Stimulated release of lactate in freely moving rats is dependent on the uptake of glutamate

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1. Physiological stimulation of neuronal activity induces an increase in extracellular lactate. Experiments were designed to determine the role of the reuptake of neuronally released glutamate in lactate delivery to the extracellular compartment.

2. *In vivo* microdialysis was used in freely moving rats. The lactate concentration in striatal dialysate was assayed using an enzyme-based on-line assay at 1 min intervals. Drugs were given locally through the dialysis probe.

3. The extracellular concentration of lactate, determined using the zero net flux method, was 346 ± 21 μM.

4. Induced grooming caused a maximal increase in lactate concentration in striatal dialysate of $58 \pm 10\%$.

5. Administration of 100 μM glutamate caused a transient increase in dialysate lactate concentration of $72 \pm 17\%$.

6. A 20 min infusion of the glutamate uptake blockers β-D,L-threohydroxyaspartate (THA) or pirrolidine-2-4-dicarboxylate (PDC) produced an increase in basal lactate, which was sustained in response to THA and transient in response to PDC.

7. Grooming induced during the infusion of PDC produced no significant increase in lactate.

8. Grooming induced after the infusion of the glutamate uptake blockers gave rise to a reduced increase in lactate.

9. These results support the hypothesis that stimulated release of lactate is dependent on the uptake of glutamate.

In a resting human subject the ratio of oxygen consumption to glucose utilization is close to 6:1, which implies the oxidation of glucose to CO_2 and H_2O (Clarke & Sokoloff, 1994). Furthermore, the rate of oxygen and glucose consumption is closely coupled to regional cerebral blood flow (rCBF) (Baron, Lebrun-Grandie, Collard, Crouzel, Mestelan & Bousser, 1982). However, there is a significant concentration of lactate in the extracellular compartment of the brain. Neuronal stimulation, which increases rCBF, local cerebral glucose utilization (rCM_{glc}) and local cerebral oxygen utilization (rCM_{O_2}) (Seitz & Roland, 1992; Marrett *et al.* 1993*a*, *b*), also causes an increase in lactate (Kuhr & Korf, 1988; Prichard *et al.* 1991; SappeyMarinier, Calabrese, Fein, Hugg, Biggins & Weiner, 1992; Fellows, Boutelle & Fillenz, 1993). There are three possible sources for this increase in lactate: the blood stream, neurones and astrocytes.

Autoradiographic measurement of cerebral lactate transport under normal and activated conditions reveals significant rate constants for lactate transport across the blood–brain barrier (Lear & Kasliwal, 1991). However, using microdialysis, Kuhr and colleagues found no significant contribution of plasma lactate to dialysate lactate under either basal or stimulated conditions (Kuhr, van den Berg & Korf, 1988). Even under conditions of ischaemia, when there is a substantial rise in brain lactate, there was no significant lactate transport from brain to blood as measured by the arterio-venous difference (Hope, Cady, Chu, Delpy, Gardiner & Reynolds, 1987). This suggests that exchange of lactate between brain and circulation in either direction is negligible.

A second possible source for the extracellular lactate is neurones. Measurements of lactate changes during hypoxia/ischaemia using ^{1}H nuclear magnetic resonance (NMR) have shown that the increase in lactate can be quantitatively accounted for by the increase in the ratio of glucose to oxygen uptake by the brain (Hope, Cady, Delpy, Ives, Gardiner & Reynolds, 1988). This suggests a switch of metabolism from oxidative phosphorylation to anaerobic

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glycolysis. However the spatial resolution is insufficient to decide whether this occurs in astrocytes or neurones. In experiments using microdialysis, local infusion of probenecid, which inhibits lactate transporters (Pardridge, Connor & Crawford, 1973), markedly reduced the increase in lactate produced by electroconvulsive shock or ischaemia (Kuhr *et al.* 1988).

A third possible source for the extracellular lactate is glial cells. There is extensive evidence from *in vitro* experiments, which has been reviewed recently (Tsacopoulos & Magistretti, 1996), that glial cells take up glucose and export lactate. In a preparation of mammalian retina, labelled glucose was taken up into glial cells and exported as lactate into the medium, from which it was taken up by the photoreceptors (Poitry-Yamate, Poitry & Tsacopoulos, 1995). Neurones can take up lactate (Dringen, Wiesinger & Hamprecht, 1993) and there is increasing evidence that not only can lactate fuel neuronal metabolism (Schurr, West & Rigor, 1988; Izumi, Benz, Zorumski & Olney, 1994), but it may be the preferred substrate (McKenna, Tildon, Stevenson & Hopkins, 1994; Poitry-Yamate *et al.* 1995).

In cultured astrocytes, glutamate added to the incubation medium causes the uptake of glucose and the release of lactate. This action of glutamate is unaffected by glutamate receptor antagonists, but is abolished by β-D,L-threo-hydroxyaspartate (THA), which blocks the Na^+-dependent glutamate transporter (Pellerin & Magistretti, 1994). The authors suggest that the cotransport of glutamate with Na^+ leads to stimulation of Na^+,K^+-ATPase, consumption of ATP and stimulation of astrocytic glycolysis. It has been proposed that this mechanism provides the coupling signal between neuronal activation and glucose uptake by astrocytes, which is then delivered as lactate for neuronal metabolism (Tsacopoulos & Magistretti, 1996). In a previous study we used microdialysis in order to test whether this process also occurs *in vivo*. We found that THA produces a large increase in basal lactate and that a tail pinch stimulus applied during THA perfusion no longer produces a statistically significant increase in lactate (Fray, Forsyth, Boutelle & Fillenz, 1996). However, THA in addition to acting as a competitive inhibitor of glutamate uptake is also a competitive inhibitor of the enzyme aspartic acid transaminase (Jenkins, 1961), which results in an accumulation of lactate. We also demonstrated that glycogenolysis induced by β-receptor stimulation could not explain stimulated lactate increases.

In the present study we have also used microdialysis in freely moving rats. The concentration of lactate in dialysate is not the true extracellular concentration; this cannot be calculated from the dialysate concentration using the *in vitro* recovery of the probe, but requires quantitative microdialysis (Morrison, Bungay, Hsiao, Ball, Mefford & Dedrick, 1991). Using the zero flow technique (Jacobson, Sanberg & Hamberger, 1985), extracellular concentrations of lactate of 1 mM (Kuhr *et al.* 1988) and 1·2 mM (Harada, Sawa, Okuda, Matsuda & Tanaka, 1993) in the hippocampus of unanaesthetized rats and 5·6 mM in the striatum of chloral hydrate-anaesthetized rats (Lada & Kennedy, 1995) have been reported. We have used the zero net flux method of Lönnroth (Lönnroth, Jansson & Smith, 1987) to determine the extracellular concentration of lactate in the striatum remote from the probe (Boutelle & Fillenz, 1996). We also examined the effect of local infusion of glutamate on basal levels of lactate. In order to examine the role of glutamate uptake in the stimulated release of lactate, we have compared the effect of THA with the effect of pirrolidine-2-4-dicarboxylate (PDC), a more selective competitive inhibitor of the glutamate transporter.

METHODS

Surgery

Male Sprague–Dawley rats weighing 200–300 g were anaesthetized, following published guidelines (Wolfensohn & Lloyd, 1994), with the following mixture: (a) Hypnorm, a combination of the neuroleptic analgesic fentanyl (0·318 mg ml^{-1}) and fluanisone (10 mg ml^{-1}), and (b) Hypnovel, the benzodiazepine midazolam (5 mg ml^{-1}), and (c) sterile water, mixed 1 : 1 : 2. This mixture was injected I.P. at a volume of 3·3 ml kg^{-1}, giving a dose of 0·25 mg kg^{-1} fentanyl, 0·8 mg kg^{-1} fluanisone and 0·4 mg kg^{-1} midazolam. Surgery typically lasted 35 min and anaesthesia was reversed by an I.P. injection of naloxone (0·1 mg kg^{-1}). As animals began to recover from the anaesthetic they were given Vetergesic, the long-lasting narcotic partial agonist buprenorphine (0·1 mg kg^{-1}, S.C.), for pain relief. Despite its long half-life, buprenorphine is eliminated within 24 h and therefore does not confound the results of the experiments. Occasionally, when surgery took longer, the animal was given a further I.M. injection of 0·1 ml Hypnorm every 10 min beyond the initial 30 min.

Once surgical anaesthesia was established, animals were placed in a stereotaxic frame and concentric microdialysis probes were implanted in the right striatum, using the following co-ordinates: 1 mm antero-posterior from bregma, 2·5 mm medio-lateral and −8·5 mm below the dura (Paxinos & Watson, 1986). Probes were secured in position using dental screws and acrylic (Associated Dental Products Ltd, Swindon, UK).

Animals were allowed 24 h recovery and were assessed for good health, using the guidelines of Morton & Griffiths (1985), after recovery from anaesthesia and at the beginning of the next day. All animals used in this study had a score of 2 or less, as defined by Morton & Griffiths; in cases where the score was 3 or more, the experiment was terminated. This work was carried out under licence in accordance with the Animals (Scientific Procedures) Act, 1986. The position of the probe in the brain was checked post mortem and in all cases the sampling membrane was located entirely within the striatum.

Microdialysis probe construction

Concentric microdialysis probes were constructed by inserting a plastic-coated silica tube (Scientific Glass Engineering, Milton Keynes, UK) and a tungsten wire (Goodfellow Metals, Cambridge, UK) into a polyacrylonitrile dialysis fibre (Hospal Industrie, Meyzieu, France) and gluing the fibre into a 12 mm stainless steel cannula (Goodfellow Metals). The tip was sealed with epoxy glue and a second silica tube was inserted into the cannula to form the

outlet. The total exposed fibre length was 4 mm and the external diameter of the sampling region of the probe was 300 μm.

Enzyme packed-bed assay for lactate

The dialysate was analysed for lactate using a flow injection enzyme-based assay (Boutelle, Fellows & Cook, 1992). In brief, lactate oxidase and horseradish peroxidase were immobilized on silica beads, which were then packed into a small bed. A phosphate buffer containing the electrochemical mediator species ferrocene (100 mM Na_2HPO_4, 1 mM EDTA and 2 mM ferrocene monocarboxylic acid, adjusted to pH 7·0 with HCl, and 0·05% Kathon CG added to prevent bacterial growth) was pumped through the bed at 0·3 ml min^{-1} by an HPLC pump (model 2248; Pharmacia LKB). Microdialysate samples were injected on-line onto the enzyme bed using a CMA 160 injector (CMA Microdialysis, Stockholm, Sweden). Lactate was oxidized by lactate oxidase, producing H_2O_2. Horseradish peroxidase converted this to H_2O and was itself regenerated by the oxidation of the ferrocene species present in the buffer. The ferrocinium produced was electrochemically reversible and was detected by reduction at a downstream electrode. The detection limit of the enzyme bed was 4 pmol, equivalent to 10 μl of 0·4 μM. The fast response of the assay allowed dialysate to be assayed at 1 min intervals.

The preoxidation system

To reduce interference by ascorbic acid and other electroactive compounds to acceptable levels, the dialysate was passed through a preoxidation system consisting of tubular electrodes (Berners, Boutelle & Fillenz, 1994). The working electrode (Pt) is kept at 600 mV *vs.* Ag–AgCl. At a flow rate of 2 μl min^{-1} this reduces ascorbate and other electroactive species normally present in the dialysate by more than 95%.

Materials

Lactate oxidase (EC 1.1.3.2) and horseradish peroxidase (EC 1.11.1.7., grade I) were from Genzyme, West Malling, Kent, UK. The silica beads used as the enzyme support were from Merck, Poole, Dorset, UK. Glutamate (monosodium salt), ferrocene monocarboxylic acid, THA and PDC were from Sigma; fentanyl–fluanisone (Hypnorm) was from Janssen Pharmaceuticals Ltd, Oxford, UK; midazolam (Hypnovel) was from Roche Products Ltd, Welwyn Garden City, UK; buprenorphine (Vetergesic) was from Reckitt and Colman Pharmaceuticals, Hull, UK; Kathon CG was from Rohm and Haas, Croydon, UK. Drugs were dissolved in artificial cerebrospinal fluid (ACSF) which had the following composition (mM): NaCl, 147; KCl, 4; $CaCl_2$, 1·2; $MgCl_2$, 1·0.

Amperometric measurements were made using EMS BP 1 potentiostats from Electrochemical and Medical Systems, Newbury, UK.

Experimental protocol

Following surgery, the animals were placed in large plastic bowls (50 cm × 55 cm) and maintained in a temperature- and light-controlled environment, with free access to food and water. Twenty-four hours after surgery experiments were carried out with the animal in its home bowl, allowing free movement. During experiments the microdialysis probe was perfused with ACSF (de Boer, Damsma, Fibiger, Timmerman, de Vries & Westerink, 1990) at a flow rate of 2 μl min^{-1} using a CMA 100 microinfusion pump (CMA Microdialysis, Stockholm, Sweden). The pH of this perfusion fluid was typically 6·5. The perfusion fluid was not buffered, since fixing the pH of the extracellular fluid (ECF) may interfere with pH-dependent physiological processes. The buffering capacity of the ECF was sufficient to cope with the slight acidity of the perfusion fluid, since the efflux dialysate had a pH close to physiological pH. Dialysate was assayed on-line using the enzyme beds as a flow injection assay. Two microlitre samples were assayed for lactate at 1 min intervals. This increased sampling rate also reduces problems due to non-linear calibration of the assay.

For the zero net flux method different concentrations of lactate were added to the perfusion medium. The difference between influx and efflux concentrations (Δin–out) were plotted against the infused concentrations and the concentration at which Δin–out was zero was derived by regression analysis.

Basal perfusate was collected for 2 h. Grooming was induced by dropping water from a plastic pipette onto the animal's nose. This stimulus was generally able to provoke 3–5 min of activity. THA and PDC were dissolved in ACSF and perfused through the microdialysis probe.

Data analysis

Lactate levels in the dialysate are expressed in micromolar units as means ± S.E.M.; n is the number of rats. Basal levels were defined as the mean of six 5 min samples collected over a period of 30–60 min prior to drug administration or stimulus application.

The response to grooming was measured either as the maximum increase in dialysate concentration expressed as a percentage of baseline or as the increase in efflux expressed as the area under the curve (AUC) measured in picomoles; this was calculated by integration over the time interval of the increase above baseline values. Statistical comparison of the data was carried out by Student's paired t test, Mann–Witney U test and the Permutation test for paired replicates (Siegel & Castellan, 1988).

RESULTS

Determination of the ECF concentration of lactate

The extracellular concentration of lactate was determined using the zero net flux method of Lönnroth (Lönnroth *et al.* 1987). Different concentrations of lactate were added to the perfusion medium. At concentrations below the ECF level, lactate is lost from the brain; at concentrations above the ECF level, lactate is taken up by the brain. Figure 1 shows a plot of influx against the difference between influx and efflux; the point at which there is neither loss to or gain from the brain is calculated by regression, and represents the lactate concentration away from the immediate vicinity of the probe. This was 346 ± 21 μM and corresponds to the extracellular concentration of lactate; the mean dialysate lactate concentration was 195 ± 15 μM and the *in vivo* recovery was 56%.

Effect of glutamate

In order to test whether the uptake of glutamate results in the release of lactate *in vivo*, we added glutamate to the perfusion medium to a final concentration of 100 μM for a period of 20 min. This produced an increase in lactate which reached a maximum of 172 ± 17% above baseline after which it declined in spite of the continued infusion of glutamate (Fig. 2). The lactate efflux above baseline in response to the infusion of glutamate was 1176 ± 140 pmol, which represents an increase of 24 ± 5% above basal efflux over the same period of time.

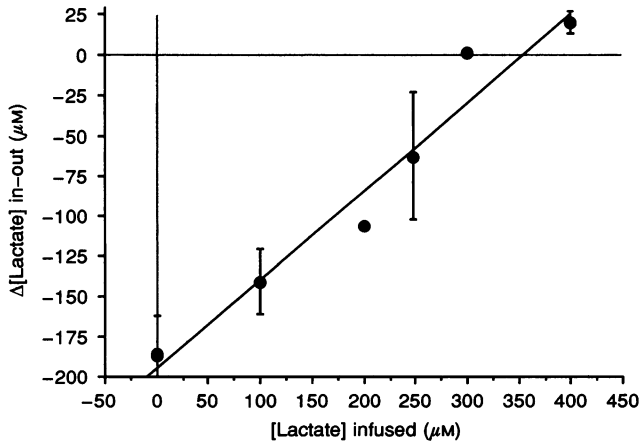

Figure 1. Regression analysis of the results of the zero net flux experiment

Concentrations of infused lactate are plotted against the difference between lactate influx and efflux. Data from 4 rats. Points are means ± S.E.M.

Effect of grooming

Lactate levels are very sensitive to the level of the activity of the rat. Since rats are nocturnal animals they are usually very quiescent during the daytime, but any spontaneous motor activity produced fluctuations in the lactate dialysate level, which were more marked with the 1 min sampling used in the present experiments than with previous 2·5 min sampling (Fellows *et al.* 1993; Fray *et al.* 1996). By dropping water onto the rat's snout, grooming was induced which lasted between 3 and 5 min. This was accompanied by a rise in lactate which reached a maximum of 158 ± 10% of basal level. The intensity and duration of grooming and the duration of the rise in lactate showed some variation. By taking a period of 12 min from the onset of grooming we calculated the mean efflux above basal in sixteen separate groomings. The time course is shown in Fig. 3 as percentage changes from basal. The calculated mean efflux above basal was 2358 ± 324 pmol lactate, which represents an increase of 39 ± 4% above the basal efflux over the same period of time.

Effect of THA and PDC

We next compared the effect of the infusion of 1 mM THA or 10 mM PDC for a period of 20 min. The concentration of THA was that used in previous *in vitro* (Pellerin & Magistretti, 1994) and *in vivo* (Fray *et al.* 1996) experiments. PDC has been shown to produce a dose-dependent increase in dialysate glutamate between 1 and 10 mM PDC, the latter raising the dialysate concentration of glutamate to 15 μM (Obrenovitch, Urenjak & Zilkha, 1996). The effect of THA and PDC infusion is shown in Fig. 4. The maximum increase in lactate was very similar in response to the two uptake blockers (236 ± 47% ($n = 7$) for THA and 280 ± 163% ($n = 6$) for PDC), but whereas the increase in response to THA was sustained, the lactate concentration during the infusion of PDC gradually returned towards preinfusion levels. The efflux of lactate was 5201 ± 1513 pmol during THA infusion and 1718 ± 720 pmol during PDC infusion. This represents a 102 ± 72% increase above basal for THA and a 51 ± 24% increase above basal for PDC.

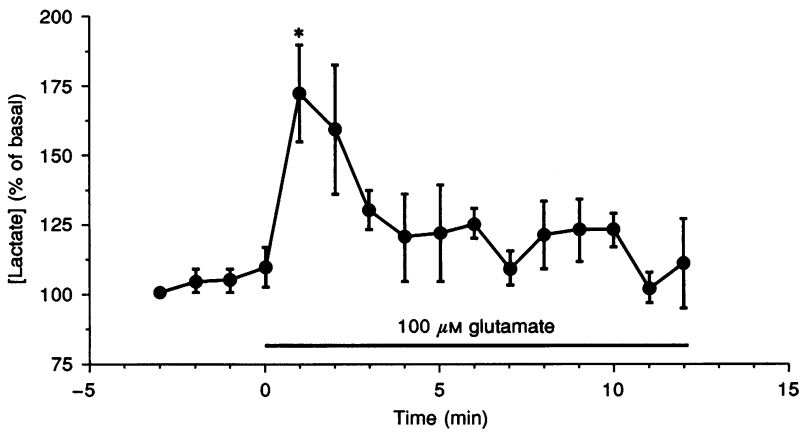

Figure 2. The effect on dialysate lactate concentration of a 20 min infusion of 100 μM glutamate

The dialysate lactate concentration is shown as a percentage of basal. Points are means ± S.E.M. * $P < 0·05$, compared with last basal value before glutamate infusion; paired t test using absolute values ($n = 3$).

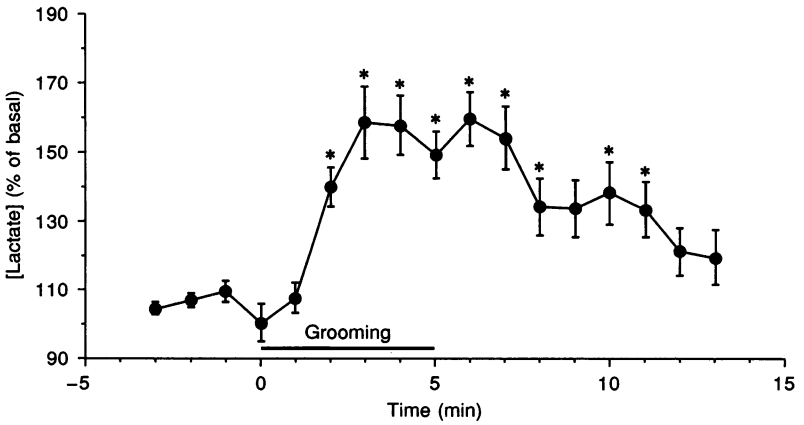

Figure 3. The effect on dialysate lactate concentration of induced grooming
The dialysate lactate concentration is shown as a percentage of basal. Points are means ± S.E.M. * $P < 0{\cdot}05$, compared with last basal value before stimulus; paired t test using absolute values ($n = 16$).

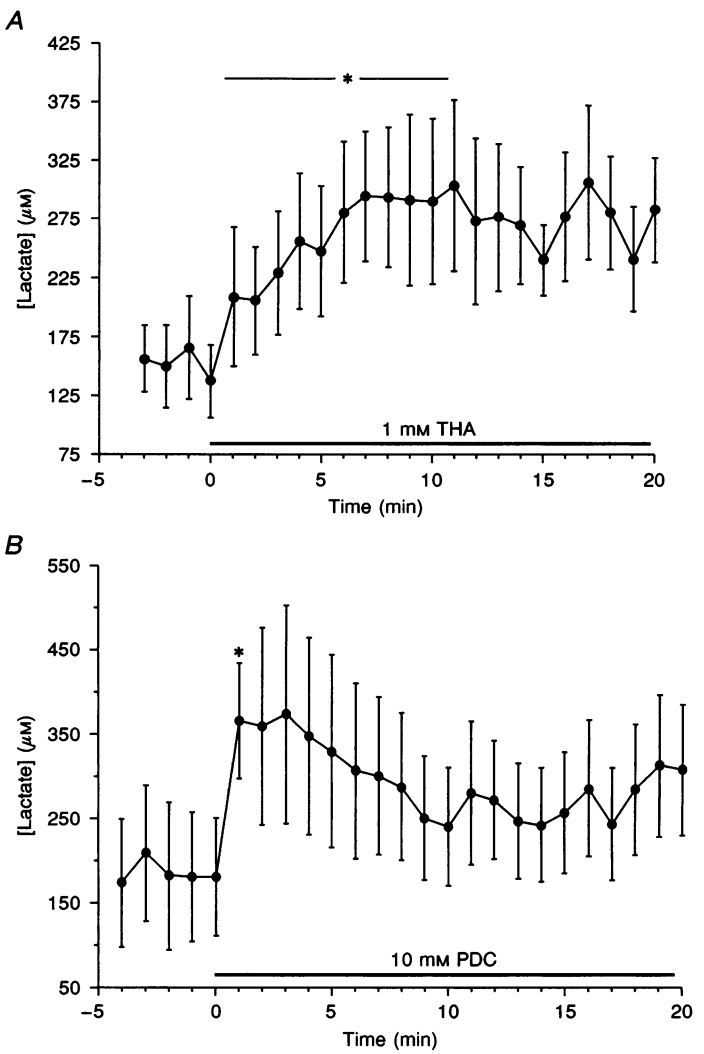

Figure 4. The effect on dialysate lactate concentration of a 20 min infusion of THA and PDC
A, 1 mM THA ($n = 7$); *B*, 10 mM PDC ($n = 6$). Points are means ± S.E.M. * $P < 0{\cdot}05$, compared with last basal value before drug infusion; paired t test.

Grooming during PDC infusion

We have previously shown that during the infusion of THA the tail pinch-induced increase in lactate is greatly reduced (Fray *et al.* 1996). In the present study grooming was induced during the infusion of PDC. An example of such an experiment is shown in Fig. 5. Because of the fluctuation of the lactate level during the infusion of PDC, it was not possible to calculate the efflux of lactate stimulated by grooming. In order to calculate the statistical significance of the reduction in the grooming response during the PDC infusion, we compared the maximum increase of lactate, calculated as a percentage of the baseline preceding the grooming response, both before and during the infusion of PDC. The reduction in the stimulated increase was statistically significant ($P = 0{\cdot}03$, $n = 5$; Permutation test for paired replicates).

Grooming after THA and PDC infusion

In order to examine to what extent the reduction in the stimulated increase in lactate concentration in response to induced grooming during the application of the glutamate uptake inhibitor was due to the already higher level of lactate, we carried out experiments with both THA and PDC in which grooming was induced during a period of reperfusion with ACSF after a 20 min period of drug infusion. We then compared the maximum increase in lactate during induced grooming before drug infusion with that evoked after drug infusion, when lactate levels had returned to the preinfusion level. We found that with both THA and PDC the postinfusion response was smaller than the pre-infusion response, provided the stimulus was applied within 60 min after infusion of the drug (THA: $P = 0{\cdot}015$, $n = 6$; PDC: $P = 0{\cdot}035$, $n = 8$).

DISCUSSION

The present experiments are an extension of the previous study concerned with the role of astrocytes in the provision of metabolic substrates for neuronal metabolism (Fray *et al.* 1996). By decreasing the sampling interval we have obtained a higher time resolution for the lactate measurement and the replacement of tail pinch with induced grooming provides a more physiological stimulus for neuronal activation.

There are at present two diametrically opposed views of the significance of lactate in the brain. According to one view, the extracellular lactate is an expression of the metabolic compartmentation in which glucose taken up by astrocytes is converted glycolytically to lactate, which is then released into the extracellular space to be taken up by neurones and metabolized by oxidative phosphorylation (Tsacopoulos & Magistretti, 1996). According to the other view, neuronal activation results in a relatively short initial period of aerobic neuronal glycolysis (Prichard *et al.* 1991; Ribeiro *et al.* 1993).

Under baseline conditions measurements of rCBF, rCM_{O_2} and rCM_{glc} in human visual cortex have the relationship expected for almost complete oxidation of glucose (Marrett, *et al.* 1993*a*). In spite of this, the basal concentration of lactate in the visual cortex has been estimated to be 0·71 mM using magnetic resonance spectroscopy (Prichard *et al.* 1991). This is somewhat higher than the striatal ECF lactate concentration of 0·35 mM determined by the zero net flux method in the present study. Using the same method, we have recently shown that the basal extracellular concentration of glutamate is 3 μM (Miele, Berners, Boutelle, Kusakabe & Fillenz, 1996*a*). Such a concentration will activate the glutamate transporter and thus may lead to the release of lactate, which could then be oxidatively phosphorylated after uptake into neurones. This could account for a substantial lactate concentration together with a ratio of oxygen : glucose utilization of 6 : 1.

The finding that both glutamate and the two competitive inhibitors of glutamate transport (which are therefore themselves transported) increase the release of lactate supports the hypothesis of metabolic compartmentation. The severe depression of the stimulated increase in lactate

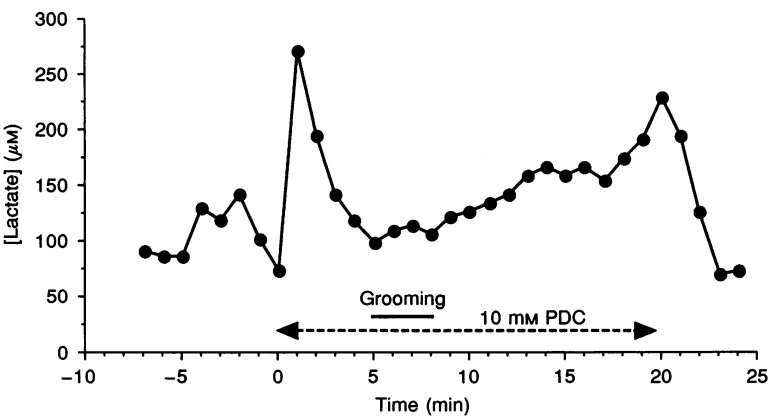

Figure 5

The effect on dialysate lactate concentration of grooming induced during a 20 min infusion of PDC (10 mM).

during the infusion of PDC gives support to the suggestion that glutamate uptake following its release provides the coupling signal between neuronal activation and increased supply of metabolic substrates. The demonstration that the stimulated release of lactate is depressed even after return to perfusion with ACSF shows that the depression of lactate release is not attributable to the fact that glycolysis is already maximal.

Although neurones use lactate as a metabolic substrate, they also take up and use glucose. We have shown previously that physiological stimulation causes an initial decrease in glucose (Fray *et al.* 1996). More recently we have found that during the stimulated decrease in glucose there is an increase in tissue oxygen, which parallels the increase in rCBF (Lowry & Fillenz, 1997). This suggests that the decrease in glucose represents an increase in utilization with no increase in oxygen utilization. The uncoupling of glucose and oxygen utilization suggests aerobic glycolysis. It is not known how much of the resulting lactate enters the extracellular compartment, but it could make a contribution to the stimulus-induced rise in lactate. However the results of the present experiments, taken together with evidence from *in vitro* experiments, such as the export of lactate from astrocytes (Dringen *et al.* 1993), the preferential release by astrocytes, and uptake by neurones, of lactate (McKenna, Tildon, Stevenson, Boatright & Huang, 1993), the uptake and utilization by photoreceptors of lactate provided by Müller cells (Poitry-Yamate *et al.* 1995), and finally the release of lactate from cultured astrocytes in response to glutamate uptake (Pellerin & Magistretti, 1994), suggest that most of the lactate comes from astrocytes in response to glutamate uptake. This glutamate uptake may come from neuronal release, giving the stimulated increases in lactate, or it may come from the high level of basal extracellular glutamate (which is probably itself derived from glial cells (Miele, Boutelle & Fillenz, 1996*b*)), and account for the basal level of lactate.

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