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Effects of phosphodiesterase inhibition on cortical spreading depression and associated changes in extracellular cyclic GMP

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Abstract

Cortical spreading depression (CSD) is a temporary disruption of local ionic homeostasis that propagates slowly across the cerebral cortex, and may contribute to the pathophysiology of stroke and migraine. Previous studies demonstrated that nitric oxide (NO) formation promotes the repolarisation phase of CSD, and this effect may be cyclic GMP (cGMP)-mediated. Here, we have examined how phosphodiesterase (PDE) inhibition, either alone or superimposed on NO synthase (NOS) inhibition, alters CSD and the associated changes in extracellular cGMP. Microdialysis probes incorporating an electrode were implanted into the frontoparietal cortex of anaesthetised rats for quantitative recording of CSD, pharmacological manipulations, and dialysate sampling for cGMP measurements. CSD was induced by cathodal electrical stimulation in the region under study by microdialysis. Extracellular cGMP increased, but only slightly, during CSD. Perfusion of either zaprinast or sildenafil through the microdialysis probe, at concentrations that inhibited both PDE5 and PDE9 (and possibly other PDE), increased significantly extracellular cGMP. Unexpectedly, these levels remained high when NOS was subsequently inhibited with N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME, 1 mM). The most interesting pharmacological effect on CSD was obtained with sildenafil. This drug altered neither CSD nor the subsequent characteristic effect of NOS inhibition, i.e. a marked widening of CSD. The fact that NOS inhibition still widened CSD in the presence of the high extracellular levels of cGMP associated with PDE inhibition, suggests that NO may promote CSD recovery, independently of cGMP formation.

Keywords: Cortical spreading depression; Nitric oxide; Cyclic GMP; Phosphodiesterase inhibition; Sildenafil; Microdialysis

1. Introduction

Cortical spreading depression (CSD) is a temporary disruption of local ionic homeostasis that propagates slowly across the cerebral cortex. This neurological abnormality is receiving renewed attention, because it has become clear that it contributes to lesion progression in experimental stroke, and possibly to the initiation of migraine attacks [1,2]. Nitric oxide (NO) formation is markedly enhanced during CSD [3], and we have demonstrated that the rapid initiation of the subsequent recovery of ionic homeostasis depends on this increased NO formation [4,5]. Cyclic GMP (cGMP) is generally considered to be the mediator through which NO exerts its actions when it is generated at physiological concentrations, subsequent to either activation of constitutive NO synthase(s) (NOS) or the application of NO donors [6]. In a recent study,[1] we have found that the cGMP analogue, 8-(4-chlorophenylthio)-cGMP (8-pCPT-cGMP) reversed concentration-dependently the characteristic widening of the CSD waves elicited under NOS inhibition, which suggested that the effect of NO on CSD recovery may be cGMP-mediated. Paradoxically, we could not reproduce the effect of NOS inhibition on CSD with soluble guanylyl cyclase (sGC) inhibitors.

To investigate further the role of NO/cGMP signalling in CSD recovery, we have examined in this study how CSD

Abbreviations: cGMP, cyclic GMP; CSD, cortical spreading depression; dc potential, direct current potential; IBMX, 3-isobutyl-1-methylxanthine; K_{Ca} channels, calcium-activated potassium channels; L-NAME, N^{ω}-nitro-L-arginine methyl ester hydrochloride; NO, nitric oxide; NOS, nitric oxide synthase; 8-pCPT-cGMP, 8-(4-chlorophenylthio)-cGMP; PDE, phosphodiesterase; sGC, soluble guanylyl cyclase.

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was altered when endogenous cGMP levels were increased by phosphodiesterase (PDE) inhibition, under normal conditions and when NOS was inhibited selectively at the site of CSD elicitation. Both zaprinast and sildenafil were used as cGMP-specific PDE inhibitors, but the concentrations that we used were likely to inhibit non-selective PDE as well [7,8]. Microdialysis probes incorporating an electrode [9] were implanted into the frontoparietal cortex of anaesthetised rats and used to carry out the following operations, within the same cortical region: (i) elicitation of individual CSD waves by cathodal electrical stimulation; (ii) recording of CSD; (iii) application of PDE inhibitors, with or without NOS inhibitor; and (iv) dialysate sample collection for the determination of changes in extracellular cGMP.

2. Materials and methods

2.1. Animals

Adult, male Sprague–Dawley rats (332 ± 11 g, mean ± S.E.M., $n = 17$; Harlan UK Ltd.) were used, with food and water available ad libitum. All animal procedures were authorised by the University of Bradford Ethical Review Panel and the British Home Office, Animals (Scientific Procedures) Act 1986, and performed in accordance with the associated guidelines. All efforts were made to minimise the number of animals used and their suffering.

2.2. Microdialysis probe implantation and CSD elicitation

Rats were anaesthetised throughout with halothane (5% for induction, 1.3–2.0% during the surgery, and 1–1.3% for maintenance) in O_2:N_2O (1:2), with the animal breathing spontaneously. Rectal temperature of animals was maintained at 37 °C throughout the experiment. Microdialysis probes incorporating a recording electrode (AN69 Hospal membrane, 0.25 mm o.d. × 1 mm fibre length; Type ME-H1, Applied Neuroscience Ltd.) [9] were implanted in the frontoparietal cortex (co-ordinates: 1.3–1.5 mm anterior to bregma, 2 mm lateral, and 1.8–2.0 mm deep from the cortical surface). The microdialysis probe/electrode was implanted slightly deeper than in the other studies in which CSD was elicited by high K-medium (i.e., 1.4–1.5 mm) [4,5], to ensure that the tip of the probe stainless-steel cannula made good contact with the exposed cortex. This was necessary because this part of the probe was later used as the cathode for electrical stimulation. Once implanted, the microdialysis probe was held in place with dental cement and two screws, but around 5 mm of the upper part of the stainless steel cannula was left free from cement to allow electrical connection. Using the stereotaxic frame as anode, the parameters for electrical induction of CSD were 7–10 mA applied for 0.5 s [10]. The stimuli were triggered and controlled by a computer-controlled constant current generator (Pulse buffer, NL510; Stimulus isolator, NL800, Neurolog System, Digitimer Ltd.).

Unless otherwise stated, microdialysis electrodes were perfused with artificial cerebrospinal fluid (ACSF; composition in mM: 125 NaCl, 2.5 KCl, 1.18 $MgCl_2$, 1.26 $CaCl_2$; pH 7.3 adjusted with 1 M NaOH, not buffered) at 1 μl/min with a syringe pump (CMA/100; CMA/Microdialysis). The microdialysis electrodes were subsequently used to carry out the following operations, simultaneously within the same cortical region [9,11]: (i) elicitation of CSD by electrical stimulation as described above; (ii) quantitative recording of CSD; (iii) collection of consecutive 20-min dialysate samples for cGMP determination; and (iv) local application of drugs. Restricting pharmacological manipulations at the site of CSD elicitation was especially suitable to avoid the possibility of peripheral effects of the NO-related drugs. In addition, it allowed circumventing the potential problem of drug penetration across the blood–brain barrier.

2.3. Electrophysiological recording

Both EEG and extracellular direct current (dc) potential were derived from the potential between the electrode built into the microdialysis probe and an Ag/AgCl reference electrode placed under the scalp of the rat [9]. This signal was first amplified with a high-impedance input, ac/dc preamplifier (NL102, Neurolog System, Digitimer Ltd.). The alternating current component in the 1–30 Hz window (5000× overall amplification) provided the EEG, and the dc component (250× or overall amplification) the extracellular dc-potential. All the recorded variables were continuously digitised; displayed; and stored using a personal computer equipped with an analogue/digital-converter. CSD was recognised as a large transient, negative shift of the dc-potential (Fig. 2).

2.4. Experimental procedure

As in our previous studies, experiments were started after a 2-h control-stabilisation period. It is relevant to note that such a delay was found sufficient for the normalisation of extracellular cGMP levels [12]. In order to minimise any possible interference of halothane anaesthesia with the biological processes under study, the halothane concentration was reduced to 1.0–1.3% (in 1:2 O_2:N_2O) during the recording period. Throughout this period, the depth of anaesthesia was monitored and adjusted through careful examination of the EEG recording and observation of the animal. Signs indicative of suitable anaesthesia included: regular and slow breathing, absence of whisker movements, and lack of reaction to brief tail pinches.

A previous study,[2] in which repeated, individual CSD were elicited by electrical stimulation showed that, except

[2] Urenjak J and Obrenovitch TP (unpublished observations).

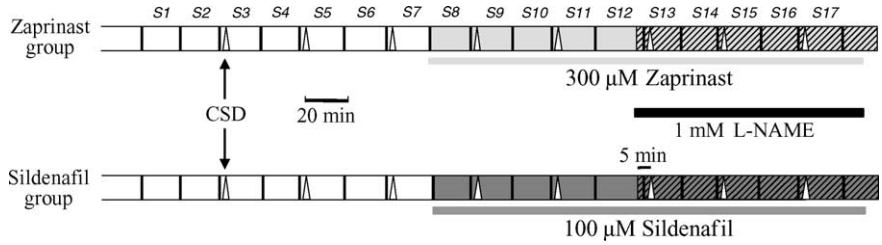

Fig. 1. Experimental procedure used to examine how PDE inhibition alters electrically-induced CSD, and the extracellular levels of cGMP. The PDE inhibitors, zaprinast and sildenafil, were perfused through the microdialysis probe/electrode, alone or with L-NAME. In both zaprinast and sildenafil groups, 17 consecutive 20-min dialysate samples (delineated by the black vertical lines) were collected, starting 2-h post implantation. Eight CSD were elicited (vertical open triangles), each followed by 40 min of recovery. No drug was applied during the 1st three CSD. Zaprinast (300 μM in 0.3% DMSO, light grey area) or sildenafil (100 μM, pH 5.0, dark grey area) was applied, starting 20 min before the 4th CSD elicitation. L-NAME (1 mM, dashed area) was co-applied with the PDE inhibitor starting 5 min prior to the 6th CSD elicitation.

for the 1st CSD, the amplitude and pattern of the elicited CSD were consistent throughout the experimental procedure under normal conditions (i.e., no drug treatment). Therefore, to reduce the number of animals used, and the cost associated with cGMP radioimmunoassay, only two groups with repeated measures were used, each for the investigation of the effects of a PDE inhibitor, zaprinast or sildenafil (Fig. 1). In both zaprinast ($n = 10$) and sildenafil ($n = 7$) groups, 17 consecutive 20-min dialysate samples were collected, starting 2-h post implantation. The 1st two samples were used to determine the basal level of extracellular cGMP. Eight CSD were then elicited by electrical stimulation, with each CSD followed by 40-min period of recovery. Whenever the 7 mA initial electrical stimulation failed to induce CSD, or produced a small CSD, a higher current (e.g., 9–10 mA) was applied for the subsequent CSD elicitation.

As one would expect cGMP production to be suppressed or reduced when cortical NOS was inhibited by N^{ω}-nitro-L-arginine methyl ester (L-NAME) (see however, Laitinen et al. [12]), we speculated that cGMP accumulation subsequent to PDE inhibition may not be detectable when it is superimposed on NOS inhibition. Accordingly, instead of attempting to reverse the effects of L-NAME on CSD by PDE inhibition, we chose to examine how a previous accumulation of cGMP due to PDE inhibition might alter the subsequent action of NOS inhibition (Fig. 1). We verified that DMSO at the concentration of 0.3% did not alter CSD (data not shown), and this DMSO concentration was unlikely to alter cGMP levels [13].

2.5. Determination of dialysate cGMP concentrations

The serial 20-min dialysate samples were stored at −80 °C, and their cGMP concentrations determined within 5 days by using a radioimmunoassay (RIA) kit (^{125}I-cGMP-RIA kit, Amersham). As this kit is designed for the analysis of 100 μl samples, the 20 μl dialysate samples were diluted five times by addition of 80 μl assay buffer. The initial range of sensitivity of the kit was 2–128 fmol/100 μl. As the concentrations of cGMP to be measured were expected to be in the low micromolar range, the range of standards was extended to include the 1 fmol/100 μl concentration. For the same reason, the optional acetylation procedure was carried out to increase the RIA sensitivity. This additional step, to be carried out prior to the addition of ^{125}I-cGMP and antibody, aims to convert the cGMP contained in the samples to be analysed to its acetylated form (in position 2), because the resulting substituted cyclic nucleotide has a greater affinity for the antibody than cGMP [14,15]. The antiserum (100 μl) was added first, then the radiolabeled tracer (^{125}I-cGMP, approximately 1500 cpm per sample), and the resulting mixture incubated at 4 °C overnight. Bound and unbound ^{125}I-cGMP were separated by addition of 500 μl charcoal mixture, immediately followed by centrifugation at $3000 \times g$ for 15 min at 4–10 °C. The charcoal mixture composition was, in mg: gelatine 100, dextran 135, and charcoal 1352 in 40 ml RIA buffer. The rationale for using dextran/gelatin-treated charcoal is that dextran and gelatin block medium and large size pores, leaving the charcoal small pores free to capture unbound cGMP. Both supernatant and pellet were then counted in a gamma counter (AutoGamma 5000 series, Canberra Packard Instruments) set for ^{125}I detection.

As the RIA kit was used in the lowest part of its concentration range, an elaborate calculation strategy was used. The averaged background (determined by counting three empty tubes) was subtracted from all supernatants and pellets counts. For each sample, the ratio of radioactivity of supernatant activity (bound ^{125}I-cGMP) versus total activity (supernatant + pellet) was then determined, to normalise the small inter-sample differences that can occur during the pipetting of the radioactive tracer and the antiserum solutions. In addition, the following modifications to the Amersham kit procedure were made: (i) the 0% displacement (α), used to determine the small amount of radioactivity that was not trapped by the charcoal, was obtained with the mixture, 200 μl assay buffer + 100 μl radioactive cGMP; (ii) the 100% displacement (β), used to determine the maximal antiserum binding, was obtained with the mixture, 100 μl assay buffer + 100 μl antiserum + 100 μl radioactive cGMP. The calibration standard values were then expressed as the percentage binding (X) of the

real maximal antiserum binding (β–α). Finally, these percentage values were transformed by using the logit function, to linearise the calibration curve, i.e. log of standards concentrations versus logit X. The same calculations were carried out to determine the percentage binding X for each dialysate sample, and the corresponding dialysate cGMP concentrations obtained from the calibration curve. Dialysate cGMP concentrations are given in Section 3 as pmol/l. The statistical analysis for cGMP data was carried out on the averaged cGMP levels, computed in each experiment for basal level, CSD alone, CSD + PDE inhibition, and CSD + PDE and NOS inhibition (Fig. 4).

2.6. Drugs and chemicals

All drug solutions were either prepared on the day of the experiment, or from frozen stock solution aliquots that were used only once. Unless otherwise stated, all drug-containing perfusion media were not buffered, but their pH were ultimately adjusted to 7.3 using 1 M NaOH. L-NAME was purchased from Tocris Cookson Ltd. and dissolved in ACSF. L-NAME media were prepared from aliquots of 1 M L-NAME stock solutions kept frozen at −20 °C. Zaprinast was purchased from Tocris Cookson Ltd. The stock solution of zaprinast (100 mM in 100% DMSO, stored at −20 °C) was diluted to 300 μM with ACSF (with or without 1 mM L-NAME). Sildenafil citrate was a generous gift from Pfizer UK Ltd. It was dissolved in acidified ACSF (pH adjusted to 5.0; with or without 1 mM L-NAME) as sildenafil precipitates in neutral media. New solutions of sildenafil were prepared before each experiment. Analytical grade activated charcoal was purchased from Merck Chemicals; dextran (average molecule weight, 73,000) was from Sigma-Aldrich; and gelatine powder was from BDH Laboratory Supplies. All other chemicals were of analytical grade.

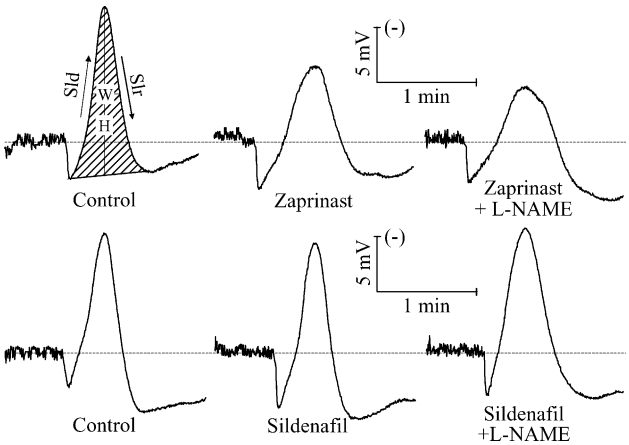

Fig. 2. Representative CSD waves elicited by electrical stimulation in controls (no drug treatment, left traces), in the presence of a PDE inhibitor (300 μM zaprinast or 100 μM sildenafil; middle traces), and when both PDE and NOS were inhibited (right traces). Note the different effects of zaprinast (300 μM) and sildenafil (100 μM) on CSD (see also Figs. 3 and 4). The left upper trace also shows the variables that were used to examine quantitatively the effects of the drugs under study on CSD. H, CSD amplitude (mV); W, CSD width (s); Sld and Slr, maximum rate of depolarisation and repolarisation, respectively (mV·min); dashed area, CSD area (mV·min).

2.7. Data presentation and statistical analysis

The polarity of the dc potential was reversed so that negative shifts (i.e., depolarisation) produce an upward deflection. The variables used to examine the drug effects on the cortex susceptibility to CSD elicitation, and on the pattern of individual CSD wave, are described in Fig. 2. All values in Section 3 are expressed as means ± S.E.M. Specific hypotheses were tested through the comparison of selected measures within each individual group, using the paired t-test. P values less than 5% were considered statistically significant.

3. Results

3.1. Electrically-induced CSD under normal conditions

Electrical stimulation using the tip of the implanted microdialysis probe as cathode produced a consistent artefact on the dc-potential signal (sudden positive shift, Fig. 2), immediately followed by a transient negative shift of the dc-potential (Fig. 2) to which a marked reduction of local EEG activity was associated (data not shown), events which are characteristic of CSD elicitation. In both zaprinast and sildenafil groups, the control CSD (i.e., 1st three elicitations) were very reproducible, except that the 1st CSD had a tendency to be slightly more pronounced than the subsequent. In the zaprinast group, CSD amplitude, width at (1/2) height, and maximum slopes of depolarisation and repolarisation for the 3rd CSD were 10.7 ± 1.2 mV, 23.1 ± 2.4 s, 53.1 ± 9.4 mV/min, and 45.2 ± 5.9 mV/min (n = 10), in respective order. Similar values (no significant difference) were obtained with the corresponding CSD in the sildenafil group.

3.2. Effects of zaprinast and sildenafil, alone, on CSD

Zaprinast (300 μM) reduced markedly and significantly the amplitude of CSD (Figs. 2–4; $P < 0.01$, comparison of 3rd CSD to the 5th CSD, paired t-test; n = 10), suppressing completely some of the 5th and subsequent CSD elicitation in 4 out of 10 experiments. Preliminary experiments showed that zaprinast (1 mM in 1% DMSO-ACSF) also abolished completely CSD elicitation induced by high K-medium, and this effect was unlikely to be due to the vehicle. Presumably as a consequence of CSD suppression, the depolarisation and repolarisation slopes were decreased, and the CSD width increased by zaprinast (Fig. 3; $P < 0.05$, $P < 0.01$ and $P < 0.05$ in respective order; comparison of the 5th CSD to the 3rd CSD using the

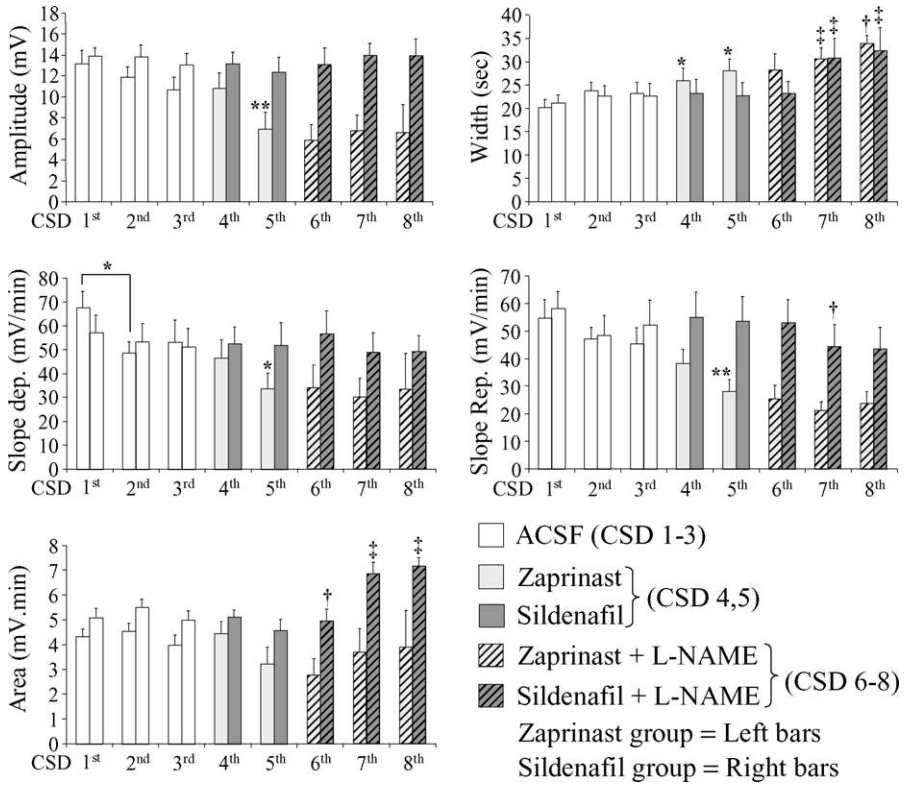

Fig. 3. Effects of the two PDE inhibitors, zaprinast and sildenafil, alone or with co-application of the NOS inhibitor, L-NAME on electrically-induced CSD. Note the different effects of the two PDE inhibitors on CSD. Zaprinast abolished CSD elicitation in 4 of 10 experiments; whenever this occurred, a null value was taken for the CSD amplitude and area, but no value was entered (i.e., missing data) for the other variables of the corresponding, suppressed CSD. Values in bar charts are means ± S.E.M. Specific measurements within individual group were compared using the paired *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$; comparison with control (i.e., 3rd CSD). $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$; comparison with CSD under PDE inhibition alone (i.e., 5th CSD).

paired *t*-test; $n = 10$). In contrast to zaprinast, sildenafil (100 μM in pH 5.0 ACSF) had no effect on CSD (Figs. 2–4).

3.3. Effects of NOS inhibition, superimposed on zaprinast and sildenafil

Superimposition of L-NAME (1 mM) on zaprinast did not alter the amplitude of CSD, but significantly increased its width (Fig. 3; $P < 0.05$, comparison of the 8th CSD to the 5th CSD with the paired *t*-test; $n = 10$). However, the delayed initiation of CSD recovery that is characteristic of NOS inhibition alone [4] was not observed (Fig. 4). The effect of NOS inhibition was more marked in the sildenafil group, possibly because CSD was not suppressed by this PDE inhibitor. In this case, both CSD width and area were increased (Fig. 3; $P < 0.01$, comparison of the 8th CSD to

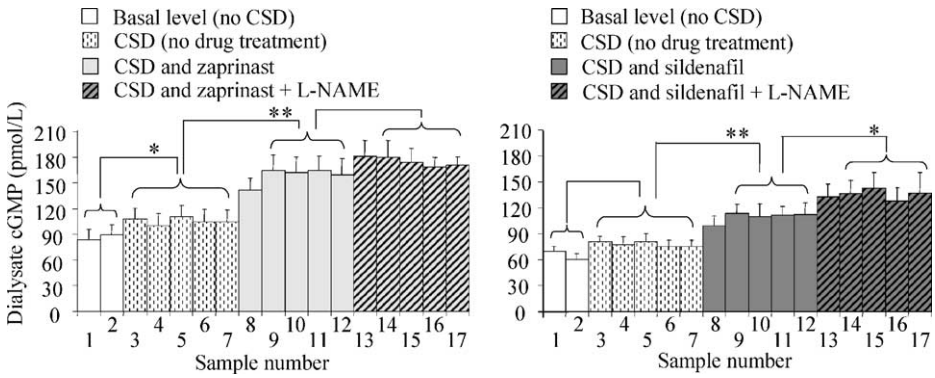

Fig. 4. Effects of PDE inhibition with zaprinast (300 μM) or sildenafil (100 μM), alone or with the NOS inhibitor, L-NAME (1 mM) on the levels of extracellular cGMP associated with electrically-induced CSD. Values in the bar charts are means ± S.E.M. Statistical analysis was carried out on the averaged cGMP levels, computed in each experiment for samples 1 and 2 (basal level), 3–7 (effect of CSD), 9–12 (PDE inhibition alone), and 14–17 (PDE and NOS inhibition). Samples 8 and 13 were not included in the corresponding averaged values to avoid the possibility that, in these 1st samples collected after the start of pharmacological manipulation, the drug treatment might not be fully effective. Specific measurements within individual group (see graphs) were compared using the paired *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

the 5th CSD, paired *t*-test; $n = 7$), and there was also a slight, but significantly decrease of the repolarisation slope (Fig. 3; $P < 0.05$, comparison the 7th CSD to the 5th CSD, paired *t*-test; $n = 7$). All these changes are those that consistently occurred with NOS inhibition alone [4,5].

3.4. Effects of CSD and PDE inhibition, alone or with NOS inhibition on dialysate cGMP levels

The basal dialysate levels of cGMP were 85.1 ± 13.4 pmol/l ($n = 10$) and 65.4 ± 5.5 pmol/l ($n = 7$) in the zaprinast and sildenafil group, respectively. There was no significant difference between these two levels. Elicitation of a single CSD in each other sample (i.e., each 40 min; see experimental procedure in Fig. 1) increased slightly the dialysate levels of cGMP (Fig. 4), by 24.3% in the zaprinast group ($P = 0.047 < 0.05$; $n = 10$), and by 19.1% in the sildenafil group, but the latter change did not reach significance ($P = 0.063$, paired *t*-test; $n = 7$).

Perfusion of zaprinast and sildenafil through the microdialysis probe, as CSD was still elicited each 40 min, produced a marked increase in dialysate cGMP, by 53.8% from a level of 105.8 ± 12.9 pmol/l in the zaprinast group, and by 42.9% from a level of 77.9 ± 7.2 pmol/l in the sildenafil group ($P < 0.01$, paired *t*-test; $n = 9$ and 6, respectively). Unexpectedly, NOS inhibition (1 mM L-NAME, superimposed on PDE inhibition) did not reduce the cGMP levels (Fig. 4), but actually increased them slightly, and this effect was significant in the sildenafil group ($P < 0.05$, paired *t*-test; $n = 6$).

4. Discussion

4.1. Methodological considerations

4.1.1. Experimental strategy

Electrical stimulation was previously used to elicit CSD and investigate its propagation [10,16,17] but, in this study, it was used to elicit CSD precisely at the implantation site of a microdialysis probe/electrode. This strategy enabled us to combine (i) quantitative CSD recording, (ii) direct, selective pharmacological manipulations, and (iii) neurochemistry (dialysate cGMP levels) at the site of CSD elicitation. In comparison to our previous studies, it allowed us to avoid the sustained perfusion of high K-medium that was used to trigger either single or repeated CSD waves [4,5]. The pattern of the CSD waves induced by electrical stimulation in this study was similar to those reported previously [10,16,17]. The 1st elicited CSD was generally slightly more pronounced than the subsequent ones, possibly because the tissue remained refractory to some degree to each subsequent CSD, despite the 40 min of recovery. The pattern of the 2nd and 3rd CSD elicited under normal conditions was consistent with that of K^+-induced CSD [4].

4.1.2. Significance of CSD and drug-induced changes in extracellular cGMP determined in the cortex of anaesthetised rats

Cyclic GMP is a key *intracellular* second messenger and, therefore, investigations into cGMP signalling should ideally include measurements of changes in cGMP at intracellular level, where cGMP is acting on its molecular targets. However, continuous or repeated measurements of intracellular cGMP are not possible. Indeed, so far, only total tissue cGMP measurements can be performed, and this requires fast fixation of the tissue under study (e.g., by microwave inactivation [18]) because of the very rapid formation and degradation of this messenger. This justifies why monitoring of dialysate cGMP has been widely used for investigations of the glutamate/NO/cGMP pathway in various brain regions (for a review, see Fedele and Raiteri [19] and references therein). It is generally assumed that extracellular cGMP levels parallel intracellular changes, because a probenecid-sensitive organic anion transporter was found to contribute to the efflux of cGMP from liver slices into the incubation medium [20], and this cGMP transport system was subsequently demonstrated to be present in the rat cerebellum [21]. Whether this assumption is still valid when CSD (and the associated increased NO formation) occurs is a difficult issue. Firstly, CSD implies a collapse of ionic homeostasis and pronounced metabolic changes that could result in a temporary dysfunction of cGMP transport across the cellular membrane. Secondly, because the organic anion transport system responsible for cGMP efflux from the cytosol may be saturable [20], and one cannot rule out the possibility that during CSD-induced activation of NO/cGMP signalling, the intracellular cGMP levels might become high enough to saturate the transporter (see, however, Ref. [22]). Nevertheless, an effective efflux of cGMP was found when cerebellar slices were exposed to a depolarising concentration of K^+ (60 mM) [21].

Previous studies showed that halothane had different effects on the NO/cGMP pathway among various brain regions but, in the cerebral cortex, this anaesthetic altered neither basal cGMP production nor its stimulation by NO donors [23]. Finally, an inherent weakness of microdialysis for the study of transient neurological abnormalities such as CSD is time resolution. In this study, the sensitivity of the cGMP RIA precluded us to use a sampling period shorter than 20 min (i.e., a duration around 10 times that of a CSD wave), and individual CSD waves were elicited only each 40 min (Fig. 1). This probably contributed to the small magnitude of the changes in dialysate cGMP that we found associated with CSD (Fig. 4).

4.1.3. PDE inhibition by zaprinast and sildenafil in our experimental conditions

PDE5, PDE6 and PDE9 are the cGMP-specific PDE that have been identified so far. PDE6 is apparently confined to the retina. With regard to the distribution of PDE5 among different organs, in mice, western blot showed that the

highest expression was in the lung, followed by heart and cerebellum, but a lower signal was evident in brain [24]. RT–PCR analysis confirmed that PDE5 is expressed in the rat brain, including cortex [25], but immunocytochemistry only detected the PDE5 protein in cerebellar Purkinje cells [26]. Therefore, PDE5 may be expressed in the cortex, but at a very low level. In contrast, PDE9A variants are highly expressed in the brain [27], including in layers II, V, and VI of the rat neocortex [28]. With regard to PDE that can use either cAMP or cGMP as substrate (i.e., non-selective PDE1, 2, 3, 10, 11) these are expressed in the cerebral cortex, especially PDE3 [29].

Sildenafil is a very potent and selective inhibitor of PDE5 (in vitro IC_{50} for PDE5, 0.0035 μM [7]), but in our study this drug probably inhibited also PDE9 because the in vitro IC_{50} of sildenafil for PDE9 is in the low micromolar range [8] and 100 μM of the drug was perfused through the microdialysis probe. The same rationale applies to zaprinast, as its in vitro IC_{50} for PDE5 and PDE9 are 1.1 and 35 μM for PDE5 and PDE9, respectively [7,8], and 300 μM of the drug was perfused through the microdialysis probe. In addition, perfusion of 100 μM sildenafil might have also inhibited non-selective PDE [7]. High concentrations of zaprinast and sildenafil were used, firstly because only a fraction of the perfusion medium drug concentration reaches the surrounding tissue in microdialysis experiments (microdialysis delivery ratio was probably 20–30% in our experimental conditions), and secondly because lower concentrations had no effect on CSD in preliminary experiments. In addition, a previous microdialysis study[3] showed that high concentrations of zaprinast (1 mM) and sildenafil (100 μM) caused a relatively small increase in cortical extracellular cGMP levels, in comparison to that produced by the non-specific PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX). There are at least two plausible explanations for the differential effects of IBMX and sildenafil/zaprinast on extracellular cGMP: (i) sildenafil and zaprinast may not penetrate well into brain cells (or be pumped out)—this is supported by the previous finding that direct treatment of rat hippocampal slices with sildenafil (0.1–100 μM) failed to increase tissue cGMP ([30], see however Ref. [25]); (ii) at the drug concentrations tested, sildenafil and zaprinast may inhibit the extrusion of cGMP from intracellular to extracellular space, where cGMP was measured by microdialysis ([31], see however Ref. [32]), whereas this may not occur with IBMX ([22], see however Refs. [33,34]).

4.2. Changes in extracellular cGMP associated with CSD—effects of PDE inhibition alone or combined with NOS inhibition

In the present study, the basal dialysate concentration of cGMP was 60–90 pmol/l, around two-fold the level found in a previous microdialysis study of the cerebral cortex, but this discrepancy is compatible with the different microdialysis flow rates that were used [12]. In contrast to the three-fold increase in tissue cGMP that was reported to occur during CSD, with a further rise to four-fold greater than controls after the CSD wavefront [35], we observed only a slight increase in dialysate cGMP with CSD (Fig. 4). This discrepancy probably arose from the methodological limitations discussed above, i.e. uncertainty related to the detection of intracellular changes in cGMP with microdialysis, and inadequate sampling frequency relative to the duration and number of CSD that were elicited.

As expected, PDE inhibition with either zaprinast (300 μM in perfusion medium) or sildenafil (100 μM) produced a significant increase in dialysate cGMP levels (Fig. 4). Taking into account the potency of these two PDE inhibitors (see above), one could have anticipated a larger increase in dialysate cGMP levels. However, as we have already mentioned, high concentrations of zaprinast (1 mM) and sildenafil (100 μM) caused a relatively small increase in cortical dialysate cGMP, in comparison to that produced by IBMX.[3] One possible explanation for this apparent discrepancy is that, at the concentrations tested, zaprinast and sildenafil also reduced the efficacy of cGMP extrusion (see above subsection). Alternatively, in our experiments, zaprinast and sildenafil could have only reduced (but not abolished) the potential of the tissue surrounding the microdialysis probe to hydrolyse cGMP.

Taking into account the length of time during which NOS was subsequently inhibited by perfusion of 1 mM L-NAME (i.e., 100 min), one would have expected the dialysate cGMP levels to be progressively reduced when NOS inhibition was superimposed on CSD and PDE inhibition, especially if one assumes that cGMP could still be hydrolysed in the region under study. However, this was not the case; the dual inhibition of PDE and NOS had even the tendency to increase further the dialysate levels of cGMP (Fig. 4).

On the one hand, this lack of effect of L-NAME on extracellular cGMP agrees with the previous observation that L-NAME did not alter the basal extracellular levels of cGMP in the frontal cortex of chloral hydrate anaesthetised rats [12]. On the other hand, the same authors (Laitenen and colleagues) subsequently found that L-NAME suppressed by 70% cGMP levels, when these were previously elevated by the PDE inhibitor, IBMX [36]. Different effects of NOS inhibition on extracellular cGMP were reported in other brain regions: in the cerebellum of chloral hydrate anaesthetised or conscious rats, L-NAME or L-NARG reduced by 74–80% the basal level of cGMP [12,37]; in the hippocampus of conscious rats, NOS inhibition markedly reduced the extracellular levels of cGMP under both normal conditions (i.e., basal levels) and PDE inhibition by IBMX [38]. All these data suggest that cGMP levels may be differently regulated among various brain regions, and that cGMP levels may not be consistently

[3] Professor E. Fedele, University of Genova, personal communication.

regulated by NO acting on sGC in the frontal cortex. Alternatively, in the cortex, the changes in intracellular cGMP may be inadequately reflected extracellularly, except when there is a marked accumulation of cGMP such as that achieved with IBMX.

4.3. Effect of NOS inhibition on the initiation of CSD recovery—role of intracellular cGMP

The most interesting and novel finding in this study was that, when NOS inhibition with 1 mM L-NAME was superimposed on PDE inhibition with sildenafil (100 μM), the widening of individual CSD wave (i.e., the key effect of NOS inhibition on CSD) [4] was still observed despite a significant increase in extracellular levels of cGMP (Fig. 4). Furthermore, in preliminary experiments, the NOS inhibition associated widening of individual CSD wave was also found when the non-specific PDE inhibitor, IBMX (1 mM) was perfused 2 h prior to, and during NOS inhibition. These findings, and the fact that the effect of NOS inhibition on CSD recovery could not be reproduced by inhibition of sGC,[1] suggest that this characteristic effect of NOS inhibition on CSD may not be linked to a subsequent, reduced cGMP formation. In other words, the role of NO in the promotion of the initiation of CSD recovery may not involve NO/cGMP signalling, but rather the *S*-nitrosation of a specific target protein by NO [39], as its formation is increased during CSD. Paradoxically, we have found that the effect of NOS inhibition on CSD recovery could be completely reversed by the cGMP analogue, 8-pCPT-cGMP.[1]

4.4. Calcium-activated potassium-channels (K_{Ca})—possible down-stream molecular target of NO and promoter of CSD recovery

Several complementary elements support this possibility. Firstly, the function(s) of K_{Ca} channels make them suitable for a potential role during the repolarisation phase of CSD. Indeed, these K^+ channels participate in action potential repolarisation and are essential to the slow after-hyperpolarisation [40], In addition, K_{Ca} channels are activated by calcium influx, i.e. a feature of the ionic changes that occur during CSD [41]. Secondly, NO was found to activate directly K_{Ca} channels (i.e., without any cGMP requirement) in both vascular smooth muscle and brain cells [42,43]. Such an action would be compatible with our finding that sGC inhibitors did not reproduce the effect of NOS inhibition on CSD,[1] and the persistent effect of NOS inhibition on CSD when extracellular cGMP is still elevated (Figs. 3 and 4). In addition, another study with dithiothreitol showed that this reducing agent also widened markedly K^+-induced CSD,[4] hence mimicking the primary effect of NOS inhibition on CSD [4]. Thirdly, K_{Ca} channels were also demonstrated to be a target of cGMP-dependent protein kinase phosphorylation [44,45]. For example, the membrane-permeable cGMP analogue, dibutyryl cGMP enhanced four- to five-fold the activity of K_{Ca} channels in cell-attached membrane patches [46]. Such an action would be compatible with our finding that 8-pCPT-cGMP reversed the effect of NOS inhibition on CSD.[1]

In short, activation of K_{Ca} channels would appear capable of initiating and promoting CSD repolarisation, and the possibility of a dual mechanism for the enhancement of their activation by NO [46] allows us to reconcile all our current and previous pharmacological findings.

[4] Urenjak J and Obrenovitch TP (unpublished observations).

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