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CENTRALLY-ACTING DRUGS AND RELATED COMPOUNDS EXAMINED FOR ACTION ON OUTPUT OF ADENINE DERIVATIVES FROM SUPERFUSED TISSUES OF THE BRAIN

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Abstract—Guinea-pig neocortical tissues were incubated with [^{14}C]adenine and superfused; after about 20 min superfusion when excess adenine was removed, the tissues lost about 0·05% of their ^{14}C/min, mainly as adenosine and its metabolites inosine and hypoxanthine. Electrical stimulation increased 2- to 5-fold the ^{14}C output, and also tissue glycolysis.

Drugs were incorporated in superfusion fluids and examined for action on ^{14}C output and on glycolysis, in unstimulated and stimulated tissues. In some instances the added compounds caused similar changes in stimulated output of ^{14}C and of lactate; these were amylobarbitone, 66 μM; desipramine, 30 and 100 μM; diphenylhydantoin, 300 μM; and chlorpromazine, 20 μM. Chlorpromazine at 100 μM, desipramine at 0·5 mM and pyridylisatogen at 20 and 50 μM augmented ^{14}C output without corresponding effect on glycolysis.

Several correlations are pointed out between these results and those of other workers who have examined the actions of drugs on cerebral cyclic AMP *in vivo*. It is concluded that liberation of adenosine contributes to features in the action of several of the compounds named above.

The cyclic AMP content of cerebral and cerebellar tissues is augmented by electrical excitation and, *in vivo* and *in vitro*, adenosine has been concluded to mediate much of this increase [1–3]. The quantities of adenosine liberated on excitation are in some cases sufficient to induce the observed increase in cyclic AMP [4] but change in the cyclic AMP of incubating tissues can be caused also by addition of other neurohumoral agents and blocked by drugs antagonistic to them [5] (see also the Discussion section). A few such compounds have now been examined for their influence on output of adenine derivatives from incubated neocortical tissues in presence and absence of electrical excitation. Their actions in these respects have been compared with reported actions of the same compounds on tissue content of cyclic AMP, with the object of detecting instances in which liberation of adenosine might contribute to the action of a drug.

MATERIALS AND METHODS

Tissue incubation, labelling and superfusion. Guinea-pig neocortical tissues were prepared promptly, mounted in quick-transfer holders, incubated and superfused as described by Pull and McIlwain [6–7]; McIlwain [8]. Four tissue samples of 50–80 mg from two animals were normally prepared together and incubated in separate tissue-holding electrodes in one experiment. Each holder was placed in an incubation vessel which contained 1 μCi of [8-^{14}C] or [U-^{14}C] adenine (54 mCi/m-mole) in 5 ml glucose–bicarbonate medium agitated by 5% CO_2 in O_2 and at 38° for 40 min. Superfusion at approx 3·5 ml/min with medium lacking adenine was then commenced, and for the first 20 min the effluent fluid, which carried excess adenine, was discarded. Subsequent effluents were collected each 2 min for up to 40 min as indicated below; added compounds, and electrical stimulation with alternating exponential pulses of peak potential 10 V (0·4 msec time constant and frequency 20 Hz) were applied to specified tissues at chosen times.

Analyses and expression of results. Determination of radioactivity and of lactic acid in superfusate samples; the preparation of tissue extracts for determination of radioactivity, potassium and adenine nucleotides and calculation of the adenylate energy charge were as previously described [6, 7, 9]. The radioactivity of tissue extracts, effluent medium samples and medium remaining in the beakers at the end of superfusion was first expressed as nCi/g tissue. Then for each 2 min collection period a rate constant for ^{14}C output (%/min) was obtained [6]. For comparison of the compounds employed in Table 1 with control tissues, each group of experiments followed a standard design. The compound under investigation was introduced to the superfusing media 8 min after beginning collection of samples and after a further 10 min, electrical stimulation was applied to the tissues for 2 min.

Materials. Diphenylhydantoin obtained from British Drug Houses Ltd., Poole, Dorset, U.K., was prepared as a 30 mM solution in 0·1 M-NaOH and 1 ml of this solution used per 100 ml superfusion medium, equilibrated before and after the addition with 5%

CO_2 in O_2 and maintained at 38°. Amylobarbitone was from the Sigma (London) Chemical Co. Ltd., London SW6, U.K., and chlorpromazine hydrochloride from May & Baker Ltd., Dagenham, Essex, U.K.

We gratefully acknowledge gifts of 2·2′-pyridylisatogen tosylate from Dr M. Spedding, School of Pharmacy, Sunderland Polytechnic, Sunderland, U.K., and of imipramine and desipramine from Geigy Pharmaceuticals, Cheshire, U.K. [8-^{14}C]Adenine (54 mCi/m-mole) and [U-^{14}C]adenine (287 mCi/m-mole) were obtained from the Radiochemical Centre, Amersham, Bucks, U.K. Other materials were from sources previously specified [6, 7, 9].

RESULTS

Tissues were prepared for the present experiments by an initial preincubation with [^{14}C]adenine, followed by superfusion during which the excess of [^{14}C]adenine was removed. A small output of ^{14}C derivatives then continued (Fig. 1), totalling about 0·05% of the tissue content/min and mainly in the form of adenosine, inosine and hypoxanthine [10]. Certain of the added compounds modified the output of ^{14}C at this stage in the experiments; Fig. 1A exemplifies this. Tissues subsequently were electrically stimulated; in the absence of added compounds, this increased the output of [^{14}C]adenine derivatives by 2- to 5-fold. More than one period of stimulation, with or without added agents, were included in some experiments (Fig. 2). At the end of superfusion, the K content of tissues was determined and in control tissues which had been superfused with normal salines, values were 70 ± 3 (S.D., 6 tissues) μat K/g initial wt tissue. It was judged valuable (see below) to determine whether a response to stimulation other than the ^{14}C output was also affected by added compounds, and for this purpose the output of lactate was measured by analysis of the effluent samples collected for ^{14}C determination. It was found (Fig. 1) that the action of a given concentration of a compound on the two responses could differ markedly.

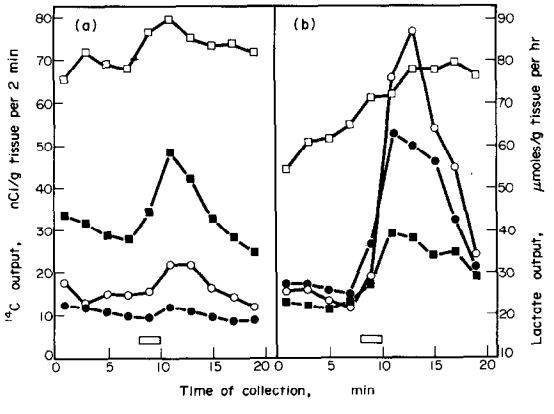

Fig. 1. Action of chlorpromazine on output: (A), of [^{14}C]adenine derivatives and (B), of lactate from neocortical tissues preincubated with [^{14}C]adenine. After preincubation, tissues were superfused with normal medium (○) or medium containing chlorpromazine at 20 μM (●), 100 μM (■) or 500 μM (□) for 20 min before beginning collection (zero time in the Fig.) of effluent medium each 2 min. Between 8–10 min tissues were electrically stimulated (bar). The K^+ contents (μequiv/g tissue) of the tissues analysed after the stimulation and a total of 64 min superfusion were: with no addition, 68; with 20 μM chlorpromazine, 70; with 100 μM chlorpromazine, 62; and with 500 μM chlorpromazine, 23.

Chlorpromazine at the greatest concentration tested, 0·5 mM, increased the tissues' output of lactate and of [^{14}C]adenine derivatives independently of excitation. This was associated with a fall in the K content of the tissue. With lower concentrations of chlorpromazine, little or no loss of K occurred and differential actions were found on the output of ^{14}C and lactate. To 20 μM chlorpromazine, output of ^{14}C was more sensitive than output of lactate: the ^{14}C of superfusates diminished before the tissue was stimulated, and during stimulation the increase in ^{14}C was about 25 per cent of that from control tissues, in comparison with a lactate output 60 per cent of normal. Chlorpromazine at 0·1 mM augmented the output of [^{14}C]adenine derivatives, with and without stimulation, while output of lactate was unchanged before stimulation and diminished during stimulation.

Amylobarbitone in the present experiments was examined at a low concentration, 66 μM, which did not affect tissue K-content (Fig. 2). This concentration inhibited the output of [^{14}C]adenine derivatives, and to a lesser extent that of lactate. In absence of excitation the ^{14}C output diminished by 30 per cent; during excitation, diminution was 50 per cent. Output of lactate was less affected by the amylobarbitone,

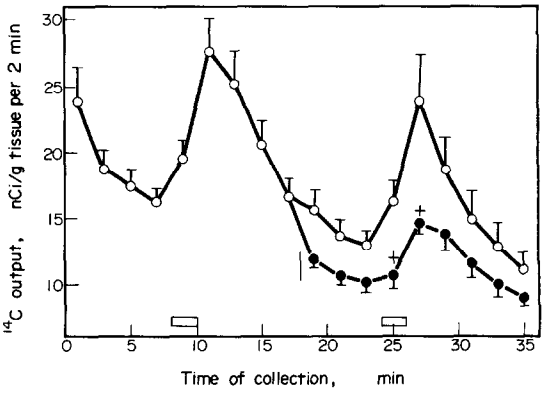

Fig. 2. Action of amylobarbitone on output of [^{14}C]adenine derivatives from neocortical tissue. Tissues were pre-incubated with [^{14}C]adenine and then superfused with normal medium for 15 min before beginning sample collection (zero time in the Fig.). At 18 min (arrow) the normal medium (○) superfusing some of the tissues was exchanged for medium containing 0·066 mM amylobarbitone (●). Between 8–10 min and 24–26 min all tissues were electrically stimulated (bars). Mean values of eight tissues prior to alteration of the medium and of four tissues subsequently are given with their S.D. shown by vertical lines. Points marked + differ significantly from controls ($P < 0·05$, Student's t-test, both tails). The K^+ contents of tissues determined at the end of superfusion with amylobarbitone did not differ from those of control tissues superfused with normal media. Output of lactate was measured and between 16 and 32 min superfusion was lower in the presence of amylobarbitone. The diminution, by a mean value of 8 per cent, was significant only in the samples collected at 28 min, then being 15 per cent.

Table 1. Added compounds on the output of ^{14}C adenine derivatives and of lactate from neocortical tissues

Expt.	Added compound (mM)	No. of tissues	^{14}C Output (% $min^{-1} \times 10^3$) Initial	Pre-stimulation	Stimulated	Lactate output (μmole/g tissue per hr) Initial	Pre-stimulation	Stimulated
1.	None	4	50 ± 10	32 ± 6	180 ± 18	14 ± 4	13 ± 4	63 ± 8
	Diphenylhydantoin (0·3)	4	52 ± 4	57 ± 12§	147 ± 11‡	10 ± 4	12 ± 2	36 ± 9§
2.	None	4	73 ± 14	49 ± 16	270 ± 75	13 ± 4	11 ± 7	64 ± 17
	Desipramine (0·03)	3	69 ± 21	42 ± 17	123 ± 29†	8 ± 1	7 ± 1	33 ± 13†
	Desipramine (0·1)	2	57, 64	39, 52	110, 198	6, 7	4, 7	30, 28
	Desipramine (0·5)	2	73, 60	218, 186	1482, 1684*	7, 8	8, 14	51, 69*
3.	None	6	54 ± 11	41 ± 9	251 ± 64	9 ± 2	7 ± 2	65 ± 20
	Pyridylisatogen (0·005)	6	64 ± 19	51 ± 9	211 ± 52	9 ± 3	9 ± 3	46 ± 13
	Pyridylisatogen (0·02)	4	65 ± 21	86 ± 6§	661 ± 40§	14 ± 3	10 ± 4	85 ± 21
	Pyridylisatogen (0·05)	1	74	155	538	21	17	48

Experiments were carried out as described in the Methods section, results are individual or mean values (±S.D.) of the number of tissues shown. Initial values given above derive from the mean output of ^{14}C and lactate of each tissue during the first four collection periods and prior to the introduction of added compounds. Pre-stimulation values are those observed after a further 10 min superfusion with or without additions and immediately prior to 2 min electrical stimulation, stimulated values (above) derive from the maximum rates of output following the electrical stimulation. Values for tissue K in Expt. 1: with diphenylhydantoin, 69 ± 5(4) μequiv/g and without, 71 ± 3(4). Significant differences from controls by Students *t*-test (two tail); †; $P < 0.05$; ‡, $P < 0.025$; §, $P < 0.01$.

* Output of ^{14}C and lactate increased continuously throughout the Expt., these values were obtained 20 min after the onset of stimulation.

the diminution becoming significant only on excitation. Diphenylhydantoin, also, was examined at a concentration, 0·3 mM, which did not affect tissue K (Table 1) and which was approaching the maximum permitted by solubility of the compound. This concentration increased the output of [^{14}C]adenine derivatives prior to stimulation, but depressed the tissues' response to stimulation. In this instance glycolytic response was more sensitive (42% inhibition) than was ^{14}C output (18% inhibition).

Imipramine and desipramine were examined in experiments similar to those of Fig. 1. The lower concentrations of desipramine, 30 and 100 μM, were without action on output of [^{14}C]adenine derivatives or lactate in absence of excitation, but depressed the tissue's response to stimulation (Table 1). At 0·5 mM, however, desipramine greatly increased the output of both ^{14}C and lactate independently of excitation. Imipramine was examined in a single experiment at 0·1 and 0·5 mM, and its effects were similar to those of the same concentrations of desipramine.

2·2′-Pyridylisatogen was examined in view of the reports [11, 27] that it antagonized actions of ATP on smooth muscle. At concentrations between 5 and 50 μM (Table 1) the pyridyl derivative modified the output of [^{14}C]adenine derivatives in a fashion similar to that shown for chlorpromazine in Fig. 1. Measurement of ATP, ADP and AMP in tissues superfused with 20 μM 2·2′-pyridylisatogen showed values little different from control tissues, with an adenylate energy charge [12] of 0·92 in each case. Theophylline, at 20 and 500 μM, was found to be without action on output of [^{14}C]adenine derivatives during the unstimulated and stimulated periods of experiments similar to those of Fig. 1.

DISCUSSION

A classification of the compounds examined

Change in tissue levels of cyclic AMP, and output of adenosine derivatives under conditions similar to those of the present experiments, are each of about 1 μmole/g per hr [10] in contrast to the rate of about 1 mmole//g per hr at which adenine nucleotide turnover can occur in association with energy metabolism. As an indication of change in energy metabolism associated with the stimulation or with the added compounds of the present experiments, output of lactate, and tissue K-content were also measured. Change in lactate output frequently parallelled change in output of [^{14}C]adenine derivatives, which suggests that in these cases the dephosphorylation in utilization of ATP, could have initiated the output of adenine derivatives (though this is not the only explanation available). In other circumstances such parallelism did not occur: thus the diminution in ^{14}C output caused by 20 μM chlorpromazine, and the increase caused by 100 μM chlorpromazine and by 20 μM pyridylisatogen, were not accompanied by a corresponding change in lactate output. Indeed 100 μM chlorpromazine diminished the output of lactate on electrical stimulation, while increasing the output of ^{14}C adenine derivatives.

These results have led us to classify the compounds examined into two categories: (1) those which change in output of adenine derivatives was in the same direction as change in lactate output, and in which the added compounds were pictured to act through modification of the tissues' energy metabolism; and (2) those in which the changed output of adenine derivatives was due to other causes, presumably more specific to stages in the metabolism of the adenine derivatives themselves. Appraisal follows of other data concerning compounds in the two categories, especially in relation to their action on cyclic AMP.

(1) *Compounds causing similar change in output of lactate and adenine derivatives.* Pentobarbital administered intraperitoneally to rats at 0·2 m-mole/kg was found [13] to lower by 15–30 per cent the cyclic AMP of four cerebral regions obtained by microwave fixation. It also opposed an increase in cyclic AMP occurring post-mortem in the brain of mice [14] by 20 per cent at 0·16 m-mole/kg. Both these are situ-

ations in which some dephosphorylation of adenine nucleotides occurs, and are thus relevant to the present findings with amylobarbitone (Fig. 1; Table 1: the two barbiturates are assumed to be of similar action). These findings showed amylobarbitone to diminish adenosine output by up to 40 per cent, especially on electrical excitation and lactate by 20 per cent. Increase of cyclic AMP in the mouse forebrain on electrical excitation *in vivo* was opposed by theophylline and caffeine, which strongly suggests adenosine to be an intermediary [3]. Similar factors appear to be involved in the action of desipramine, for here also we found an increased output of [^{14}C]adenine derivatives. Berndt [15] found a partial inhibition by theophylline of an increase in cerebral cyclic AMP caused by the drug *in vivo*; *in vitro*, Kodama *et al.* [16] found almost complete antagonism of a desipramine-induced increase in cyclic AMP, by theophylline.

Diphenylhydantoin as an anticonvulsant *in vivo* opposes effects of several agents which cause convulsions or seizure discharges [17]. These include discharge from lesions caused by local cerebral freezing, which are accompanied by increase in sodium–potassium activated adenosine triphosphatase [18]. Diphenylhydantoin at 0·2 μmole/kg intraperitoneally in rats opposed this increase in the NaK–ATPase and presumably, therefore, would diminish the formation of adenosine: an action to be compared with the 20 per cent diminution caused by 0·3 mM diphenylhydantoin in output of [^{14}C]adenine derivatives from electrically stimulated tissues in the present experiments (Table 1).

(2) *Compounds of more specific action on output of adenine derivatives.* Sedation of rats induced by chlorpromazine was found [15] to be correlated in time course with an increase in cyclic AMP content of samples of their frontal cortex. Theophylline antagonized both the increase and the sedation, and blocking by theophylline of an increase in cyclic AMP induced by chlorpromazine has been reported also *in vitro* [19]. This cannot reflect an action of theophylline on the cyclic nucleotide phosphodiesterase, for cyclic AMP levels would then be further increased. Situations in which theophylline diminishes cyclic AMP appear to be limited to those involving adenosine [1, 2, 3]. The present experiments have indeed shown a phase in the action of chlorpromazine in which it causes liberation of adenosine (Fig. 1), though this is not the most sensitive of its *in vitro* actions.

Greater sensitivity to chlorpromazine was shown by the isolated tissues when they were electrically stimulated (Fig. 1); 20 μM chlorpromazine then diminished the resulting increase in output of adenosine. This situation also has a counterpart in the brain *in situ*, for Uzunov and Weiss [20] found that prior administration of chlorpromazine to rats diminished a post-mortem rise to their cerebellar content of cyclic AMP. Cyclic AMP does not yet appear to have been examined in relation to the action of 2,2′pyridylisatogen, but the concentrations of the compound now found to affect ^{14}C output are similar to those which interact with ATP in smooth muscle relaxation [11].

Comment on measurement of cyclic AMP in interpretation of drug action

Determinations of the cyclic AMP of target-organs have recently been widespread in studies of drug-action [21], but only rarely have the measurements been accompanied by a measure of the energy-metabolism or adenosine of the tissues involved. Present data suggest that such additional measures are advisable, particularly in organs known to carry adenosine-activated adenylate cyclase systems, as does the brain [3, 22].

It is especially noteworthy that the present study has shown that compounds which evoke changes in cyclic AMP and are of a variety of pharmacological types, can markedly alter the output of adenine derivatives from cerebral tissues. Adenine derivatives are not the only compounds released on excitation of isolated cerebral tissues and capable of augmenting cyclic AMP: other biogenic amines are also released [23, 24], and may contribute to the present results. Mediation which includes adenosine is however supported in the five instances quoted in which change in cyclic AMP, or in the overt action of a drug, was opposed (not augmented: see above) by theophylline. Sattin *et al.* [28] published after the present work was completed, a detailed appraisal of how the effects on cyclic AMP of neurohumoral agents and related drugs contribute to their pharmacological actions; and postulated in the brain a category of α-adrenergic receptors which require the copresence of adenosine. Correspondingly, it appears likely that certain drugs acting on the brain may owe their efficacy to their modifying the release of more than one neurohumoral agent, even when these agents act on a single category of receptor. Chlorpromazine is one of the compounds concluded by the preceding discussion to include adenosine-activated adenylate cyclases in its action. Study of chlorpromazine in relation to noradrenaline and dopamine and prior to knowledge of the involvement of adenosine, had already led to the conclusion that multiple actions on adenylate cyclase system(s) must be invoked in understanding its action [20, 25, 26].

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