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IN VIVO EFFECTS OF APOMORPHINE AND 4-(3-BUTOXY-4-METHOXYBENZYL)-2-IMIDAZOLIDINONE (RO 20-1724) ON CYCLIC NUCLEOTIDES IN RAT BRAIN AND PITUITARY

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Abstract—The effect of apomorphine or of 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO 20-1724), a potent phosphodiesterase inhibitor, on levels of cyclic AMP and cyclic GMP *in vivo* was examined in the pituitary, cerebellum, corpus striatum and nucleus accumbens–olfactory tubercle. RO 20-1724 was also tested in combination with apomorphine to determine whether this drug could potentiate the effect of apomorphine. Rats were injected with vehicle or RO 20-1724 (30 mg/kg) 30 min prior to an injection of saline or apomorphine hydrochloride (1 or 10 mg/kg). The animals were killed by microwave irradiation 7 min after the second injection. RO 20-1724 increased levels of cyclic AMP in all four regions, especially in the pituitary. RO 20-1724 increased levels of cyclic GMP in the cerebellum, but not in the pituitary. Apomorphine increased cyclic AMP in the pituitary, and cyclic GMP in all four regions. RO 20-1724 did not produce supra-additive effects with apomorphine. The system most responsive to either drug was cyclic AMP in the pituitary, where cyclic AMP increased approximately 10-fold after either apomorphine or RO 20-1724.

The primary molecular sites of action of the drugs apomorphine and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO 20-1724) are thought to be different, but the cyclic nucleotides, cyclic AMP and cyclic GMP, have been postulated to be involved in the mechanism of action of both drugs.

Apomorphine is considered to be a dopamine (DA) agonist that directly stimulates the DA receptor [1, 2]. Stimulation of dopamine receptors by dopamine or apomorphine may result in activation of dopamine-sensitive adenylate cyclases. Dopamine-stimulated adenylate cyclases have been demonstrated in many brain regions including the caudate nucleus, nucleus accumbens, olfactory tubercle, substantia nigra, and frontal cortex [3–8]. *In vivo*, apomorphine has been shown to increase striatal cyclic AMP levels [9–12], as well as cerebellar cyclic GMP levels [13–15].

RO 20-1724 has been shown to be a potent phosphodiesterase inhibitor *in vitro* [16–21].

RO 20-1724 potentiates elevations in cyclic AMP in guinea pig cortical slices in response to adenosine, histamine, norepinephrine and glutamate [22–24]. RO 20-1724 also increases basal cyclic AMP levels in guinea pig cerebellar slices [25]. We were interested in determining the *in vivo* cyclic nucleotide responses to RO 20-1724, and also whether RO 20-1724 could serve as a pharmacological amplifier for *in vivo* effects of a neurotransmitter receptor agonist such as apomorphine.

Measurements of *in vivo* levels of cyclic nucleotides require rapid tissue inactivation methods to minimize post-mortem changes in these compounds [26]. Microwave irradiation is a rapid fixation technique that leaves the brain in a condition suitable for regional dissection. Using this method, we have been able to measure *in vivo* levels of cyclic AMP and cyclic GMP from the same rat brain regions [27, 28].

In this report we describe the effects of apomorphine and RO 20-1724 on *in vivo* levels of cyclic AMP and cyclic GMP in the pituitary and three regions of rat brain: cerebellum, combined nucleus accumbens–olfactory tubercle and corpus striatum.

MATERIALS AND METHODS

Animals. Male albino rats (275–312 g), WRC stock from the Walter Reed Army Institute of Research colony, were used in all experiments.* The animals had free access to food and water, and were maintained in a 12-hr light–dark cycled room. For 1 week prior to death, animals were handled twice a day and habituated to entering an open-ended plexiglass cylinder similar to the microwave applicator cylinder.

Solutions. Apomorphine hydrochloride was obtained from Merck & Co., Inc. (Rahway, NJ) and dissolved in isotonic saline with 1 mg/ml ascorbic acid. The dose was calculated as the hydrochloride. Animals were injected i.p. with apomorphine HCl at various doses, as described in the specific experiment, or with saline containing 1 mg/ml ascorbic acid.

The phosphodiesterase inhibitor RO 20-1724 was a gift from Hoffman–La Roche, Inc. (Nutley, NJ). A 10 mg/ml solution was prepared by dissolving 75 mg

* In conducting the research described in this report, the investigators adhered to the *Guide for Laboratory Animal Facilities and Care*, as promulgated by the Committee of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council.

of RO 20-1724 in 0.3 ml of warm ethanol. Warm propylene glycol (2.25 ml) and warm saline (4.95 ml) were then added. A similar vehicle solution was prepared as above without RO 20-1724. The solutions were kept at 55° in a shaking water bath for the duration of the experiment. RO 20-1724 or vehicle was administered i.p. 30 min prior to apomorphine or saline.

Microwave death. Animals were killed by microwave irradiation at 2450 MHz. The rats were placed in a plexiglass holder and inserted in a hole in the short-circuiting endplate of a WR 430 waveguide exposure chamber, in such a manner that the longitudinal axis of the head was perpendicular to the microwave E field. The power source and waveguide were modified to achieve greater uniformity and efficiency of inactivation [29, 30]. Animals were exposed for 5 sec with 2.5 kW forward power (1–3 per cent reflected). The power source was a Varian PPS-2.5, modified with electronic control for precise timing and leveling of output power. Frequency output was verified at 2440 ± 20 MHz with a spectrum analyzer. Prior to exposure, each animal was impedance-matched over the range 2420–2460 MHz to a low power (10 mW/cm^2) signal from a sweep generator, using a double-stub tuner.

Sample preparation. Following microwave irradiation, the heads were cooled briefly on dry ice for ease of handling. The brain was then removed carefully and the desired regions were dissected as described previously [31]. The tissue pieces were weighed and then sonicated with a Heat Systems model 185 in 50 mM sodium acetate buffer (pH 6.2). The sonicates were then centrifuged at 25,000 *g* for 15 min. The supernatant fractions were stored at −70° until assayed.

Cyclic nucleotide assay. Cyclic AMP and cyclic GMP levels were determined by a slight modification of the radioimmunoassay described by Steiner *et al.* [32]. The reaction volume of 0.5 ml differed in that it contained 200 μg of rabbit gamma globulin (Schwarz/Mann, Orangeburg, NY). After 16 hr at 4°, separation of free and bound cyclic nucleotides was accomplished by the addition of 1.5 ml of ice-cold 50 mM sodium acetate (pH 6.2) with 16% Carbowax 6000 and 1 mg/ml rabbit gamma globulin, followed by centrifugation at 4° for 10 min at 2000 *g* with subsequent aspiration of the supernatant fraction.

For measurement of the cyclic nucleotides in the smaller brain regions, a further modification of the method described by Harper and Brooker [33] was employed. Standards and brain samples were acetylated at the 2′0 position in 50 mM sodium acetate (pH 4.8), using a freshly prepared 2:1 mixture of triethylamine/acetic anhydride. Following the acetylation procedure, the radioimmunoassay proceeded as noted above.

The data were analyzed by computer, using a non-linear four-parameter logistic model weighted for non-uniformity of variance [34]. The respective sensitivities (minimal detectable amounts) for cyclic AMP and cyclic GMP were 0.10 and 0.025 pmole/assay tube for the routine assay, and 3 fmoles for the acetylated assay. Phosphodiesterase treatment of tissue extracts reduced cyclic AMP and cyclic GMP to undetectable levels, representing a reduction greater than 95 per cent for cyclic AMP and 80 per cent for cyclic GMP in each region.

RESULTS

Time course of apomorphine effects. Rats were injected with saline or 10 mg/kg apomorphine hydrochloride and killed by microwave irradiation after 1, 3, 5, 7 or 15 min. As shown in Table 1, apomorphine rapidly elevated both cyclic AMP and cyclic GMP in all three brain regions. Significant increases in cyclic AMP were seen as early as 3 min after 10 mg/kg apomorphine. Increases in cyclic AMP were maximal after 5 or 7 min. While significant increases in cyclic GMP were seen as early as 3 min in all regions, the highest measured levels were seen 15 min after 10 mg/kg apomorphine.

Effect of RO 20-1724 *and apomorphine on cyclic AMP and cyclic GMP in pituitary, cerebellum, nucleus accumbens–olfactory tubercle and corpus striatum.* Rats were injected i.p. with RO 20-1724 (30 mg/kg) or vehicle 30 min prior to a second injection of saline or apomorphine hydrochloride (1 or 10 mg/kg). Animals given RO 20-1724 were observed to be behaviorally depressed. The animals were killed by microwave irradiation 7 min after the second injection.

As seen in Table 2, RO 20-1724 increased cyclic AMP levels in all four regions tested, but especially in the pituitary where cyclic AMP levels increased over 10-fold. The elevations were statistically significant ($P < 0.05$) in the pituitary, cerebellum and nucleus accumbens–olfactory tubercle. Apomorphine significantly increased cyclic AMP levels only in the pituitary where there was a greater increase seen after 10 mg/kg apomorphine than after 1 mg/kg apomorphine ($P < 0.05$).

As shown in Table 3, RO 20-1724 significantly increased levels of cyclic GMP only in the cerebellum ($P < 0.05$), although levels of cyclic GMP in the striatum and nucleus accumbens–olfactory tubercle were increased in all three RO 20-1724 treatment groups. Apomorphine (10 mg/kg) increased levels of cyclic GMP in all four regions.

Inspection of the data in Tables 2 and 3 revealed that the effects of RO 20-1724 and apomorphine were approximately additive.

DISCUSSION

In vivo, the phosphodiesterase inhibitor RO 20-1724 increased levels of cyclic AMP in all four tested regions: pituitary, cerebellum, nucleus accumbens–olfactory tubercle and corpus striatum. The elevation was over 10-fold in the pituitary, which appears to have a very responsive cyclic AMP system. RO 20-1724 also increased cyclic GMP levels *in vivo* in cerebellum, nucleus accumbens–olfactory tubercle and corpus striatum, but not in the pituitary. Experiments performed *in vitro* with RO 20-1724 have suggested that the drug is a more effective inhibitor of cyclic AMP phosphodiesterase activity than cyclic GMP phosphodiesterase activity [16, 21]. Our results demonstrate that RO 20-1724 *in vivo* increases both

Table 1. Time course of increases in cyclic nucleotides in three brain regions following apomorphine injection*

Group	Cyclic AMP (pmoles/mg wet weight ± S.E.M.)			Cyclic GMP (pmoles/mg wet weight ± S.E.M.)		
	Striatum	Accumbens–tubercle	Substantia nigra	Striatum	Accumbens–tubercle	Substantia nigra
Saline	0.440 ± 0.031	0.750 ± 0.017	0.656 ± 0.024	0.051 ± 0.006	0.107 ± 0.004	0.084 ± 0.010
Apomorphine (1 min)	0.516 ± 0.034	0.851 ± 0.048†	0.721 ± 0.028	0.058 ± 0.005	0.090 ± 0.018	0.115 ± 0.015
Apomorphine (3 min)	0.558 ± 0.050†	0.875 ± 0.057†	0.818 ± 0.040†	0.070 ± 0.008†	0.144 ± 0.009†	0.131 ± 0.009†
Apomorphine (5 min)	0.592 ± 0.037†	0.949 ± 0.076†	0.843 ± 0.030†	0.082 ± 0.006†	0.145 ± 0.009†	0.153 ± 0.009†
Apomorphine (7 min)	0.569 ± 0.049†	0.931 ± 0.050†	0.930 ± 0.100†	0.093 ± 0.011†	0.177 ± 0.004†	0.132 ± 0.013†
Apomorphine (15 min)	0.539 ± 0.021†	0.891 ± 0.011†	0.912 ± 0.081†	0.121 ± 0.011†	0.191 ± 0.019†	0.228 ± 0.036†

* Rats were injected with 10 mg/kg apomorphine HCl and killed by microwave irradiation 1, 3, 5, 7 or 15 min later (N = 5 per group). In addition, five rats were injected with saline and one saline animal was killed at each time point.

† Significantly different from saline group ($P < 0.05$) by Student's *t*-test (one-tailed).

Table 2. Effects of apomorphine and RO 20-1724 on cyclic AMP levels *in vivo* in cerebellum, striatum, nucleus accumbens–olfactory tubercle and pituitary*

Drug treatment		Cyclic AMP (pmoles/mg wet weight ± S.E.M.)			
First injection	Second injection	Cerebellum	Striatum	Accumbens–tubercle	Pituitary
Vehicle	Saline	0.884 ± 0.119	0.891 ± 0.128†	0.789 ± 0.037	1.73 ± 0.36
Vehicle	Apomorphine HCl (1 mg/kg)	0.920 ± 0.136	0.851 ± 0.085	0.830 ± 0.054	3.56 ± 0.80‡
Vehicle	Apomorphine HCl (10 mg/kg)	0.882 ± 0.051	0.817 ± 0.082	0.861 ± 0.030	14.72 ± 0.74‡
RO 20-1724	Saline	1.381 ± 0.198‡	1.200 ± 0.181	1.040 ± 0.041‡	21.38 ± 1.80‡
RO 20-1724	Apomorphine HCl (1 mg/kg)	1.402 ± 0.367	1.411 ± 0.166‡	1.139 ± 0.081‡	29.43 ± 3.97‡
RO 20-1724	Apomorphine HCl (10 mg/kg)	1.694 ± 0.111†,‡	1.106 ± 0.134	1.252 ± 0.092‡	31.14 ± 1.82‡

* Each animal was injected with vehicle or RO 20-1724 (30 mg/kg) 37 min prior to being killed. Each rat then received a second injection of saline or apomorphine 7 min prior to death (N = 4 per group). Comparisons between selected treatment groups were made by Student's *t*-test (one-tailed).

† N = 3.

‡ Significantly different from vehicle–saline groups ($P < 0.05$).

Table 3. Effects of apomorphine and RO 20-1724 on cyclic GMP levels *in vivo* in cerebellum, striatum, nucleus accumbens–olfactory tubercle and pituitary*

Drug treatment		Cyclic GMP (pmoles/mg wet weight ± S.E.M.)			
First injection	Second injection	Cerebellum	Striatum	Accumbens–tubercle	Pituitary
Vehicle	Saline	1.071 ± 0.131	0.086 ± 0.007	0.132 ± 0.011	0.121 ± 0.015
Vehicle	Apomorphine HCl (1 mg/kg)	1.709 ± 0.451	0.095 ± 0.012	0.160 ± 0.013	0.093 ± 0.008
Vehicle	Apomorphine HCl (10 mg/kg)	3.484 ± 0.782†	0.118 ± 0.007†	0.225 ± 0.019†	0.178 ± 0.017†
RO 20-1724	Saline	2.084 ± 0.251†	0.110 ± 0.017‡	0.158 ± 0.014	0.135 ± 0.009
RO 20-1724	Apomorphine HCl (1 mg/kg)	3.778 ± 1.125†	0.116 ± 0.008†	0.197 ± 0.010†	0.183 ± 0.030
RO 20-1724	Apomorphine HCl (10 mg/kg)	3.514 ± 0.350†	0.145 ± 0.025†	0.280 ± 0.011†	0.121 ± 0.018

* Each animal was injected with vehicle or RO 20-1724 (30 mg/kg) 37 min prior to being killed. Each rat then received a second injection of saline or apomorphine 7 min prior to death (N = 4 per group). Comparisons between selected treatment groups were made by Student's *t*-test (one-tailed).
† Significantly different from vehicle–saline group ($P < 0.05$).
‡ N = 3.

cyclic AMP and cyclic GMP in particular brain regions. The *in vivo* effects on cyclic GMP must also be considered in assessing the biochemical pharmacology of this drug.

Apomorphine increased levels of cyclic AMP and/or cyclic GMP in some regions but not others. Cyclic GMP levels *in vivo* were increased by apomorphine in substantia nigra (Table 1), in cerebellum and pituitary (Table 3), and in striatum and accumbens–tubercle (Tables 1 and 3). Pituitary cyclic GMP levels were not increased by apomorphine in the RO 20-1724 treatment groups. Seven minutes after 10 mg/kg apomorphine, cyclic AMP levels were unchanged in cerebellum (Table 2), increased or unchanged in striatum and accumbens–tubercle (Tables 1 and 2), increased in substantia nigra (Table 1), and greatly increased in pituitary (Table 2). The failure to find elevated cyclic AMP in striatum after apomorphine in the second experiment could be explained by the somewhat higher 'control' cyclic AMP levels in vehicle-saline injected rats (Table 2) vs the saline injected controls used in the experiments shown in Table 1. However, in other experiments in our laboratory (unpublished data), we have found that the cyclic AMP response to apomorphine *in vivo* in striatum is generally variable and not robust. This has also been observed by other investigators [35]. The major responses to apomorphine were increased cyclic GMP levels in cerebellum and nucleus accumbens–olfactory tubercle and a very large cyclic AMP increase in the pituitary. DA receptors have been demonstrated in the nucleus accumbens–olfactory tubercle, but none are found in the cerebellum [36]. It has been postulated that the apomorphine-induced cyclic GMP elevation in the cerebellum might be mediated via a multi-synaptic pathway originating outside the cerebellum [13, 15].

Pituitary cyclic AMP was remarkably responsive to apomorphine or RO 20-1724. Levels of cyclic AMP increased approximately 8-fold after apomorphine, and 12-fold after RO 20-1724. Pituitary cyclic GMP levels were slightly increased by apomorphine and unaffected by RO 20-1724. The turnover of cyclic AMP in the pituitary must be relatively high and the system very sensitive to demonstrate such large increases.

The pituitary has been shown to contain DA receptors and it has been suggested that dopamine may act directly on the pituitary to inhibit prolactin release [37, 38]. The administration of apomorphine *in vivo* decreases prolactin [38, 39]. Schmidt and Hill [40] recently reported that adenylate cyclase activity in intact pituitaries *in vitro* was unaffected by apomorphine, although striatal adenylate cyclase activity was stimulated under the same experimental condition. Mowles *et al.* [41] have shown that dopamine and apomorphine inhibit prolactin secretion but do not affect cyclic nucleotide levels in pituitary cell cultures. Our results, however, show that *in vivo* pituitary cyclic AMP is elevated by apomorphine. Either apomorphine is able to stimulate pituitary DA receptors *in vivo* but not *in vitro*, or possibly apomorphine affects pituitary cyclic AMP through activation of a system originating outside the pituitary.

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