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Brain cAMP response to phosphodiesterase inhibitors in rats killed by microwave irradiation or decapitation

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The level of cAMP in tissue depends on the relative activities of the enzymes adenylate cyclase (AC) and 3′,5′-cyclic nucleotide phosphodiesterase(s) (PDE). When the brain tissue is left intact after the animal's death, rapid and extensive changes in the level of cAMP take place, thus preventing the determinations of *in vivo* levels by conventional methods.

Decapitation shock may imbalance the AC/PDE relation, most probably in favour of the AC activity. PDE inhibitors would then be expected to raise cAMP levels. We have therefore developed a microwave irradiation (MWI) device for *in vivo* cAMP determination. Since stress has been reported to influence cAMP levels in the cerebral cortex of rats [1, 2], restraint of the experimental animal should be avoided. One way of overcoming this problem would be to use a short-acting, strong and focused MWI pulse. cAMP levels in different brain regions of rats killed by MWI, which were taken as *in vivo* levels, were therefore compared with rats sacrificed by decapitation. Two PDE inhibitors were examined more closely—rolipram, a potential antidepressant [3], and IBMX, a classical methylxanthine inhibitor.

Methods and results

Animal treatment. Male Wistar rats (140–190 g) were injected via the tail vein 1 or 2 min before sacrifice with suspensions of PDE inhibitors in saline containing 10% (w/v) Cremofor EL. ICI 63.197 (2-amino-6-methyl-8-propyl-2,3-dihydro-[1,2,4]-triazolo[4,3-a]pyrazin-7-oxide) was a gift from Imperial Chemical Industries (U.K.) and Ro 20-1724 [4-(3-butoxy-4-methoxy-benzyl)-2-imidazolidone] was donated by Hoffmann-La Roche (Basel, Switzerland). Rolipram [ZK 62711, 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone] and its stereoisomers were synthesized by Dr. R. Schmiechen, Schering. IBMX (3-isobutyl-1-methylxanthine) and all other compounds were obtained from commercial suppliers.

Procedures. The MWI device (Püschner GmbH, Schwanewede, F.R.G.) had a variable output power of up to 10 kW at 2450 MHz. Untrained rats were positioned to run into the polypropylene tube topped by a Plexiglas cone. At the end of the cone, the rat triggered the onset of irradiation by interrupting an infrared light beam. MWI of 1 sec, 6 kW was chosen. After MWI the rats were decapitated and the heads cooled on crushed ice. Brain parts were homogenized in 1 mmole/l. formic acid in 75% ethanol. After centrifugation at 5000 *g* for 15 min, aliquots of the supernatant were lyophilized. Tissue derived from decapitated or inadequately irradiated animals was frozen on dry ice within 2 min of sacrifice. cAMP was determined by a protein binding method [4]. cAMP PDE activity was measured in the 30,000 *g* supernatant of whole forebrain (20 mmole/l. Tris–HCl, 5 mmole/l. $MgCl_2$, pH 7.5) with 1 μmole/l. [^{32}P]cAMP (New England Nuclear, Boston, MA) by a two-step method [5, 6]. cAMP was absorbed onto charcoal, and the liberated [^{32}P]-phosphate quantified in the supernatant. The frontal cortex was defined by a vertical cut at the level of the anterior commissure. Data were subjected to analysis of variance, followed by Scheffe or Dunnett tests.

Time course of post-mortem cAMP accumulation. The cAMP content in the cerebellum rapidly increased between 0.5 and 10 min after decapitation. Related to zero-time accumulation (defined by 1 sec MWI), a half-maximal increase was reached at 30 sec, with a maximum level (*ca.* 4-fold) 4 min after decapitation.

Time course of enzyme inactivation by MWI. The cAMP PDE activity and cAMP levels in the cerebrum and cerebellum were determined after MWI lasting 0.6, 0.8, 1.0 or 1.2 sec, or after decapitation (zero time). As shown in Fig. 1, after MWI of 0.8 sec there was some residual PDE activity in the cerebrum. After 1 sec MWI, the PDE activity in both parts of the brain fell below the detection limit. A constant level of cAMP in the cerebellum was achieved after 1 sec. In the cerebrum the cAMP level was less dependent on the radiation time and, in contrast to the cerebellum, increased slightly with prolonged MWI.

Decapitation vs MWI (Fig. 2). Whereas in the frontal cortex the cAMP levels were identical both in animals killed by MWI or decapitation, in the cerebellum and the remainder of the cerebrum cAMP increased significantly after decapitation (140 and 50%, respectively). In the striatum the cAMP level decreased by 50% after decapitation. For verification of stable conditions after MWI, the tissue was left at room temperature for 30 min. The regions of the brain investigated did not differ from the MWI group that was assayed immediately.

Effect of treatment with rolipram and IBMX. The cAMP response to different treatments depended on the mode of tissue fixation and the brain area investigated (Table 1). The brain tissue of decapitated animals exhibited elevated cAMP levels after treatment with both PDE inhibitors. IBMX, a very potent *in vitro* PDE inhibitor [7], at 3 mg/kg raised the cAMP levels in all areas except that most responsive to the decapitation stimulus, the cerebellum. In the decapitation group rolipram was qualitatively similar but somewhat more potent than IBMX, and produced a pronounced cAMP accumulation even in the cerebellum.

The *in vivo* level of cAMP, as defined by MWI tissue fixation, gave clearly different pictures for rolipram and IBMX. IBMX at the doses administered did not change the *in vivo* levels in three of the four areas, while rolipram showed enhanced cAMP levels in these areas even at the lower dose. Table 2 summarizes the *in vivo* effects of the stereoisomers of rolipram and some other PDE inhibitors on cerebral cAMP levels. (−)-Rolipram was clearly more active than the (+)-isomer in this regard and about equally active to the racemate, consistent with the stereospecific reduction of neurotropic effects in the rat [8]. The cAMP-enhancing effect of Ro 20-1724 is in accord with the recent findings of Kant *et al.* [9].

Discussion

Since its first application by Stavinhoa *et al.* [10], microwave inactivation of brain tissue has become the most reliable method for determining the *in vivo* levels of cAMP. An additional advantage of MWI is the preservation of gross structural integrity of most brain areas, and the ease of dissection and further analytical processing. Since the strongest microwave apparatus to date (7.3 kW, 2450 MHz) has been limited to mice [11], we have developed a device suitable for rats. It was optimal at a power output of 6–8 kW for 1 sec. With this irradiation schedule, damage of the hypothalamic area and some loss of olfactory lobe tissue had to be taken into account. Higher energy densities caused severe vacuolization of the tissue at the base of the brain, and the expulsion of the adjacent tissue through the olfactory sinus. Besides the need for reproducible posi-

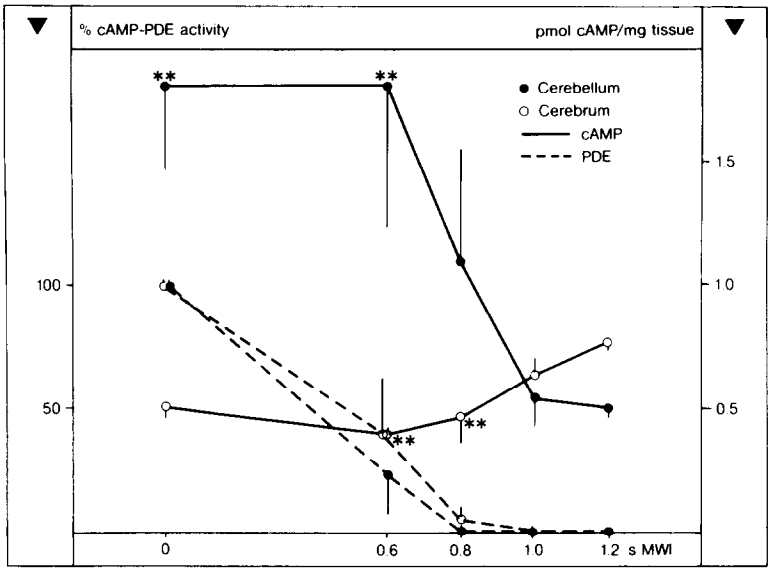

Fig. 1. cAMP level and cAMP PDE activity in rat cerebrum and cerebellum after various times of MWI (6 kW). 0 sec = decapitation. Tissue samples were prepared immediately after sacrifice. Shown are the means ± S.D., n = 5. **P < 0.01 vs 1.0 sec MWI.

tioning of the rat brain at the point of MWI focus, we also used the running tube to avoid causing stress to the animals by immobilization. Although we did not observe altered cAMP levels in the whole brain following 10 min restraint of the rat in a wire cage (data not shown), this could have been due to already elevated stress levels. We did not succeed in testing this possibility by adapting the rats to the procedure, because they would not repeatedly enter the running tube. The mechanism of changes in cAMP after decapitation is not known, but an imbalance of AC and PDE activity could be brought about through inhibition of PDE by an endogenous factor [12], or through the release of stimulatory or inhibitory factors for AC activity. Undetectable PDE activity at shorter irradiation times did not necessarily imply stable levels of cAMP, as can be seen after 0.8 sec MWI in the cerebellum (Fig. 1, and ref. [13]). At this time cAMP levels were higher than after 1 sec MWI, indicating residual AC activity to be the most likely cause in post-decapitation cAMP changes. Thus for the interpretation of elevated cAMP levels in response to any substance, factors other than their PDE inhibitory activity also have to be considered. In decapitation experiments the most obvious is the influence on cAMP levels of adenosine, which increases markedly during hypoxia [14]. The adenosine sensitivity of AC shows regional heterogeneity [15, 16]. A considerable blockade of adenosine uptake into rat brain synaptosomes has been reported recently for most PDE inhibitors [17]. Of the drugs tested in the present investigation, Ro 20-1724 was the most effective with an IC_{20} of 10 nmole/l. Rolipram, ICI 63.197 and papaverine were *ca.* 10–30 times less active [17]. Alkylxanthines are known to antagonize adenosine stimulation as well as the inhibition of AC [18, 19]. These processes will either enhance or depress cAMP production, depending on the relative abundance of the respective AC within a particular brain area. This may be the reason for the effectiveness of the methylxanthines IBMX and theophylline only in the post-decapitation cAMP accumulation.

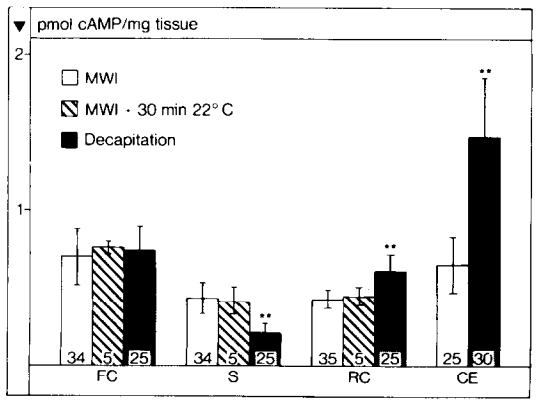

Fig. 2. cAMP content of rat brain regions which were frozen immediately after MWI, 30 min after MWI, or immediately after decapitation. FC: Frontal cortex; S: striatum; RC: remainder of cerebrum; CE: cerebellum. Shown are the means ± S.D.; n as indicated in the columns. **P < 0.01 vs MWI group.

The ability of rolipram, ICI 63.197 and Ro 20-1724 to raise cAMP *in vivo* levels and other pharmacological data [20, 21] strongly suggests that these substances exert their characteristic behavioural effects through cAMP signalling pathways. This group of substances has in common the ability to inhibit preferentially cAMP rather than cGMP degradation [22–24], in contrast to, for example, the unselective methylxanthine PDE inhibitors. For a selective PDE inhibitor, extrapolation of *in vitro* data obtained with crude PDE preparations to the *in vivo* situation is inappropriate: besides pharmacokinetic differences, selective inhibition of a PDE isoenzyme which is quantitatively of minor importance *in vitro* but functionally coupled closely to *in vivo* AC activity, as has been discussed [25], will appear weak *in vitro*. There are several lines of evidence which support this assumption: (1) the inhibitory potency of Ro 20-1724 on PDE from erythrocytes is three orders of magnitude higher than in cerebral cortex [26]; (2) the high inhibitory potency of ICI 63.197 is selective for only one of five PDEs isolated by isoelectric focusing [27]; and (3) rolipram exhibits potent yet only partial PDE inhibition in rat brain supernatant [22].

While the enhancement of cAMP accumulation in the brain tissue of decapitated animals reflects the sum of PDE inhibitory action and interference with artefactually

Table 1. cAMP (pmole/g tissue) in rat brain regions after decapitation or MWI

Treatment i.v., 2 min	Dose mg/kg	Frontal cortex decap.	Frontal cortex MWI	Striatum decap.	Striatum MWI	Remainder of cerebr. decap.	Remainder of cerebr. MWI	Cerebellum decap.	Cerebellum MWI
Vehicle (decap.: n = 20; MWI: n = 28)		680	670	190	450	590	400	1440	680
Rolipram (decap.: n = 10; MWI: n = 9)	0.3	900	1030**	260	680**	840**	630**	2630	770
	3.0	1290**	1380**	440**	800**	1120**	800**	3090**	920**
IBMX (decap.: n = 5; MWI: n = 9)	0.3	670	730	230	410	650	450	1270	740
	3.0	1060**	640	320**	450	1010**	560**	1610	660

Shown are the means of n animals as indicated. S.E.M. was on average 7.5%, with an extreme of 15%, of the corresponding value. **Significantly different from vehicle, $P < 0.01$.

Table 2. cAMP in rat cerebrum after treatment with PDE inhibitors and MWI

Treatment i.v., 1 min	Dose mg/kg	cAMP, % of controls
(±)-Rolipram	0.3	130**
(−)-Rolipram	0.3	138**
(+)-Rolipram	0.3	98
Ro 20-1724	0.3	131
Ro 20-1724	3.0	225**
ICI 63.197	3.0	208**
IBMX	0.3	98
IBMX	3.0	111
IBMX	10.0	135*
Papaverine	100	129
Theophylline	100	112

n = 3–10. Significantly different from vehicle; *$P < 0.1$; **$P < 0.01$.

involved AC activity, the level of cAMP in MWI tissue of drug-treated animals reflects the direct *in vivo* action on PDE and/or AC systems. Since cAMP levels rapidly increase following treatment with rolipram, ICI 63.197 and Ro 20-1724, it is proposed that the activity of these PDE inhibitors is directed to an PDE isoenzyme involved in the rapid turnover of a specific compartment of cAMP.

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Desferrioxamine: a scavenger of superoxide radicals?

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The iron chelating agent desferrioxamine is frequently used to inhibit the iron-catalyzed production of hydroxyl radicals ($OH^{\cdot}$) by superoxide generating systems [1–5]. This effect of desferrioxamine can be related either to iron chelation or to the intervention of an iron-independent mechanism, such as a direct scavenging effect on reactive oxygen radicals. Whereas desferrioxamine was shown to scavenge $OH^{\cdot}$ in certain conditions [6], the present study was performed to test a possible reactivity of desferrioxamine on superoxide radicals ($O_2^{\cdot}$).

Materials and methods. Desferrioxamine methane sulfonate (Desferal) was from Ciba-France. Xanthine oxidase (EC 1.2.3.1), horseradish peroxidase (EC 1.11.1.7), nitroblue tetrazolium (NBT), ferricytochrome c, xanthine sodium salt, phenol red, diethylenetriaminepentaacetic acid (Detapac) were obtained from Sigma Chemical Co., St Louis, MO, U.S.A. Other reagent grade chemicals were from Merck, Darmstadt, F.R.G.

The ability of desferrioxamine at different concentrations to scavenge $O_2^{\cdot}$ and thereby inhibit reactions mediated by $O_2^{\cdot}$ was assayed by measuring the inhibition of the reduction of ferricytochrome *c* or NBT mediated by the aerobic action of xanthine oxidase on xanthine [7, 8]. Reductions of ferricytochrome *c* to ferrocytochrome *c* and of NBT to formazan were measured at 550 nm and 560 nm, respectively, at 25°C, in an Uvikon model 820 recording spectrophotometer. Xanthine oxidase was itself assayed by following the conversion of xanthine to urate at 292 nm. Detapac was added, when quoted, to the assay mixture in order to chelate trace amounts of iron and prevent thereby the production of $OH^{\cdot}$ radicals by the xanthine–xanthine oxidase which occurs in presence of traces of iron [9].

H_2O_2 production in the xanthine–xanthine oxidase system was measured in presence and in absence of desferrioxamine. After incubation during 15 min, 100 μl of reactive media were removed and H_2O_2 measured according to [10] after addition of a buffered solution (140 mM NaCl, 10 mM potassium phosphate buffer pH 7.0, 5.5 mM dextrose, 0.28 mM phenol red and 8.5 U/ml of horseradish peroxidase). The samples were incubated for 5 min at room temperature and brought to pH 12.5 by addition of NaOH. Absorbance was measured at 610 nm and compared to H_2O_2 standards and appropriate reagent banks.

Results and discussion. We studied the ability of desferrioxamine to compete with ferricytochrome *c* for a flux of $O_2^{\cdot}$ generated by the action of xanthine oxidase on xanthine. Figure 1 shows that desferrioxamine inhibited in a concentration-dependent fashion this reduction of ferricytochrome *c* mediated by $O_2^{\cdot}$. Further experiments using NBT, a substrate involving no iron, instead of ferricytochrome *c* showed that desferrioxamine inhibits NBT reduction by $O_2^{\cdot}$ in the same way as ferricytochrome *c* reduction (results not shown). This finding excludes the intervention of iron chelation in the observed effects.

A direct inhibitory action of desferrioxamine on xanthine oxidase can be excluded as desferrioxamine does not modify the rate of urate formation from xanthine as shown previously by Gutteridge *et al.* [1] and confirmed in our experimental conditions (results not shown).

As the results were consistent with desferrioxamine acting as a scavenger of $O_2^{\cdot}$ like ferricytochrome *c* itself, we studied the competition between desferrioxamine and cytochrome *c* for $O_2^{\cdot}$. As it has been shown recently that the familiar Lineweaver–Burk plot can be used to describe the trapping by ferricytochrome *c* of $O_2^{\cdot}$ generated by the xanthine–xanthine oxidase system [11], we used this plot to interpret the results with desferrioxamine addition. As

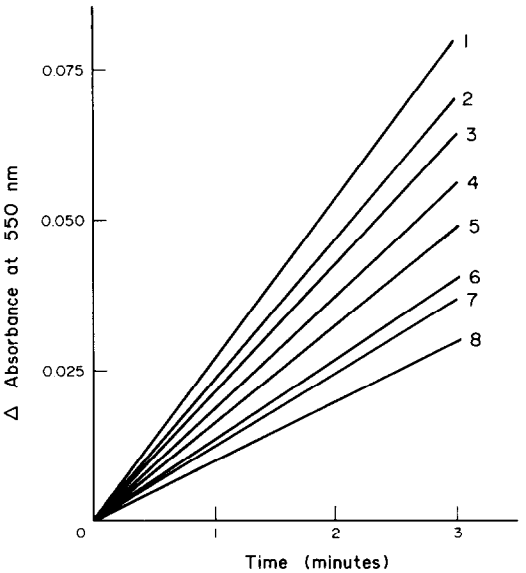

Fig. 1. Effect of desferrioxamine on the ferricytochrome *c* reduction mediated by the aerobic action of xanthine oxidase on xanthine. Reaction mixtures contained 0.1 mU/ml of xanthine oxidase, 1×10^{-5} M ferricytochrome *c* and 5×10^{-5} M xanthine in 3.0 ml of 0.05 M potassium phosphate buffer, pH 7.8, containing 1 mM detapac. Results are expressed as the change in absorbance at 550 nm vs time either in the absence (1) of desferrioxamine or with increasing concentrations of this compound (2, 3, 4, 5, 6, 7, 8 = 0.2, 0.5, 1, 1.5, 2.25, 4.12, 5.5 mM, respectively). Each reaction was carried out four-fold giving almost identical results.