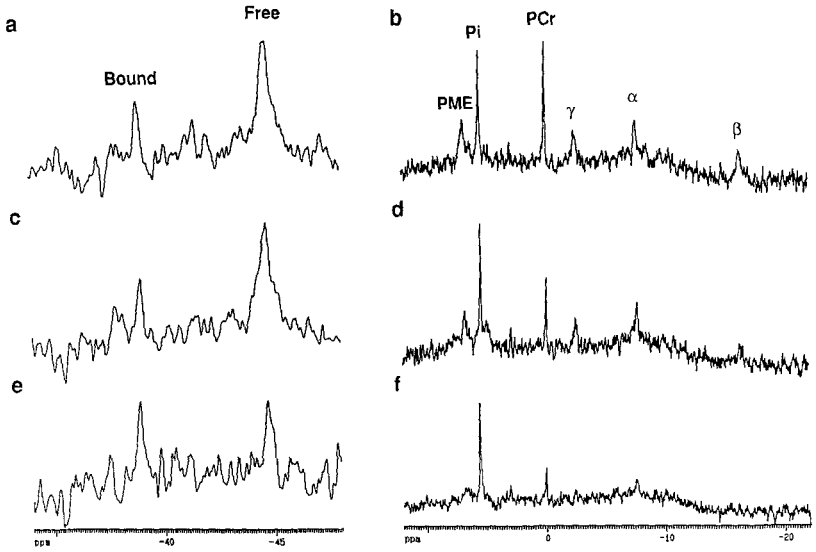

FIG. 3. ^{19}F (**a, c,** and **e**) and ^{31}P NMR (**b, d,** and **f**) spectra of guinea-pig cerebral cortical slices superfused in control media (a and b), then hypoxic media (c and d), followed by hypoxic media containing 0.5 m*M* glucose (e and f). The experimental conditions are described in the text. The ^{19}F NMR spectra show no change in $[Ca^{2+}]_i$ after 30 min of hypoxia (b) and a 100% increase after exposure for 120 min to the combined insult (e). The ^{31}P NMR spectra show the decrease in PCr but not ATP content after 50 min of hypoxia (d) with marked decreases in PCr and ATP levels after exposure for 85 min to the combined insult (f). PME, phosphomonoester.

caused a significant increase in $[Ca^{2+}]_i$ to 596 ± 164 n*M*. The ^{31}P NMR spectra of Fig. 4 confirmed the small decrease (b and d) in PCr (and not of ATP) content in hypoxia alone and that low glucose in the hypoxic medium caused a further decrease in levels of both metabolites (f). PCr content was then 25–30% of control, and the ATP resonances were lost in the noise and so could not be quantified. Reversing the procedure (experiment B) gave virtually identical results (Fig. 4) in that an increase in $[Ca^{2+}]_i$ to 541 ± 108 n*M* was only observed when the low glucose medium was rendered hypoxic. There was therefore no difference in the ultimate effect of the order in which the sequential insults were given, and the overall value of $[Ca^{2+}]_i$ for the two series of combined sequential insults (experiments A + B) was 572 ± 131 n*M*, a 100% increase above control. The data of Table 1 also confirm that the effect on PCr content was similar in the absence and presence of the calcium indicator. PCr content fell to 25–30% as a result of the combined sequential insults and recovered on return of the tissues to control media only to ~45%.

We were able to assess $[Ca^{2+}]_i$ on return to control media in only few experiments because by the time the sequential ^{19}F and ^{31}P spectra had been accumulated most of the 5FBAPTA had washed out of the tissues (see Materials and Methods). However, in those experiments where we could measure the BAPTA resonances the $[Ca^{2+}]_i$ fell slightly on return to control media but was always well above normal values.

When the tissues were subjected to the combined

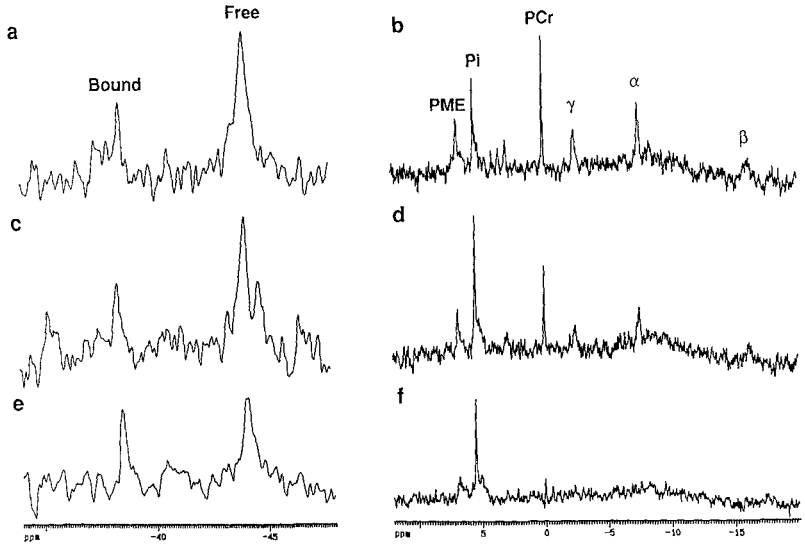

FIG. 4. ^{19}F (**a, c,** and **e**) and ^{31}P NMR (**b, d,** and **f**) spectra of guinea-pig cerebral cortical slices superfused in control media (a and b), then media containing 0.5 m*M* glucose (c and d), followed by hypoxic media containing 0.5 m*M* glucose (e and f). The experimental conditions are described in the text. The ^{19}F NMR spectra show no change in $[Ca^{2+}]_i$ after 20 min of hypoglycaemia (b) and a 100% increase after exposure for 60 min to the combined insult (e). The ^{31}P NMR spectra show the small decreases in PCr and ATP content after 25 min of hypoglycaemia (d) with marked decreases in both after exposure for 25 min to the combined insult (f). PME, phosphomonoester.

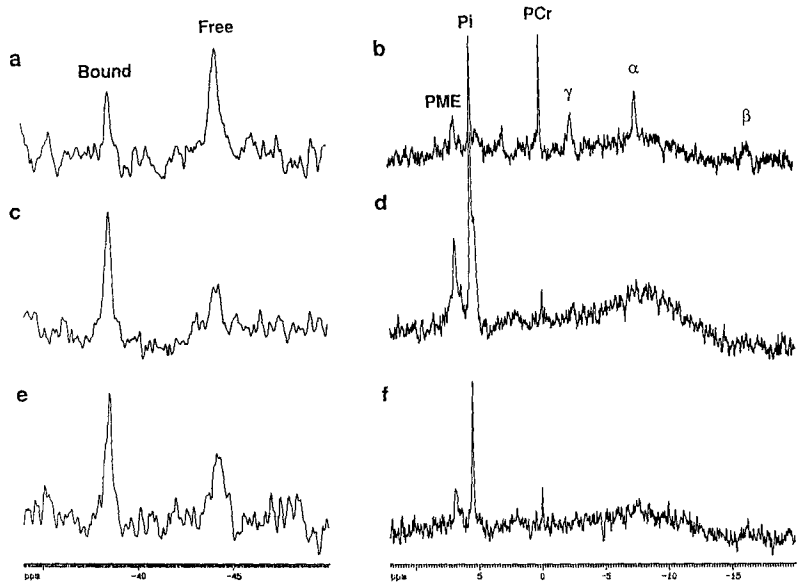

FIG. 5. ^{19}F (**a, c,** and **e**) and ^{31}P NMR (**b, d,** and **f**) spectra of guinea-pig cerebral cortical slices superfused in control media (a and b), then immediately in hypoxic media containing 0.5 m*M* glucose (c and d), and finally on return to control media (e and f). The experimental conditions are described in the text. The ^{19}F NMR spectra show a large fivefold increase in $[Ca^{2+}]_i$ after 40 min of exposure to the double insult (c) with no detectable recovery after a further 60 min in control media (e). The ^{31}P NMR spectra show the large decreases in PCr and ATP content after 1 h of exposure to the double insult (d) with a slight recovery of PCr level after a further 30 min in control media (f). PME, phosphomonoester.

insult (Fig. 5) immediately after acquisition of the control spectra (a), the measured $[Ca^{2+}]_i$ rose within the first 15 min to ~1,300 n*M* (Table 1) and remained high thereafter. The ^{31}P spectra of Fig. 5d showed a marked decrease in PCr content (to 20–25% of control) with ATP undetectable. On return to normal conditions (f) the PCr content recovered slightly to ~30% of control (similar to that reported by Kauppinen and Williams, 1990), and there was no detectable normalization of $[Ca^{2+}]_i$ towards control levels (e).

DISCUSSION

In this study we chose concentrations of glucose and O_2 that are known to interfere with synaptic electrical activity but that exert only mild effects on metabolism. The O_2 concentration of our hypoxic media was measured, using a Jenway 9015 oxygen meter, to be 65 ± 5 μM. This concentration has previously been shown to affect cell excitability, levels of PCr and lactate, and release of neurotransmitters but has little effect on the redox state as measured by the NADH/NAD^+ ratio (for review, see Bachelard, 1990). The significant decrease in $[Ca^{2+}]_i$ that was observed in the first spectrum obtained during the hypoxic insult was unexpected, as it was the first time we have detected a decrease in $[Ca^{2+}]_i$ following a metabolic perturbation. We believe it to be a real change, because not only is it statistically significantly different, but the decrease was seen in all five experiments performed. The calcium eventually returned to control levels, for which we can advance no obvious explanation because there was no decrease in ATP level [in line with previous studies on hypoxia (Cox et al., 1983)], and the fact that the PCr content did not decrease further with time suggests that a new metabolic steady state has arisen. Although Lee et al. (1990) observed a slow, steady increase in $[Ca^{2+}]_i$ in cultured chromaffin cells in the presence of 100% N_2, their measured O_2 concentration (12 μM) was much lower than ours (65 μM). They found that this level of O_2 inhibited the K^+-evoked release of catecholamines with no further increase in $[Ca^{2+}]_i$, and they suggested that the effect of the severe hypoxia was due to inhibition of calcium transport through voltage-dependent channels. In the present study, the fall in $[Ca^{2+}]_i$ may possibly be related to such an inhibition of transport.

A moderately low glucose level (0.5 m*M*) caused a slight decrease in both PCr and ATP levels and no significant change in $[Ca^{2+}]_i$. However, the calculated value for $[Ca^{2+}]_i$ was much more variable, as reflected in the much higher SD values (Table 1). During exposure to more clearly hypoglycaemic concentrations of glucose (0.2 m*M*) in earlier preliminary unpublished experiments performed in Cambridge, we had observed a similar "instability" in $[Ca^{2+}]_i$, and thought at that stage that it was due to technical difficulties. However, this variability was quite consistent in the present studies, and again we believe it to be a real result.

Only when the tissues were exposed to the combined sequential insult was there any significant increase in $[Ca^{2+}]_i$ (~200% of control), similar to the effects of K^+-induced depolarization (Bachelard et al., 1988). This increase in $[Ca^{2+}]_i$ in the combined sequential insult is relatively small compared with the large fivefold increase observed when the combined insult was immediate. This study reports the first direct measurement of $[Ca^{2+}]_i$ in moderate conditions of hypoxia and hypoglycaemia in an actively metabolising whole tissue preparation of the cerebral cortex

and suggests that neither of these two insults alone causes any significant increase in $[Ca^{2+}]_i$, which can be detected only when the tissues are exposed to both insults. The marked contrast between the sequential combined insult and the immediate combined insult on the effects on $[Ca^{2+}]_i$, and on the energy state, indicates that the tissue adapts protectively by some unknown mechanism to a hypoxic or a hypoglycaemic insult, entering a new metabolic steady state, when it is then better able to withstand further metabolic damage. The basis for this is not clear, especially as the metabolic consequences of hypoxia and of hypoglycaemia, e.g., in terms of lactate, ATP, and the NADH/NAD^+ ratio (Bachelard, 1990), are so different. This adaptation may be related to the "ischaemic preconditioning" observed in the perfused heart, where brief exposure to hypoxia (or ischaemia), followed by a short period of perfusion under normal conditions, protects the heart against subsequent exposure to the same insult (reviewed by Lawson and Downey, 1993). Evidence has also been reported for the protective effects of brief pretreatment with sublethal ischaemia against subsequent ischaemic damage to rat and gerbil hippocampal neurones (Kato et al., 1991; Liu et al., 1992).

Our results, particularly on the differential effects on PCr and ATP content caused by the variations in the insults, also suggest that a large proportion, but not all, of the cells are suffering permanent damage. It is possible also that only a proportion of the cells is capable of this metabolic adaption. These are clearly new and unexpected observations that have implications for clinical understanding and perhaps management of coma and stroke.

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REFERENCES

Bachelard H. S. (1990) The molecular basis of coma and stroke, in *The Metabolic and Molecular Basis of Acquired Disease* (Cohen R. D., Lewis B., Alberty K. J. M. M., and Denman A. M., eds), pp. 1354–1380. Bailliere Tindall, London.

Bachelard H. S., Cox D. W. G., and Drower J. (1984) Sensitivity of guinea-pig hippocampal granule cell field potentials to hexoses *in vitro:* an effect on cell excitability. *J. Physiol. (Lond.)* **352,** 91–102.

Bachelard H. S., Cox D. W. G., Feeney J., and Morris P. G. (1985) ^{31}P-Nuclear-magnetic-resonance studies on superfused cerebral tissues. *Biochem. Soc. Trans.* **13,** 835–839.

Bachelard H. S., Badar-Goffer R. S., Brooks K. J., Dolin S. J., and Morris P. G. (1988) Measurement of free intracellular calcium in the brain by ^{19}F-nuclear magnetic resonance spectroscopy. *J. Neurochem.* **51,** 1311–1313.

Badar-Goffer R. S., Bachelard H. S., and Morris P. G. (1990*a*) Cerebral metabolism of acetate and glucose studied by ^{13}C-NMR spectroscopy: a technique for investigating metabolic compartmentation in the brain. *Biochem J.* **266,** 133–139.

Badar-Goffer R. S., Ben-Yoseph O., Dolin S. J., Morris P. G., Smith G. A., and Bachelard H. S. (1990*b*) Use of 1,2-bis-(2-amino-5-fluorophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (5FBAPTA) in the measurement of free intracellular calcium in the brain by ^{19}F-nuclear magnetic resonance spectroscopy. *J. Neurochem.* **55,** 878–884.

Ben-Yoseph O., Bachelard H. S., Badar-Goffer R. S., Dolin S. J., and Morris P. G. (1990) Effects of *N*-methyl-D-aspartate on $[Ca^{2+}]_i$ and the energy state in the brain by ^{19}F- and ^{31}P-nuclear magnetic resonance spectroscopy. *J. Neurochem.* **55,** 1446–1449.

Brooks K. T. and Aaronson P. I. (1991) The measurement of intracellular calcium using FURA-2 in the rat cerebral cortex and the effect of hypoxia or aglycaemia. *Soc. Neurosci. Abstr.* **17,** 1269.

Carroll J. M., Toral-Barza L., and Gibson G. (1992) Cytosolic free calcium and gene expression during chemical hypoxia. *J. Neurochem.* **59,** 1836–1843.

Cheung J. Y., Bonventre J. V., Malis C. D., and Leaf A. (1986) Calcium and ischaemic injury. *N. Engl. J. Med.* **314,** 1670–1676.

Choi D. W. (1988) Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci.* **11,** 465–469.

Cox D. W. G., Morris P. G., Feeney J., and Bachelard H. S. (1983) ^{31}P-n.m.r. studies on cerebral energy metabolism under conditions of hypoglycaemia and hypoxia in vitro. *Biochem. J.* **212,** 365–370.

Dubinsky J. and Rothman S. M. (1991) Intracellular calcium concentrations during "chemical hypoxia" and excitotoxic neuronal injury. *J. Neurosci.* **11,** 2545–2551.

Duchen M. R., Valdeomillos M., O'Neill S. C., and Eisner D. A. (1990) Effects of metabolic blockade on the regulation of intracellular calcium in dissociated mouse sensory neurons. *J. Physiol. (Lond.)* **424,** 411–426.

Gibson G. E., Manger T., Toral-Barza L., and Freeman G. (1989) Cytosolic-free calcium and neurotransmitter release with decreased availability of glucose or oxygen. *Neurochem. Res.* **14,** 437–444.

Harrison S. M. and Bers D. M. (1987) The effect of temperature and ionic strength on the apparent Ca-affinity of EGTA and the analogous Ca-chelators BAPTA and dibromo-BAPTA. *Biochim. Biophys. Acta* **925,** 133–143.

Jurkowitz-Alexander M. S., Altschuld R. A., Hohl C. M., Johnson J. D., McDonald J. S., Simmons T. D., and Horrocks L. A. (1992) Cell swelling, blebbing, and death are dependent on ATP depletion and independent of calcium during chemical hypoxia in a glial cell line (ROC-1). *J. Neurochem.* **59,** 344–352.

Kato H., Liu Y., Araki T., and Kogure K. (1991) Temporal profile of the effects of pretreatment with brief cerebral ischemia on the neuronal damage following secondary ischemic insult in the gerbil: cumulative damage and protective effects. *Brain Res.* **553,** 238–242.

Kauppinen R. A. and Williams S. R. (1990) Cerebral energy metabolism and intracellular pH during severe hypoxia and recovery: a study using ^{1}H, ^{31}P and ^{1}H [^{13}C] nuclear magnetic resonance spectroscopy in the guinea pig cerebral cortex in vitro. *J. Neurosci. Res.* **26,** 356–369.

Kauppinen R. A., McMahon H. T., and Nicholls D. G. (1988) Calcium-dependent and calcium-independent glutamate release, energy status and cytosolic free calcium concentration in isolated nerve terminals following metabolic inhibition—possible relevance to hypoglycaemia and anoxia. *Neuroscience* **27,** 175–182.

Kirschenlohr H. L., Metcalfe J. C., Morris P. G., Rodrigo G. C., and Smith G. A. (1988) Ca^{2+} transient, Mg^{2+} and pH measurement in the cardiac cycle by ^{19}F-NMR. *Proc. Natl. Acad. Sci. USA* **85,** 9017–9021.

Lawson C. S. and Downey J. N. (1993) Preconditioning: state of the art of myocardial protection. *Cardiovasc. Res.* **27,** 542–550.

Lee K., Miwa S., Koshimura K., Hasegawa H., Hamahata K., and Fujiwara M. (1990) Effects of hypoxia on the catecholamine release, Ca^{2+} uptake, and cytosolic free calcium concentration in cultured bovine adrenal chromaffin cells. *J. Neurochem.* **55,** 1131–1137.

Lemasters J. J., Diguiseppi J., Nieminem A.-L., and Herman B. (1987) Blebbing, free Ca^{2+} and mitochondrial membrane potential preceding cell death in hepatocytes. *Nature* **325,** 78–81.

Liu Y., Kato H., Nakata N., and Kogure K. (1992) Protection of rat hippocampus against ischemic neuronal damage by pretreatment with sublethal ischemia. *Brain Res.* **586,** 121–124.

Siesjö B. K. and Bengtsson F. (1989) Ca^{2+} fluxes, Ca^{2+} antagonists, and Ca^{2+}-related pathology in brain ischaemia, hypoglycaemia and spreading depression: a unifying hypothesis. *J. Cereb. Blood Flow Metab.* **9,** 127–140.

Siesjö B. K. and Deshpande J. K. (1987) Electrolyte shifts between brain and plasma in hypoglycemic coma. *J. Cereb. Blood Flow Metab.* **7,** 789–793.

Simon R. P., Schmidley J. W., Meldrum B. S., Swan J. H., and Chapman A. G. (1986) Excitotoxic mechanisms in hypoglycaemic hippocampal injury. *Neuropathol. Appl. Neurobiol.* **12,** 567–576.

Uematsu D., Greenberg J. H., Reivich M., and Karp A. (1989) Cytosolic free calcium, NAD-NADH redox state and hemodynamic changes in the cat cortex during severe hypoglycemia. *J. Cereb. Blood Flow Metab.* **9,** 149–155.