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In vivo microdialysis study of a specific inhibitor of soluble guanylyl cyclase on the glutamate receptor/nitric oxide/cyclic GMP pathway

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1 Nitric oxide (NO) is known to stimulate soluble guanylyl cyclase, thereby eliciting an elevation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in target cells. Recently, a selective inhibitor of soluble guanylyl cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), has been identified and characterized *in vitro*. We have investigated the *in vivo* effects of ODQ on the glutamate receptor/NO/cyclic GMP pathway by monitoring extracellular cyclic GMP during microdialysis of the cerebellum or the hippocampus of freely-moving adult rats.

2 Intracerebellar administration of ODQ (1-100 μM) via the microdialysis probe inhibited, in a concentration-dependent manner, the basal extracellular level of cyclic GMP. The maximal inhibition, measured after a 20 min perfusion with 100 μM ODQ, amounted to 80% and persisted unchanged as long as ODQ was perfused. When ODQ was removed from the perfusion stream after 20 min, the levels of cyclic GMP started to recover, suggesting reversibility of guanylyl cyclase inhibition by ODQ.

3 The cyclic GMP response evoked in the cerebellum by NMDA (200 μM) or by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA; 100 μM) was largely attenuated by 100 μM ODQ. The pattern of the inhibition curves suggests competition for guanylyl cyclase between ODQ and the NO generated by NMDA or AMPA receptor activation.

4 ODQ (100 μM) prevented the elevation of extracellular cyclic GMP levels provoked by intracerebellar infusion of the NO generator S-nitroso-N-acetylpenicillamine (SNAP; 1 mM). The inhibition of the SNAP effect was rapidly relieved when ODQ was removed from the perfusion fluid. However, ODQ (100 μM) was unable to affect the cyclic GMP response elicited by 5 mM SNAP, in keeping with the proposed idea that ODQ binds to the 'NO receptor' in a reversible and competitive manner.

5 Infusion of ODQ (10, 100 or 300 μM) into the hippocampus of freely-moving rats diminished the basal extracellular level of cyclic GMP. The maximal inhibition amounted to 50% and was produced by 100 μM ODQ.

6 The cyclic GMP response observed when 1 mM SNAP was perfused in the hippocampus, similar in percentage terms to that seen in cerebellum, was dramatically reduced during co-infusion of 100 μM ODQ.

7 ODQ appears to act *in vivo* as a selective, reversible and possibly competitive inhibitor of the soluble guanylyl cyclase targeted by NO. This enzyme may generate most (about 80%) of the cyclic GMP found under basal conditions in the extracellular space of the cerebellum. In the hippocampus, about 50% of the basal cyclic GMP does not seem to originate from the ODQ-sensitive soluble guanylyl cyclase.

Keywords: NMDA receptors; AMPA receptors; nitric oxide; guanylyl cyclase; cyclic GMP; ODQ; microdialysis; cerebellum; hippocampus

Introduction

Nitric oxide (NO), either synthesized endogenously from L-arginine through the catalysis of NO synthase or originating from exogenously administered NO donors, binds to and activates soluble guanylyl cyclase, thereby producing an increase of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in target cells. In the CNS, endogenous NO is synthesized in response to activation of ionotropic glutamate receptors of the N-methyl-D-aspartate (NMDA) or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) type (Garthwaite *et al.*, 1988; Bredt & Snyder, 1989; Knowles *et al.*, 1989; Garthwaite, 1991; Maura *et al.*, 1995).

Understanding the functional significance of a complex pathway like that comprising activation of NMDA/AMPA receptors, of NO synthase and of guanylyl cyclase requires the availability of selective tools able to dissect out the various steps of the pathway. Although several pharmacological tools are available for studying the glutamate receptor/NO synthase/cyclic GMP pathway, a selective inhibitor of the soluble form of guanylyl cyclase, the NO 'receptor', has been missing until recently, and this has hindered the investigation of the functions of cyclic GMP in NO signal transduction. Garthwaite & coworkers (1995) have just introduced a new compound, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), which, based on a careful *in vitro* study, appears to be the first selective and potent inhibitor of soluble guanylyl cyclase.

Given the potential role that ODQ may play in further elucidating the pathophysiology of the NO-cyclic GMP system, it was important to validate *in vivo* the characteristics of the novel compound. Previous studies showed that monitoring extracellular cyclic GMP during *in vivo* microdialysis of the cerebellum (Vallebuona & Raiteri, 1993; 1995; Luo *et al.*, 1994) or the hippocampus (Vallebuona & Raiteri, 1994; 1995) permits the study of the glutamate receptor/NO synthase/cyclic GMP pathway in freely-moving rats. In the present work we investigated the effects of ODQ on cerebellar or hippocampal

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extracellular levels of cyclic GMP released under basal conditions, during activation of NMDA or AMPA glutamate receptors and following administration of a NO generator.

Methods

Neurosurgery and dialysis procedure

Male Sprague-Dawley rats (250–300 g, CD-COBS, Charles River, Calco, Italy) were anaesthetized with Equitesin 3 ml kg^{-1}, placed in a stereotaxic frame (David Kopf Instruments) and implanted with a microdialysis probe which was transversely positioned in the cerebellum or the dorsal hippocampi according to the following coordinates: cerebellum AP = −2.3, H = +6.0; hippocampus AP = +3.8, H = +6.5 from the interaural line (Paxinos & Watson atlas, 1986). A piece of dialysis fibre made of a co-polymer of acrylonitrile sodium methallyl sulphonate (AN69HF Hospal S.p.A., Bologna, Italy; 0.3 mm o.d. with more than 40,000 mol. wt cut-off) was covered with epoxy glue to confine dialysis to the area of interest (8 and 10 mm glue-free zone for the cerebellum and the hippocampi, respectively). The skull was exposed and two holes were drilled on the lateral surface at the level of cerebellar cortex or dorsal hippocampi. One dialysis probe, held straight by a tungsten wire inside, was inserted transversely into the brain so that the glue-free zone was exactly located in the target area. The tungsten wire was withdrawn and stainless steel cannulae (22-gauge diameter, about 15 mm long) were glued to the ends of the fibre. These ends were bent up and fixed vertically to the skull with dental cement and modified Eppendorf clips. After a 24 h recovery period, rats were placed in perspex cages and the probes perfused at a flow rate of 5 $\mu l\ min^{-1}$ (CMA/100 microinjection pump, Carnergie Medicine, Stockholm, Sweden) with artificial cerebrospinal fluid (artifical CSF) containing (in mM): NaCl 145, KCl 3, $CaCl_2$ 1.26, $MgCl_2$ 1, buffered at pH 7.4 with 2 mM phosphate buffer. Consecutive samples were collected every 20 min following a washout period of 1 h and assayed for their cyclic GMP content by a commercially available radioimmunoassay kit (Amersham dual range, Amersham Radiochemical Centre, Buckinghamshire, U.K.). At the end of the experiment, rats were killed by euthanasia and the correct position of the probe was verified by histological examination of the fibre tract.

Statistics and expression of results

The data presented are expressed as percentages of basal values. The cyclic GMP content of the first 2 – 3 samples collected before drug treatments was averaged and considered as the basal value. Differences between control (artificial CSF alone) and drug-treated animals were analysed by two-way ANOVA with repeated measures over time followed by Newman-Keuls multiple comparison test. Differences were considered significant at the level of $P<0.05$.

Materials

N-methyl-D-aspartic acid (NMDA), (RS)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), S-nitroso-N-acetylpenicillamine (SNAP) and 1H-[1,2,4]oxadiazole[4,3-a] quinoxaline-1-one (ODQ) were purchased from Tocris Cookson (Bristol, U.K.). AMPA (100 mM) was dissolved in the minimum amount of NaOH (0.1 M), the pH adjusted to 7.4 with HCl (0.1 M) and the desired volume achieved by addition of 0.01 M phosphate buffer: subsequent dilutions were made in artificial CSF. ODQ (10 mM) was dissolved in dimethyl sulphoxide (DMSO); DMSO at the concentration of 3% (present in the solution of ODQ 300 μM) slightly (approx. 20%), though not significantly, increased cyclic GMP basal levels only in the 20 min-fraction following its addition to the perfusion artificial CSF. Basal cyclic GMP levels then returned to control values in all the following fractions (data not shown). However, 1% DMSO did not modify cyclic GMP basal levels and was not included in the aCSF used for controls.

The experimental procedures *in vivo* were approved by the Ethics Committee of the Institute of Pharmacology and Pharmacognosy, University of Genoa, according to European legislation on the use and care of laboratory animals (CEE 86/609).

Results

Decrease by ODQ of basal extracellular cyclic GMP levels in the cerebellum of freely-moving rats

Local application of varying concentrations of ODQ (1, 5, 10, 50 or 100 μM) caused a concentration-dependent decrease in basal dialysate cyclic GMP levels (Figure 1). The nucleotide levels were halved during infusion of 5 μM ODQ. The maximal inhibition observed amounted to 80%; it was obtained during the first 20 min of infusion with 100 μM ODQ and maintained as long as ODQ was administered. When the administration of ODQ was interrupted following 20 min, the extracellular levels of cyclic GMP started to recover towards control levels (Figure 4a).

Blockade by ODQ of the cyclic GMP responses evoked by NMDA or AMPA in the cerebellum

A dramatic elevation of extracellular cyclic GMP efflux was observed when the cerebellum was perfused for 20 min with 200 μM NMDA (Figure 2a) or with 100 μM AMPA (Figure 2b), in medium containing 1 mM Mg^{2+}. Local infusion of 100 μM ODQ, from 20 min before administration of the excitatory amino acids until the end of the experiment, largely attenuated the NMDA- or AMPA-evoked cyclic GMP response.

ODQ prevents the cerebellar cyclic GMP response elicited by the NO generator SNAP

As shown in Figure 3, administration of SNAP (1 mM) via the dialysis probe caused a pronounced increase of extracellular cyclic GMP. This effect was relatively slow with respect to that

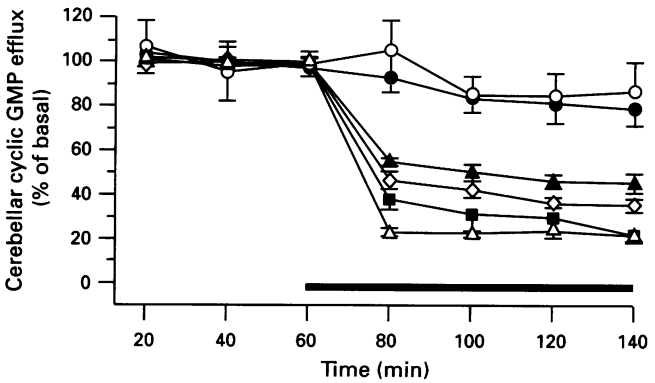

Figure 1 Effect of the guanylyl cyclase inhibitor ODQ on basal extracellular levels of cyclic GMP in the cerebellum of freely-moving rats. Time-course pattern of the inhibition of cyclic GMP efflux by ODQ 1 (●), 5 (▲), 10 (◇), 50 (■) and 100 μM (△) which was perfused locally for the time indicated by the horizontal solid bar; (○) controls (aCSF alone). Cyclic GMP absolute basal levels (not corrected for the *in vitro* recovery) amounted to 185.7 ± 20.3 fmol $100\ \mu l^{-1}$ ($n=50$). Each point represents the mean ± s.e.mean of 4 different experiments. For sake of clarity, statistical symbols have been omitted but ODQ-induced effects were always significant at the level of $P<0.05$ at least. For further technical details see Methods.

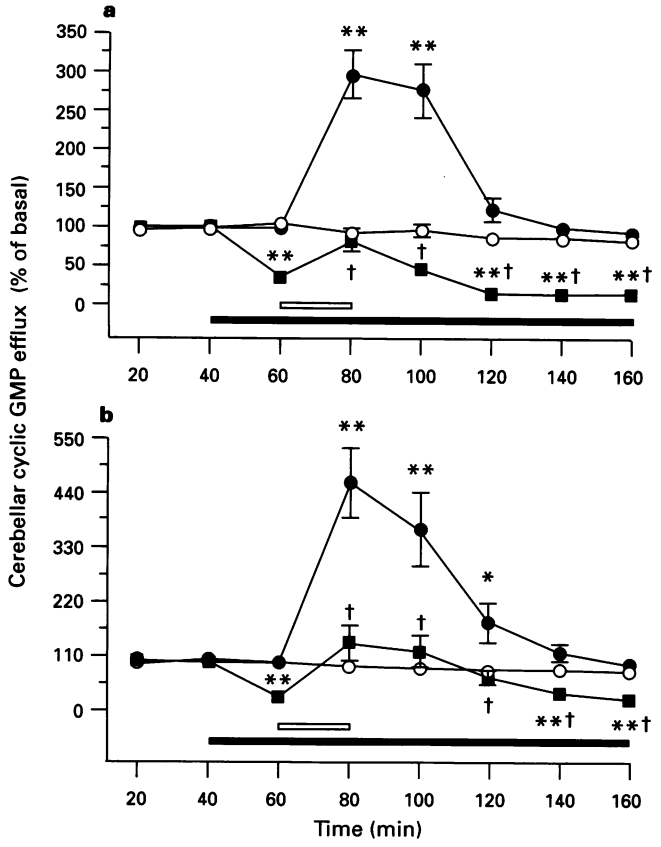

Figure 2 Blockade by ODQ of the NMDA- or AMPA-induced increase in cyclic GMP extracellular levels in the cerebellum of freely-moving animals. NMDA 200 μM (●; a) or AMPA 100 μM (●; b) were perfused through the probe for 20 min (open horizontal bar) whereas ODQ 100 μM (■; solid horizontal bar) was present one fraction before and together with NMDA or AMPA; (○) controls (aCSF alone). Each point represents the mean ± s.e.mean of 4–5 different experiments. *$P<0.05$ and **$P<0.01$ *vs.* controls and basal values; †$P<0.01$ *vs.* 200 μM NMDA or 100 μM AMPA.

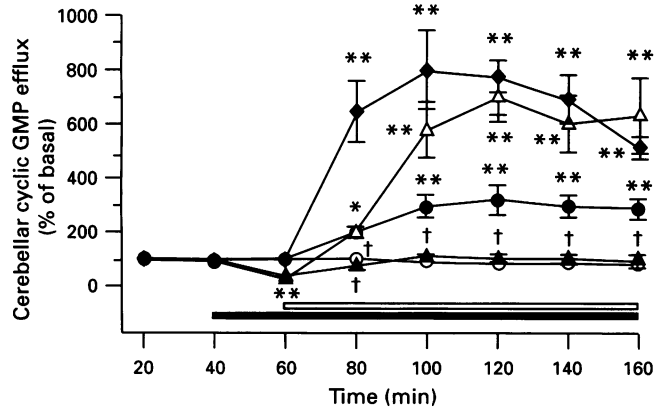

Figure 3 Effects of ODQ on the SNAP-induced increase in cyclic GMP extracellular levels in the cerebellum of freely-moving rats. SNAP 1 mM (●), SNAP 1 mM + ODQ 100 μM (▲); SNAP 5 mM (◆) or SNAP 5 mM + ODQ 100 μM (△) were perfused locally through the dialysis probe for the time indicated by the horizontal open bar. ODQ 100 μM (solid horizontal bar) was present in the perfusion stream one fraction before and together with SNAP; (○) controls (aCSF alone). Each point represents the mean ± s.e.mean of 4 different experiments. *$P<0.05$ and **$P<0.01$ *vs.* controls and basal values; †$P<0.01$ *vs.* 1 or 5 mM SNAP.

evoked by NMDA or by AMPA. The SNAP (1 mM)-evoked cyclic GMP response was almost nullified by co-infusing 100 μM ODQ. When SNAP was perfused at higher concentrations (5 mM), ODQ (100 μM) was able to attenuate the cyclic GMP response only during the first 20 min, while the inhibition was completely overcome in all the following fractions. As shown in Figure 4b, following a 20 min pretreatment with 100 μM ODQ plus 20 min of SNAP (1 mM) and ODQ (100 μM) co-infusion, removal of ODQ permitted rapid restoration of the cyclic GMP response evoked by SNAP (cf. Figures 3 and 4b).

Basal extracellular levels of cyclic GMP in the hippocampus of freely-moving rats: inhibition by ODQ

Local application of varying concentrations of ODQ (10, 100 or 300 μM) into the hippocampus diminished the efflux of cyclic GMP observed under basal conditions. The maximal inhibition of the basal extracellular levels of the nucleotide was rapidly reached in the first 20 min fraction collected during infusion with 100 or 300 μM ODQ (Figure 5). This maximal effect (50%) was significantly lower than that (about 80%) produced in the cerebellum by 50 μM ODQ (cf. Figures 5 and 1).

ODQ prevents the SNAP-induced cyclic GMP response in hippocampus

Figure 6 shows that SNAP (1 mM), infused into the dorsal hippocampi of freely-moving rats, induced a slow but pronounced increase in the extracellular levels of cyclic GMP. Co-administration of 100 μM ODQ largely prevented the cyclic GMP response evoked by the NO donor.

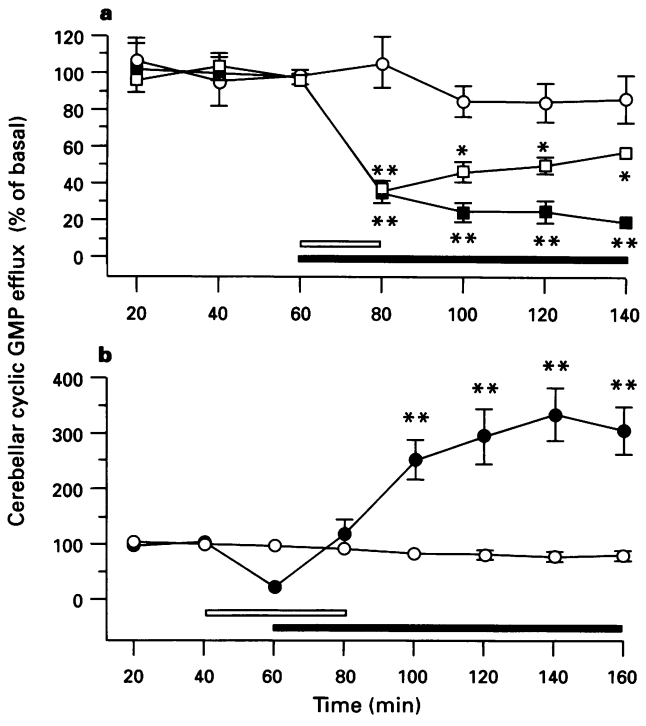

Figure 4 ODQ reversibly inhibits basal and SNAP-induced cyclic GMP efflux in the cerebellum of freely-moving rats. (a) ODQ (50 μM) was perfused into the cerebellum for 80 (■; data redrawn from Figure 1) or 20 min (□) as indicated by the horizontal solid and open bars, respectively. (b) SNAP (1 mM; ●) was present in the perfusion stream for the time indicated by the horizontal solid bar. ODQ (100 μM) was present one fraction before and one fraction together (open bar) with SNAP and was then removed from the perfusion fluid; (○) controls (aCSF alone). Each point represents the mean ± s.e.mean of 3–4 different experiments. *$P<0.05$ and **$P<0.01$ *vs.* controls and basal values.

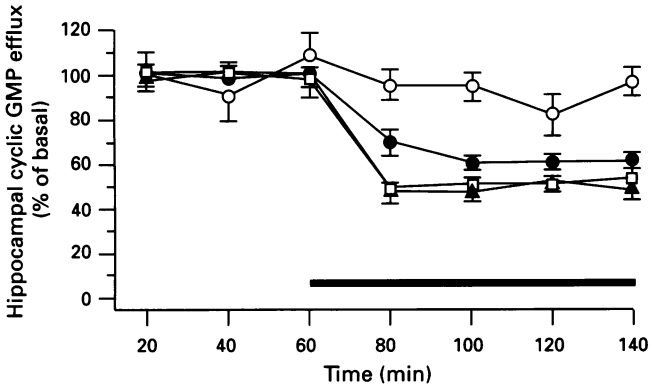

Figure 5 Effect of ODQ on basal extracellular levels of cyclic GMP in the hippocampus of freely-moving rats. Time-course pattern of the inhibition of cyclic GMP efflux by ODQ 10 (●), 100 (▲) and 300 μM (□) which was perfused locally for the time indicated by the horizontal solid bar; (○) controls (aCSF alone). Cyclic GMP absolute basal levels (not corrected for the *in vitro* recovery) amounted to 18.30 ± 1.93 fmol $100\,\mu l^{-1}$ ($n=24$). Each point represents the mean ± s.e.mean of 4 different experiments. For sake of clarity, statistical symbols have been omitted but ODQ-induced effects were always significant at the level of $P<0.05$ at least.

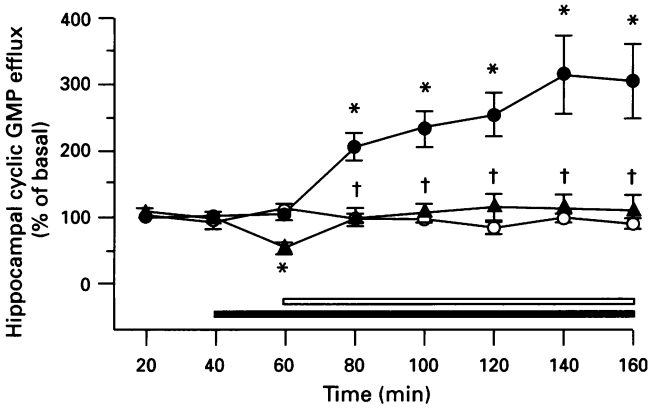

Figure 6 SNAP-induced elevation of cyclic GMP extracellular levels is prevented by ODQ in the hippocampus of freely-moving rats. SNAP (1 mM; ●) was perfused in the dorsal hippocampi for the time indicated by the horizontal open bar. ODQ (100 μM; ▲) was present in the perfusion stream one fraction before and together with SNAP (horizontal solid bar); (○) controls (aCSF alone). Each point represents the mean ± s.e.mean of 4 different experiments. *$P<0.01$ *vs.* controls and basal values; †$P<0.01$ *vs.* 1 mM SNAP.

Discussion

Results from an *in vitro* study show that ODQ is a potent inhibitor of NO-sensitive soluble guanylyl cyclase activity, without actions on the particulate form of the enzyme. ODQ does not seem to interfere with any of the steps leading to NO formation; furthermore, it does not inhibit constitutive or inducible NO synthase activity and it does not inactivate NO (Garthwaite *et al.*, 1995).

In freely-moving rats, the baseline level of cyclic GMP in the cerebellar dialysate was maximally lowered to about 20% by ODQ. In contrast to the cerebellum, the extracellular levels of cyclic GMP under basal conditions in the hippocampus could be reduced by ODQ only to a maximum of 50% (cf. Figures 1 and 4). Membrane-bound and soluble forms of guanylyl cyclase have been identified (Chinkers & Garbers, 1991; Koesling *et al.*, 1991). It is known that NO-induced cyclic GMP synthesis occurs via activation of the soluble form of the enzyme (Nakane *et al.*, 1990; Ignarro, 1991). Thus, assuming that ODQ acts also *in vivo* as a selective inhibitor of soluble guanylyl cyclase, our data suggest that: (a) brain extracellular cyclic GMP released under basal conditions originates both from NO-activated soluble guanylyl cyclase and from NO-insensitive membrane-bound guanylyl cyclase; (b) while 80% of the basal cyclic GMP released in the cerebellum is produced by NO acting at soluble guanylyl cyclase, about 50% of the basal cyclic GMP released in the hippocampus originates from the particulate enzyme activated by agents different from NO, including atrial natriuretic factor. The increase in cyclic GMP levels elicited by this factor, possibly in astroglial cells (Friedl *et al.*, 1989; De Vente *et al.*, 1990), has been found to be ODQ-insensitive (Garthwaite *et al.*, 1995). Results from previous microdialysis experiments are consistent with the present data concerning ODQ and the conclusions drawn here. In fact, the basal extracellular level of cyclic GMP in the cerebellum of freely-moving rats was reduced to about 20% by administration of the NO synthase inhibitor, N^G-nitro-L-arginine (Vallebuona & Raiteri, 1993) whereas, in the hippocampus, the maximal inhibition of the basal level of cyclic GMP did not exceed 50% (Vallebuona & Raiteri, 1994). Support for the present findings also comes from studies of hybridization histochemistry of soluble guanylyl cyclase mRNA in the rat brain. These studies show that cells in the granular layer of the cerebellum contained high levels of transcripts, whereas only low levels of soluble guanylyl cyclase mRNA were found in the hippocampus, mainly localized in the pyramidal layer of CA1-3 and in the granular layer of the dentate gyrus (Burgunder & Cheung, 1994).

In *in vitro* experiments performed with cerebellar slices of immature (8-day-old) rats, the inhibition by ODQ of the cyclic GMP response induced by NMDA was found to be fully reversible after a 1 h washout period (Garthwaite *et al.*, 1995). ODQ also appears to behave as a reversible inhibitor of soluble guanylyl cyclase in the cerebellum of adult living rats. As shown in Figure 4a, inhibition of basal efflux of cerebellar cyclic GMP caused by ODQ started to be relieved as soon as drug administration was terminated. Full recovery of extracellular cyclic GMP levels was not observed, since the experiment was interrupted after 140 min of perfusion; at this time the cyclic GMP curve was steadily rising towards control values. The washout of ODQ is clearly slower *in vivo* than *in vitro*, probably because of tissue diffusion of the drug away from the probe. That ODQ is a reversible inhibitor of NO-sensitive guanylyl cyclase is also indicated by the data illustrated in Figure 4b. In these experiments ODQ was first administered alone for 20 min, then it was coinfused with SNAP for an additional 20 min period; finally, ODQ was removed from the perfusion fluid while SNAP administration was continued. The cyclic GMP response elicited by 1 mM SNAP was fully restored soon after removal of ODQ (cf. Figures 4b and 3), as one would expect if guanylyl cyclase is inhibited by ODQ in a reversible manner.

On the other hand, continuous infusion of ODQ (100 μM) dramatically inhibited the SNAP (1 mM)-induced cyclic GMP elevation; the observation that ODQ, at a concentration which should maximally block guanylyl cyclase under basal conditions (Figure 1), was not able to prevent completely the increase caused by 1 mM SNAP suggests competition between the newly generated NO and the guanylyl cyclase inhibitor. This view is corroborated by the results obtained when SNAP was infused at higher (5 mM) concentrations; in this case, ODQ partly diminished the cyclic GMP increase during the first 20–40 min of SNAP infusion but such inhibition was clearly surmounted in the following fractions. These data are compatible with the view that newly generated NO can displace ODQ from its binding site on soluble guanylyl cyclase and that the site where ODQ binds may coincide with the NO recognition site on the enzyme.

Local administration of the excitatory amino acids NMDA or AMPA was reported to produce elevations of extracellular cyclic GMP in the cerebellum of freely-moving adult rats

subjected to microdialysis (Vallebuona & Raiteri, 1993; Luo *et al.*, 1994; Fedele & Raiteri, 1996). The effects of both NMDA and AMPA could be totally abolished by the NO synthase inhibitor N^G-nitro-L-arginine (Vallebuona & Raiteri, 1993; Fedele & Raiteri, 1996) indicating the involvement of NO in the cyclic GMP response. As shown in Figure 2, ODQ largely attenuated the cyclic GMP responses to NMDA or to AMPA confirming *in vivo* the observations previously made in cerebellar (Garthwaite *et al.*, 1995) and hippocampal (Boulton *et al.*, 1995) slices. This result, together with the sensitivity to NO synthase inhibition, indicates that both responses are mediated via stimulation by NO of soluble guanylyl cyclase activity. The patterns of the curves showing NMDA- or AMPA-evoked cyclic GMP release in the presence of ODQ deserve a brief comment. In fact, in the absence of excitatory amino acids, these curves would be very similar to the curve illustrating the inhibition of basal cyclic GMP efflux caused by 100 μM ODQ, i.e. by the maximally effective concentration of the inhibitor (Figure 1). Indeed, a 20 min ODQ pretreatment caused a marked inhibition of cyclic GMP control values. However, during infusion of NMDA or AMPA for 20 min, extracellular cyclic GMP returned towards basal levels (Figures 2a and b) suggesting that the NO produced following glutamate receptor activation could partly displace ODQ from its binding site on soluble guanylyl cyclase.

Before the identification of ODQ, two other compounds have been used as guanylyl cyclase inhibitors, namely methylene blue and LY-83583. The action of these two compounds as inhibitors of the enzyme has never been established convincingly. Indeed, methylene blue appears to be a generator of superoxide anions and a NO synthase inhibitor (Mayer *et al.*, 1993), while LY-83583 is reported to inhibit NO release (Mulsch *et al.*, 1988) and to display other effects apparently unrelated to guanylyl cyclase inhibition (Barbier & Lefebvre, 1992; Kontos & Wei, 1993; Luond *et al.*, 1993). For these reasons, the results obtained with these drugs are sometimes difficult to interpret. ODQ, a compound that inhibits soluble guanylyl cyclase in a potent, selective and reversible manner, both *in vitro* and *in vivo*, will be of great help in further elucidating the pathophysiology of the NO-cyclic GMP system.

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