Two different constituents of plasma increase cytosolic calcium selectively in neurons or glia of primary rat cerebellar cultures

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1. The ability of several serum fractions to increase the cytosolic calcium concentration ($[Ca^{2+}]_i$) was tested in rat cerebellar cells maintained in primary culture.
2. Serum filtered through an ultrafiltration membrane with 3000 Da molecular mass cut-off (filtered serum, FS) selectively stimulated neurons whereas dialysed serum (DS) selectively stimulated glia.
3. The effects of FS were due to glutamate as they were reproduced by *N*-methyl-D-aspartate (NMDA), blocked by NMDA receptor antagonists and prevented by enzymatic removal of glutamate.
4. The effects of DS on glia were not reproduced by platelet-activating factor, thrombin or bradykinin. They were not lost on heating or extraction with diethyl ether. They were reproduced by a methanol–chloroform–HCl extract from DS and by several commercial fraction V plasma albumins.
5. These $[Ca^{2+}]_i$-increasing factors present in blood could contribute to brain damage during ischaemia if they reached the brain interstitium on disruption of the blood–brain barrier.

Ischaemic damage to the brain is usually regarded as derived from the combined effect of hypoxia, hypercapnia, glucose deprivation, acidosis and noxious actions of released substances, such as potassium, glutamate or NO (Siesjö, 1988; Choi, 1988; Martin, Lloyd & Cowan, 1994; Szatkowski & Attwell, 1994; Choi, 1995). Increasing cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) has been implicated as playing a central role in triggering neuronal death, and among the mechanisms leading to this $[Ca^{2+}]_i$ increase, Ca^{2+} entry secondary to activation of glutamate receptors has received particular attention (Choi, 1995). Postsynaptic effects of glutamate would be potentiated by partial depolarization and decreased Ca^{2+} extrusion secondary to energy shortage. With regard to the origin of glutamate, both massive activation of glutamatergic synapses and non-synaptic release of glutamate either from neurons, or from glia by reversal of the glutamate transporter, have been suggested (Szatkowski & Attwell, 1994). Serum-induced increases of $[Ca^{2+}]_i$ in neurons and glia have been reported previously (Van den Pol, Finkbeiner & Cornell-Bell, 1992). However, the possible damaging effect of blood factors reaching the brain's extracellular fluid upon disruption of the blood–brain barrier by hypoxia have scarcely been considered. In addition to the normal plasma constituents, activation of humoral (contact system, coagulation, complement) and cellular (leukocytes, platelets) mechanisms, which could occur on blood stasis, may give rise to potential activators of neurons and glia. Here we have studied the effects of serum and several serum fractions on $[Ca^{2+}]_i$ of cerebellar neurons and glia maintained in primary culture.

METHODS

Cerebellar cells were obtained from 5- to 7-day-old Wistar rat pups killed by cervical dislocation followed by decapitation. Cells were prepared essentially as described by Levi, Aloisi, Ciotti & Gallo (1984) except that dispase (5 mg ml^{-1}) was used instead of trypsin. Cells were plated on poly-L-lysine-coated 11 mm diameter coverslips and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum and 500 μg ml^{-1} gentamicin for 2 days. Then the culture medium was removed and replaced by Sato's medium (Bottenstein & Sato, 1979), in order to avoid excessive proliferation of glia, and the culture was continued for a further 2–4 days. Finally, 10% horse serum was added 24–48 h before the experiment.

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$[Ca^{2+}]_i$ measurements were performed as described previously (Villalobos, Fonteriz, Lopez, García & García-Sancho, 1992; Lopez, García, Artalejo, Neher & García-Sancho, 1995). Briefly, cell-coated coverslips loaded with fura-2 AM (the acetoxymethyl ester form of fura-2; Grynkiewicz, Poenie & Tsien, 1985) were placed under the inverted microscope (Nikon Diaphot, ×40 oil-immersion objective lens) and fluorescence images were processed using an Applied Imaging MagiCal image processor (Sunderland, UK). Consecutive frames obtained at 340 and 380 nm excitation (about 5 s time resolution) were ratioed pixel by pixel and $[Ca^{2+}]_i$ was estimated by comparison with fura-2 standards. Standard incubation medium had the following composition (mM): NaCl, 145; $MgCl_2$, 1; $CaCl_2$, 1; glucose, 10; K-Hepes, 10; pH 7·4. The temperature was 36 °C.

For differential identification of neurons and glia, the single-cell contents of β-tubulin III and glial fibrillary acidic protein (GFAP) were assayed by indirect immunofluorescence (Raff, Fields, Sakomori, Mirsky, Pruss & Winter, 1979) in the same coverslips

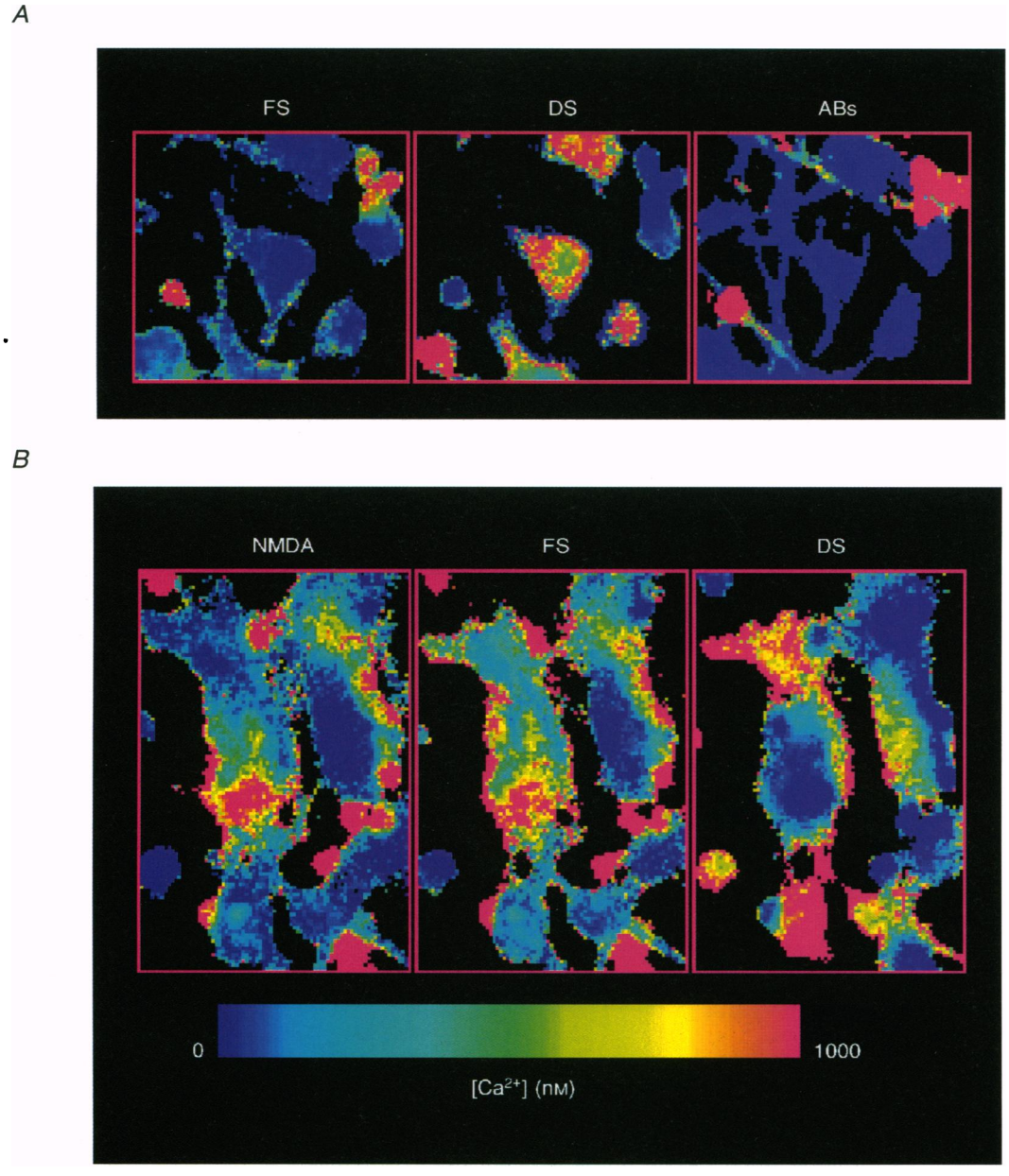

Figure 1. Effects of filtered serum (FS) and dialysed serum (DS) on cerebellar cell cultures

Both FS and DS were tested at 1 : 30 dilution. The left-hand and centre panels in *A* compare the effects of FS and DS on $[Ca^{2+}]_i$, coded in pseudocolour. The right-hand panel (antibodies (ABs)) shows identification of neurons and glia in the same microscope field. Images obtained for β-tubulin III and GFAP fluorescences were ratioed pixel by pixel and coded in pseudocolour. Cells in red correspond to neurons (high β-tubulin : GFAP ratio) and cells in blue to glia. Four neurons, 3 grouped in the upper right corner and 1 near the lower left were present. *B* compares the effects of NMDA (100 μM with 10 μM glycine added), FS and DS on $[Ca^{2+}]_i$. The $[Ca^{2+}]$ calibration scale is shown at the bottom and applies to both *A* and *B*.

Table 1. Comparison of the responses of neurons and glia to NMDA, glutamate, FS and DS

	Percentage of cells responding to:			
	NMDA	Glu	FS	DS
Neurons	98	98	98*	2
Glia	0	5	18**	98

* 0% responded to GPT-treated serum.
** 7% responded to GPT-treated serum.

Analysis of the responses in 71 neurons and 64 glial cells identified by immunocytochemistry in 4 different experiments: stimulation for 30 s with 100 μM NMDA and 10 μM glycine; 15 μM glutamate and 30 μM glycine; FS; or DS (both at 1 : 30 dilution). GPT, glutamate–pyruvate transaminase.

used for $[Ca^{2+}]_i$ measurements. The field of interest was located by positioning a cross engraved in the coverslip as in the $[Ca^{2+}]_i$ experiment, and two images (one for each antibody) were captured with the image processor.

Fresh human blood and serum was obtained from healthy volunteers through the University Hospital Blood Bank. Filtered serum (FS) was obtained by filtering human serum through an ultrafiltration membrane (Microcon 3; molecular mass cut-off, 3000 Da; Amicon, Beverly, MA, USA). Dialysed serum (DS) was obtained by dialysing human serum against 1000 vol. of standard incubation medium. Other treatments of FS and DS are described in the figure legends.

Bradykinin, bradykinin antagonists, thrombin, hirudin, mouse anti-β-tubulin III, rabbit anti-GFAP and fluorescein-coupled goat anti-mouse immunoglobulin G (IgG) were from Sigma. Goat anti-rabbit IgG labelled with Cascade Blue and fura-2 AM were obtained from Molecular Probes. Platelet-activating factor (PAF) was from Calbiochem. MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imin maleate) was a generous gift from Merck, Sharp & Dohme. BN-52031 and WEB-2086 were generous gifts from Institute Henry Beaufour, Paris, France and Boehringer Ingelheim KG, Germany, respectively. Other chemicals were obtained either from Sigma or from E. Merck, Darmstadt, Germany.

Unless otherwise stated, values are given as means ± S.E.M.

RESULTS

Addition of serum (1 : 30 dilution) to the cerebellar cultures produced an increase in $[Ca^{2+}]_i$ in most of the cells present in the microscope field. Both neurons and glia (immunocytochemically identified by the presence of β-tubulin III or GFAP, respectively) responded. The increase in $[Ca^{2+}]_i$ was blocked by 80 μM D-2-amino-7-phosphonoheptanoate (AP7; a blocker of NMDA-type glutamate receptors, Watkins & Olverman, 1987) in neurons but not in glia (see below), suggesting that activation by serum may occur by different mechanisms in these two cell types.

In order to separate the substances responsible for the effects in neurons and glia, serum was fractionated by several procedures. Figure 1 compares the effects of DS and FS. The effects of these two fractions were complementary (compare the left-hand and centre panels in Fig. 1*A*). FS selectively stimulated cells which were later identified as neurons by immunocytochemistry (as shown in the right-hand panel of Fig. 1*A*) whereas DS selectively stimulated glia. The pattern of response to FS closely overlapped with that obtained by stimulation with *N*-methyl-D-aspartate (NMDA), whereas DS increased $[Ca^{2+}]_i$ in NMDA-insensitive cells (Fig. 1*B*). This is best evidenced in the cells located at the periphery of cell groups; in central areas of these groups several different cells may overlap, producing more complex images.

The traces in Fig. 2*A* and *B* exemplify the typical time course of the responses to both serum fractions in a single neuron and a single glial cell, respectively. Traces in *C* compare the mean responses of all the neurons (continuous line, $n = 27$) and glia (dotted line, $n = 13$) present in the same microscope field. Again, FS stimulated only neurons whereas DS selectively stimulated glia. Essentially the same results were obtained when cells were separated into NMDA-sensitive and -insensitive groups (not shown). This outcome was not surprising, as the NMDA-sensitive and -insensitive cell groups in our cultures overlapped with neurons and glia, respectively (Table 1). Occasionally, some neurons showed a delayed $[Ca^{2+}]_i$ increase following stimulation with DS, which is reflected in the mean trace by a small and late increase in $[Ca^{2+}]_i$ (Fig. 2*C*). In all cases, the $[Ca^{2+}]_i$ increase in neurons, which was often oscillatory, was preceded by a $[Ca^{2+}]_i$ increase in the neighbouring glia (Fig. 2*D*). This delayed response in neurons was facilitated by adding glycine to the incubation medium and was never observed in the presence of AP7.

The effect of FS in neurons was blocked by NMDA-type glutamate receptor antagonists such as AP7 (80 μM, Fig. 2*E*, $n = 25$), ketamine (40 μM, not shown) or MK-801 (1 μM, not shown). The effects of FS were reproduced by filtered plasma (FP, Fig. 2*E*, $n = 25$). Assays of glutamate (Lowry & Passonneau, 1972) in serum, undiluted FS or FP showed concentrations ranging from 40 to 90 μM. Stimulation with L-glutamate at concentrations similar to those reached by addition of diluted serum reproduced the effects of FS (Glu, Fig. 2*F*, $n = 28$). Finally, enzymatic removal of serum glutamate by addition of pyruvate and glutamate–pyruvate transaminase prevented the effect on neurons (FS + GPT, Fig. 2*F*, $n = 28$).

Most of the glial cells were unresponsive to FS, but a small $[Ca^{2+}]_i$ increase was observed in 18% (Table 1). About 40% of the glia responding to FS (7% of all the glia) also responded to FS treated with glutamate–pyruvate transaminase and an additional 30% (5% of all the glia) responded to glutamate (but not to NMDA, see Table 1). In the remaining 30% of the glia responding to FS the effects could not be attributed either to glutamate or to the direct effect of other FS constituents. In any case, the $[Ca^{2+}]_i$ increase induced by FS in responding glial cells was much smaller than that observed in neurons.

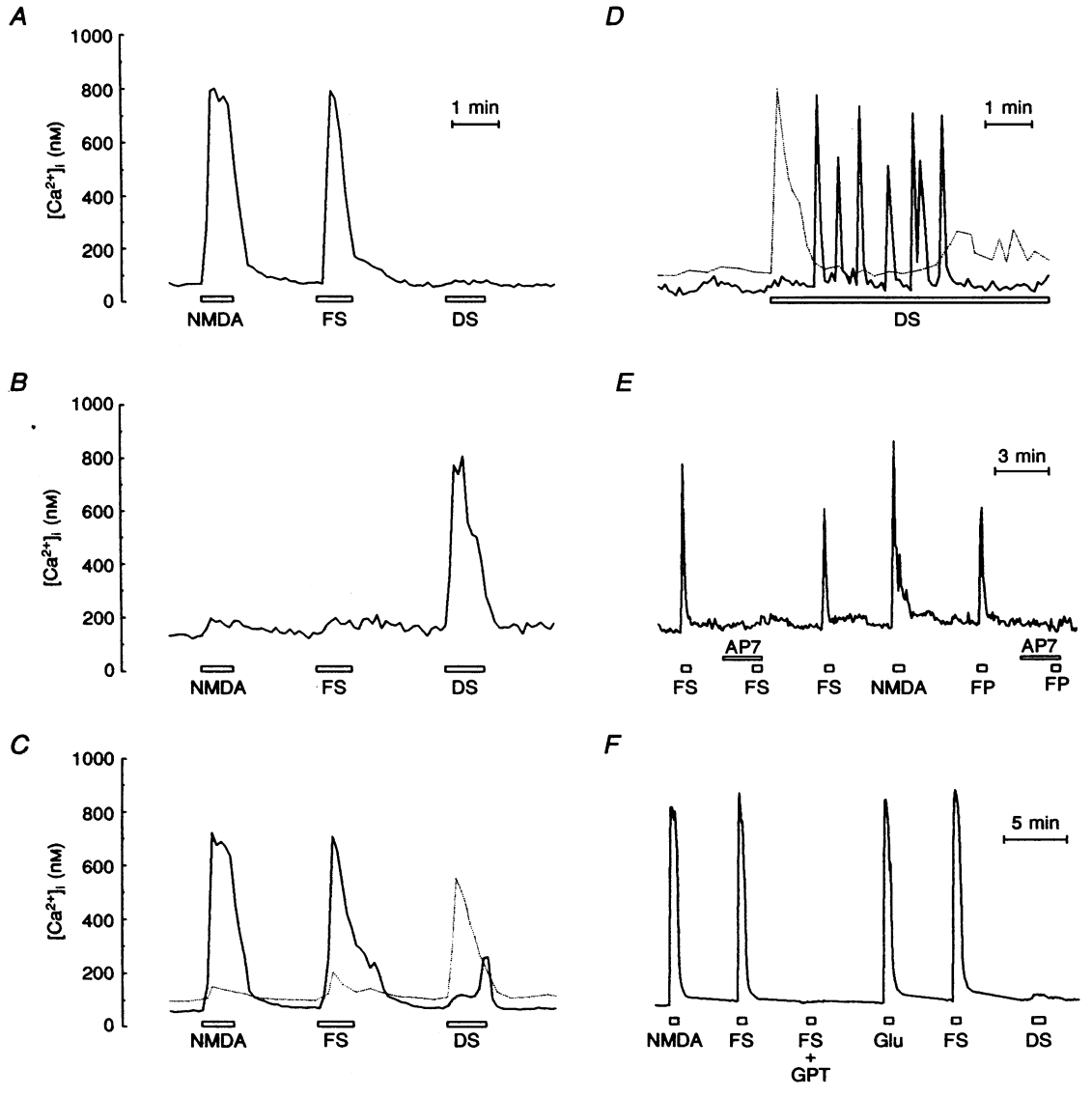

Figure 2. Comparison of the effects of FS and DS in neurons and glia

Additions of FS, DS and NMDA were as in Fig. 1. Traces correspond to a single neuron (*A*), a single glial cell (*B*) and the mean of all the neurons (continuous line; $n = 27$) and glia (dotted line; $n = 13$) present in the microscopic field of view (*C*). Trace *D* compares the time course of the response to DS in a single neuron (continuous line) and a single glial cell (dotted line) which were very close to each other in the microscope field. The incubation medium contained 10 μM glycine in order to facilitate possible effects of released glutamate. In trace *E*, the effects of 80 μM AP7 on the response of 25 neurons to FS and filtered plasma (FP) is shown. FP was prepared by dialysis of plasma obtained from blood anticoagulated with EDTA against 1000 vol. of Ca^{2+}-free incubation medium (0·1 mM EGTA added). In trace *F*, the effects of exogenous L-glutamate (Glu, 3 μM with 7 μM glycine added) and of glutamate removal from serum (FS + GPT) are shown for 28 neurons. For glutamate removal, pyruvate (10 mM) and glutamate–pyruvate transaminase (5 U ml^{-1}) were added to the serum 90 min before filtration. The treatment reduced the glutamate content of the serum from 80 to < 4 μM. Addition of pyruvate only did not modify the effect of FS. Experiments *A–C* are representative of 12 similar ones performed with different batches. Experiments *D–F* are representative of 3 similar ones.

On the other hand, the stimulation of glia by DS was reproduced by dialysed plasma obtained from blood anti-coagulated with either EDTA, citrate or heparin and by DS obtained from coagulated plasma (dialysis against 1000 vol. of Ca^{2+}-free medium, results not shown). Bradykinin (20 nM), thrombin (0·5 U ml^{-1}) or platelet-activating factor (10 nM) were able to activate some of the glia present in the microscope field, but they did not reproduce the effects of DS. Antagonists of bradykinin (des-Arg^9,[Leu^8]-bradykinin, 200 nM and [$Thi^{5,8}$,D-Phe^7]-bradykinin, 20 nM), thrombin (hirudin, 8 U ml^{-1}) and PAF receptors (BN-52031, 20 μM and WEB-2086, 2 μM) did not block the effects of DS (results not shown).

Figure 3 shows the effects of several treatments of DS on its $[Ca^{2+}]_i$-increasing action in glial cells. Much of the activity (70 ± 4%, $n = 101$) remained after heating at 90 °C for 10 min (HDS, Fig. 3*A*). Although the response was somewhat smaller, the same cells responded to sequential addition of DS and HDS (Fig. 3*C*). Extraction with diethyl ether did not modify the effect of DS ($n = 44$ cells, results not shown), but much of the $[Ca^{2+}]_i$-increasing

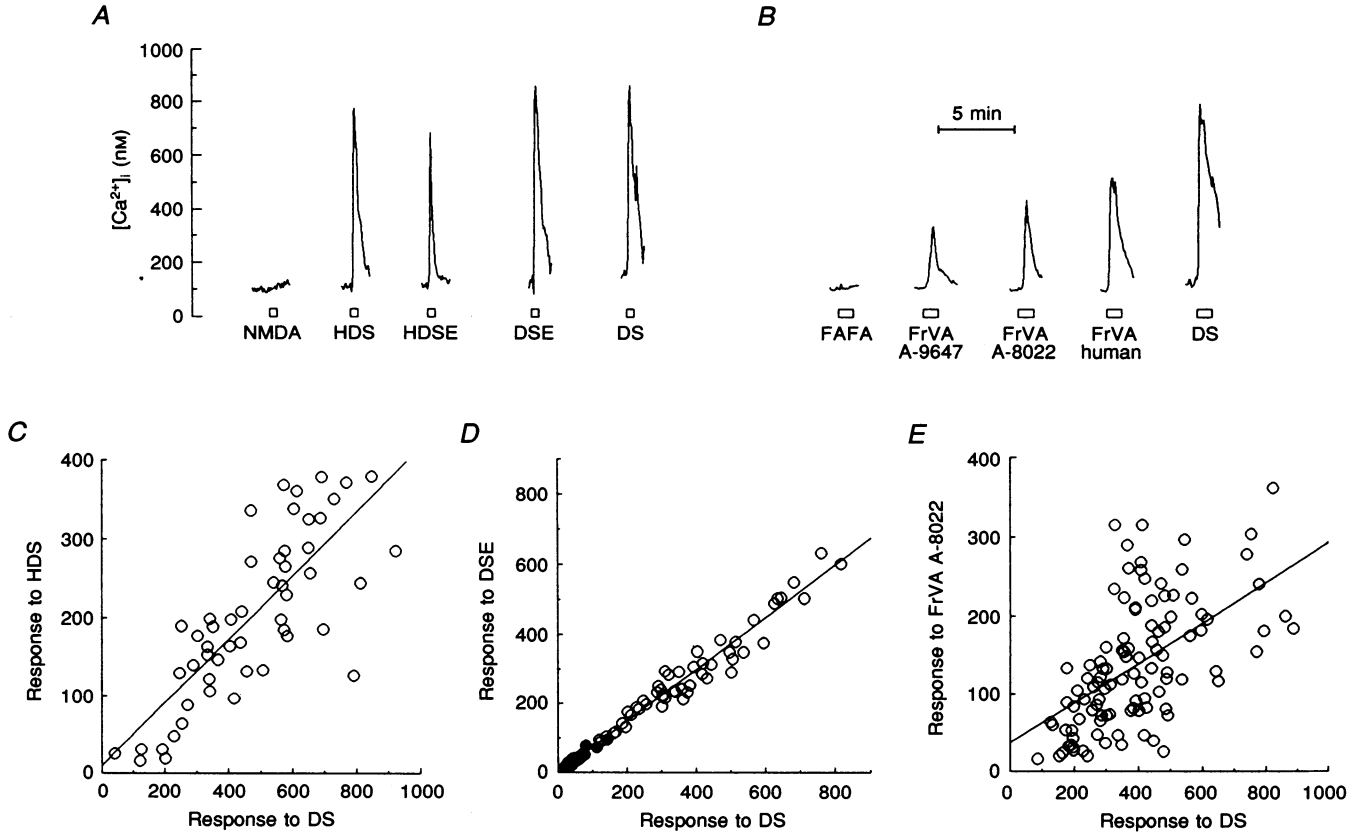

Figure 3. Comparison of the effects of DS, heated DS, a chloroform–methanol–HCl extract from DS or heated DS and several serum albumins on $[Ca^{2+}]_i$ of NMDA-insensitive cells

Most of these cells could be positively identified as glia by immunocytochemistry (see Table 1). In trace *A*, the mean response of 51 cells present in the same microscopic field, selected by not responding to NMDA, is shown. Heated DS (HDS) was prepared by heating DS at 90 °C for 10 min. For preparation of the extract from DS (DSE), 2·4 ml of methanol, 2·4 ml of chloroform and 1·2 ml of 1 N HCl were added to 1 ml of serum. After vigorous shaking, the chloroform phase was evaporated to dryness and redissolved in 1 ml of standard incubation medium containing 2 mg ml^{-1} defatted albumin. A similar extract was prepared from HDS (HDSE). DS, HDS, DSE and HDSE were all assayed at 1 : 30 dilution. *B* shows the response to several serum albumins, all tested at a final concentration of 2 mg ml^{-1}. FAFA, fatty acid-free bovine serum albumin (Sigma, A-6003); FrVA, serum albumin fraction V. Three types of FrVA were used: A-9647 and A-8022 (bovine) from Sigma, and human albumin from the Centre National de Transfusion Sanguine, Paris. Experiments are representative of 3 similar ones. *C–E*: correlations between the responses to DS and either HDS, DSE or FrVA A-8022. Responses are expressed as the mean $[Ca^{2+}]_i$ increase during the first 20 s following agonist addition, which was estimated in every single cell as the mean of all the $[Ca^{2+}]_i$ values during this integration period minus the mean $[Ca^{2+}]_i$ value before agonist addition. Lines were fitted by the least-squares procedure. The values for the correlation coefficients (r) were: *C*, 0·81 ($n = 51$); *D*, 0·99 ($n = 51$); *E*, 0·60 ($n = 108$). In *D* the values for 41 neurons present in the same microscope field are shown by filled circles for comparison.

activity was recovered in a chloroform–methanol–HCl extract (Bligh & Dyer, 1959) from DS or from HDS (DSE and HDSE, respectively, Fig. 3*A*). The responses to DS and to the chloroform–methanol–HCl extract (DSE) occurred in the same cells and were closely correlated (Fig. 3*D*). Finally, fraction V serum albumin (FrVA) at 2 mg ml^{-1} reproduced the effects of serum, whereas defatted serum albumin (FAFA) did not (Fig. 3*B*). Again, stimulation by FrVA and DS took place in the same cells (Fig. 3*E*).

DISCUSSION

Our results indicate that serum contains two different constituents able to increase $[Ca^{2+}]_i$ selectively in either neurons or glia, respectively. The neuron-stimulating factor was identified as glutamate on the basis of its presence in serum, its size, the reproducibility of the effects by exogenous glutamate or NMDA, their blocking by NMDA receptor antagonists and their prevention by enzymatic removal of glutamate. A minor fraction of the glia (18%, Table 1) gave a weak response to FS. The response in a subfraction of the cells (5% of all the glia) may be accounted for by glia having glutamate receptors other than NMDA. Glial responses to non-NMDA receptors have been described previously (Wyllie & Cull-Candy, 1994). Another subfraction (7% of all the glia) responded to an unidentified filterable plasma component different from glutamate, since response was not prevented by treatment with GPT. For the remaining glial cells subfraction (6% of all the glia) it is difficult to trace what they were responding to. The possibility that they were responding to some factor released from the neurons stimulated by FS should be considered, but this can only be suggested as a working hypothesis. In any case, the response of glial cells to FS was, when present, very small compared with the response of the neurons.

DS primarily stimulated glial cells, identified by their reactivity to GFAP antibodies. Stimulation of neurons by DS was sometimes observed (e.g. Fig. 2*D*), but was preceded by stimulation of surrounding glia. It has been reported that stimulation of glia with bradykinin produces release of glutamate from these cells, which secondarily stimulates neighbouring neurons (Parpura, Basarsky, Liu, Jeftinija, Jeftinija & Haydon, 1994). A similar mechanism could operate here. As a matter of fact, stimulation of neurons on addition of DS was never observed in the presence of glutamate receptor antagonists.

The identity of the glia-stimulating factor (GSF) present in DS is not clear. It is not bradykinin, thrombin or PAF because, although these agonists were able to increase $[Ca^{2+}]_i$ in some glial cells, the pattern was different. On the other hand, pharmacological blockers were unable to prevent its action. GSF is not formed by the activation of blood humoral or cellular mechanisms, as dialysed plasma had the same action as DS. GSF did not pass through ultrafiltration membranes (3000 Da cut-off) nor was it lost by dialysis (10 000 Da cut-off), suggesting that either it is a high molecular mass substance or it is strongly bound to plasma proteins. GSF is heat stable and not removed by diethyl ether, but it *is* extracted by chloroform–methanol–HCl mixtures. These results are compatible with GSF being a polar lipid which binds strongly to plasma proteins.

The effects of GSF documented here are reminiscent of those reported recently for a methanol-soluble factor attached to plasma albumin, which is able to induce calcium spikes and stimulate proliferation in astrocytes (Nadal, Fuentes, Pastor & McNaughton, 1994, 1995). This factor was present in most commercial fraction V serum albumins, but not in fatty acid-free preparations. It was non-dialysable, heat stable, inactivated by treatment with lipase and exhibited the extraction characteristics of a polar lipid (Nadal *et al.* 1995). The observation that the effects of serum GSF are reproduced by fraction V serum albumin, but not by defatted albumin (Fig. 3), strongly reinforces the idea that the GSF described here is the same substance as the methanol-soluble factor attached to serum albumin described before (Nadal *et al.* 1994, 1995).

An important cytotoxic role has been attributed to substances released from brain cells, especially when their action is combined with energy-depleting mechanisms (see the Introduction). It has been shown, for example, that combining added glutamate with either hypoxia or glucose deprivation kills cultured neurons within a few minutes (Novelli, Reilly, Lysko & Henneberry, 1988). In the intact animal suffering ischaemic damage, the glutamate is assumed to come from brain cells, either neurons or glia (Martin *et al.* 1994; Szatkowski & Attwell, 1994). Our results point out, however, that the neighbouring blood contains enough glutamate and GSF to produce a strong $[Ca^{2+}]_i$ increase in both neurons and glia. In particular, the concentration of glutamate in plasma, 50–100 μM, would be enough to kill neurons when combined with hypoxia or hypoglycaemia (Novelli *et al.* 1988). These considerations lead to speculation on the possible role of plasma factors in the brain damage following ischaemic accidents. Disruption of the blood–brain barrier, perhaps by hypoxia, might allow rapid entry and accumulation of blood constituents into the brain interstitial fluid. These substances may have cytotoxic effects, especially when acting jointly with hypoxia and hypoglycaemia resulting from the interruption of blood flow.

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