Analogs of Cyclic Adenosine Monophosphate: Correlation of Inhibition of Purkinje Neurons with Protein Kinase Activation

Abstract. *Cyclic adenosine monophosphate (cyclic AMP) and 11 derivatives were applied to rat cerebellar Purkinje cells by iontophoresis. Cyclic AMP inhibited 63 percent of the cells, while the 8-parachlorophenylthio- and 8-benzylthio- analogs of cyclic AMP inhibited the spontaneous firing of 92 and 80 percent of cells, respectively. The ability of the 11 analogs to inhibit neuronal firing correlated ($r = +.78$) with their reported potency in activating cyclic AMP-dependent protein kinase. These results extend previous studies, pointing to the mediation by cyclic AMP of the noradrenergic inhibition of Purkinje neurons, and provide new physiological evidence that protein phosphorylation is a major step in the action of cyclic AMP.*

Adenosine 3′,5′-monophosphate (cyclic AMP) has been implicated as an intracellular second messenger of central catecholaminergic neurotransmission on evidence from: (i) biochemical studies (*1*) showing catecholamine-evoked elevations of cyclic AMP in most brain regions; (ii) immunocytochemical studies showing an increase of cyclic AMP bound in cerebellar Purkinje cells after noradrenergic stimuli (*2*); (iii) electrophysiological studies showing a modulation of the catecholaminergic inhibitions of several neuron types by drugs known to interact with the cyclic AMP system (*3, 4*), and a similarity between responses to catecholamines and cyclic AMP applied by iontophoresis (*4, 5*).

Although cyclic AMP might affect membranes directly (*6*), a major hypothesis (*7*) suggests that cyclic AMP acts by regulation of protein kinase, which phosphorylates specific brain proteins (*8*), thus altering the biophysical properties of membranes (*9*). However, the role of cyclic AMP in noradrenergic inhibition has been questioned on the grounds that the percentage of cerebellar Purkinje cells depressed by cyclic AMP does not match the high percentage depressed by norepinephrine (*10*). This difference has been attributed to technical factors which impede iontophoretic release (*11, 12*) and to the necessity for cyclic AMP (a sparingly permeable and enzymatically labile agent) to reach intracellular protein kinase (*12*). Recently, analogs of cyclic AMP, some of which are more potent than cyclic AMP in activating brain protein kinase (*13, 14*), have become available (*15*). We now report that analogs of cyclic AMP depress Purkinje neurons in correlation with their ability to activate protein kinase. These findings further substantiate the concepts that protein kinase as well as cyclic AMP are involved in noradrenergic neurotransmission.

Eighteen male albino rats of (120 to 300 g) were anesthetized with 1 percent halothane, mounted in a stereotaxic frame, and maintained at 37°C. Techniques of craniotomy, dura removal, and identification of Purkinje cells during extracellular recording with five-barrel glass micropipettes have been described (*16*). Drugs (*17*) were iontophoretically applied (by anionic current) to individual neurons with the use of a circuit having continuous automatic neutralization of currents, with a driving electromotive force of ± 100 volts (*18*). In another series of experiments designed to control for possible current artifacts inherent in iontophoresis (*19*), cyclic AMP and nine of its derivatives were ejected with cationic bulk flow using the technique of electroosmosis (*17, 20*).

The precautions used in testing neuronal responsivity to drugs have been discussed (*19, 21*). Cyclic AMP and 11 derivatives were tested on 211 Purkinje cells and 13 unidentified cells. Responses to the agents were classified for effect on spontaneous activity as depressant, excitatory, or biphasic and reversible. When tested by iontophoresis, cyclic AMP and four other derivatives depressed a majority of Purkinje cells (Fig. 1 and Table 1). Rapid, strong inhibitions to cyclic AMP were often observed, although prolonged (1 to 5 minutes) responses with a long latency (30 to 60 seconds) also occurred (*21*) (Fig. 1B). Depressions produced by cyclic AMP were occasionally weak, and in 50 percent of the cases required currents of 80 na or more.

The percentage of Purkinje cells depressed by cyclic AMP (63 percent of 57 neurons) is similar to that (61 percent) reported previously (*3*). However, 80 percent or more of the Purkinje cells were depressed by three of the cyclic AMP derivatives substituted at C-8, namely 8-parachlorophenylthio- (8-PCPT-), 8-benzylthio-, and 8-amino- analogs (Table 1). The first two derivatives depressed firing more strongly and rapidly than cyclic AMP, and at lower ejection currents (Fig. 1, B and C). The N^6-monobutyryl derivative depressed the same percentage of Purkinje cells as cyclic AMP (*22, 23*).

The effects of cyclic AMP and nine derivatives were tested by electroosmosis (*20, 24*). In these experiments (Table 1), cyclic AMP depressed only 43 percent of the Purkinje cells, as would be expected from the smaller amounts of cyclic AMP released by electroosmosis as compared to iontophoresis (*11*). However, four derivatives, 8-PCPT-, 8-benzylthio-, N^6-monobutyryl-, and N^6,$O^{2'}$-dibutyryl- cyclic AMP still depressed 50 percent or more of the cells. As with iontophoresis, the 8-PCPT- analog depressed a large percentage (82 percent); 8-PCPT- and 8-benzylthio- derivatives generally produced striking depressions at low ejection currents (5 to 80 na).

Two derivatives, $O^{2'}$-monobutyryl and 2′-deoxy cyclic AMP were ineffective in depressing activity and, in fact, excited two-thirds of the Purkinje cells (Fig. 1B). Alterations of the $O^{2'}$ position of the ribose moiety inactivate the ability of cyclic AMP to stimulate protein kinase (*14, 25*).

If the proportion of Purkinje cells inhib-

Table 1. Response of Purkinje cells to cyclic AMP and its analogs. Protein kinase (PK) activity is scaled from 0 to 4.

Cyclic AMP or analog	PK	Iontophoresis (%)*					Electroosmosis (%)*				
		Cells (No.)	↓	↑	0	⇵	Cells (No.)	↓	↑	0	⇵
8-Parachloro-phenylthio	4	12	92	0	0	8	38	82	3	13	3
8-Benzylthio	4	20	80	0	5	15	35	69	17	9	6
8-Amino	3	11	82	0	9	9					
Cyclic AMP	3	57	63	11	16	10	23	43	17	39	0
N^6-Monobutyryl	3	29	62	7	31	0	26	65	23	8	4
8-Bromo	4	29	48	14	35	3	8	38	38	12	12
8-Isopropylthio	3	19	47	11	26	16	23	48	30	0	22
8-Methylthio	4	19	42	32	10	16	16	31	37	13	19
N^6,O^2-Dibutyryl	0	20	40	0	40	0	11	54	18	7	0
8-Methylamino	2	19	21	53	21	5	17	35	47	0	18
O^2-Monobutyryl	0	14	14	64	14	7	7	0	71	29	0
2′-Deoxy	0	16	6	69	6	18					

*Effect of iontophoresis (anionic current) and electroosmosis (cationic) expressed as percent of testable cells. ↓, Depression of spontaneous discharge; ↑, acceleration or speeding; 0, no effect; ⇵, biphasic or reversible. Compounds are arranged in decreasing order of percentage inhibitions by iontophoresis. Protein kinase (PK) refers to relative potency in activating protein kinase of bovine brain (*25*), as reported by Meyer and Miller (*14*).

ited are compared with the relative values of brain protein kinase activation for each of the derivatives published by Meyer and Miller (*14*), a Pearson moment correlation can be computed (*26*). With increasing protein kinase activation scaled relatively from 0 to 4 (cyclic AMP = 3), percent inhibitions of Purkinje cells are significantly correlated: ($r = +.78$ and $+.72$; $P < .01$ and .02, respectively, for the iontophoresis and electroosmosis experiments). Correlation coefficients computed (*26*) for percent excitations and protein kinase activation were negatively significant ($r = -.85$ and $-.78$; $P < .001$ and .01, respectively, for iontophoresis and electroosmosis). The regression lines (*26*) are shown in Fig. 1E. Dibutyryl cyclic AMP is excluded from the correlation, since tissue deacylases convert it to N^6-monobutyryl cyclic AMP with a concomitant change in relative protein kinase activation from 0 to 3, respectively (*14, 27*).

Two points are apparent from these correlations. First, even though other contributory factors such as membrane permeability, degradation by phosphodiesterase and release transport numbers are ignored, cyclic AMP analogs with strong protein kinase stimulating ability are more likely to depress a large percentage of Purkinje cells. Second, analogs with little protein kinase activity generally excite Purkinje cells, suggesting that excitation by cyclic AMP and its derivatives result from an action unrelated to protein kinase activation. Thus, the outcome of an iontophoretic test depends on the competition between the excitatory and inhibitory effects of the molecule, the latter determined by protein kinase stimulating potency.

These results with derivatives of high protein kinase activating capability strengthen the link between adrenergic inhibition of Purkinje cells and its intracellular mediation by cyclic AMP (*3*). Several analogs studied here depressed percentages of cells approaching those seen with norepinephrine. One, the 8-PCPT-analog, depressed 92 percent of Purkinje cells, very near the 98 percent reported for norepinephrine depressions (*16*). These findings thus overcome the objection (*10*) that a cell-by-cell mimicry of norepinephrine effects is not produced by cyclic AMP.

Our results constitute the first direct correlation between the electrophysiological effects of analogs of cyclic AMP in the central nervous system and their relative ability to activate protein kinase. The results thus add further credence to the suggestion by Kuo and Greengard (*7*) that protein kinase activation is an important step in the physiology of cyclic AMP.

The approach used here might be useful in testing hypotheses of similar links between cyclic nucleotides and neurohormones in other systems. The continued use of dibutyryl cyclic AMP as the sole agent to test these hypotheses now seems unwise, since it must be enzymatically altered to affect intracellular protein kinase (*27*). Iontophoresis studies of other neuron systems using cyclic AMP analogs such as 8-PCPT- and 8-benzylthio-, which have potent protein kinase activating ability seems warranted, as would a similar reevaluation of those neuron systems where cyclic AMP and its dibutyryl analog are reported (*28*) not to reproduce the responses to catecholamines.

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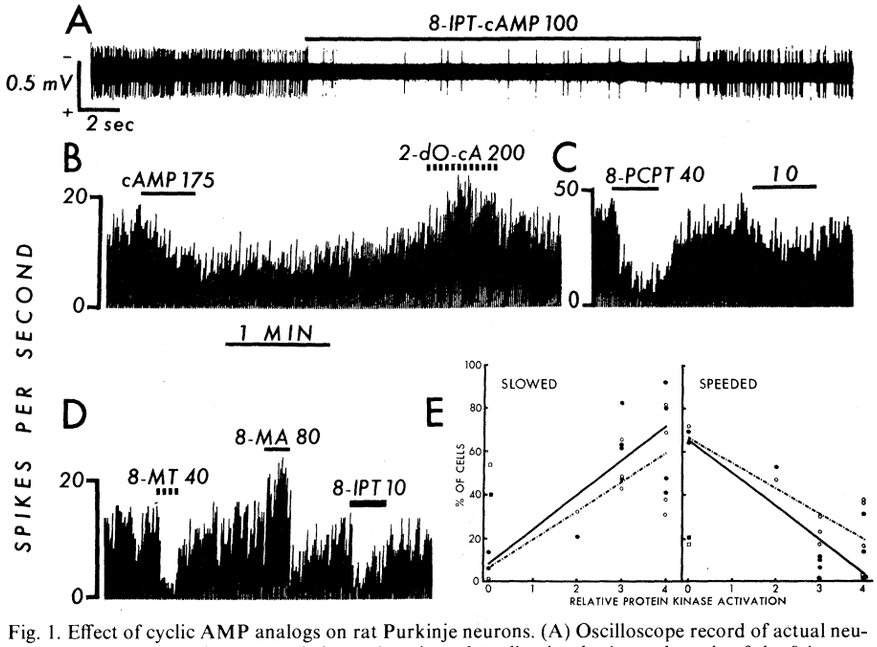

Fig. 1. Effect of cyclic AMP analogs on rat Purkinje neurons. (A) Oscilloscope record of actual neuronal discharge. Bar above record shows duration of application by iontophoresis of the 8-isopropylthio- analog of cyclic AMP (100 na). Most spikes occurring during inhibition by the drug are climbing fiber responses, which are not affected by cyclic AMP analogs. (B) Polygraph record of neuronal discharge rate (integrated over 1-sec intervals) showing weak, slow inhibition by cyclic AMP (175 na) and rapid excitation by cyclic 2′-*O*-deoxy AMP (200 na). (C) Rate record of another Purkinje cell showing rapid, strong inhibitions by the 8-parachlorophenylthio analog of cyclic AMP (40 na and 10 na, applied by electroosmosis from a 0.02*M* solution). (D) Rate record of same cell as in panel A, showing depression of firing by the 8-methylthio- analog (40 na) and the 8-isopropylthio-analog (10 na) of cyclic AMP and excitation by the 8-methylamino- analog of cyclic AMP (80 na), all by iontophoresis. Time calibration bar pertains to panels B, C, and D. (E) Regression plots of Pearson correlation comparing percentages of cells inhibited (slowed) and excited (speeded) by cyclic AMP and the analogs, scaled according to their ability to activate bovine brain protein kinase relative to cyclic AMP (= 3). Protein kinase values taken from Meyer and Miller (*14*). Filled circles and solid regression lines refer to cyclic AMP and ten analogs applied by iontophoresis; open circles and dotted line refer to the electroosmosis experiments. The squares apply to N^6,O^2-dibutyryl cyclic AMP, whose values were excluded from the calculations (see text).

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17. The sources of the drugs used in this study are as follows: cyclic AMP, Calbiochem; 8-parachlorophenylthio-cyclic AMP, ICN; 8-benzylthio-cyclic AMP, ICN; 8-isopropylthio-cyclic AMP, ICN; 8-methylthio-cyclic AMP, ICN; 8-methylamino-cyclic AMP, ICN; 8-amino cyclic AMP, ICN; 8-bromo-cyclic AMP, Sigma; N^6-monobutyryl cyclic AMP, Sigma; $N^6,O^{2'}$-dibutyryl cyclic AMP, Sigma; $O^{2'}$-monobutyryl cyclic AMP, Sigma; 2'-deoxy-cyclic AMP, ICN. In iontophoresis experiments the drugs were used in a concentration of 0.25*M* in distilled water, except 8-PCPT cyclic AMP, which was 0.02 to 0.04*M*. In electroosmosis experiments, the compounds were dissolved in normal saline at 0.02 to 0.05*M*.
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21. If the following criteria were not satisfied, tests of Purkinje cells were discarded: (i) regular, spontaneous neuronal activity at rates less than 70 per second; (ii) continuous neutralization of iontophoretic currents within 0 to 15 na; (iii) constant spike amplitude without evidence of injury. Since the interpretation of negative results with iontophoresis is difficult, a classification of "no effect" was given a cell only when the drug barrel had been "warmed up" by several pulses of at least 100 na applied over a 2- to 5-minute period (*11, 12*), and when the neuron then did not respond by a change in rate of a least 10 percent to at least two consecutive pulses of 200 na each applied for at least 1 minute. Had large ejection currents and long durations of application not been used, many responses to cyclic AMP would have been overlooked. These considerations could in part account for the low frequency of cyclic AMP-induced depressions of Purkinje cells reported by Lake and Jordan (*10*).
22. Several unidentified cells were tested which did not have the characteristics of Purkinje cells, such as climbing fiber bursts and regular firing patterns of simple spikes at rates greater than 10 to 15 per second. Of 33 complete tests with several of the more potent derivatives, only 6 percent of these neurons displayed depressions of rate, 39 percent were speeded, and 55 percent were uneffected. Here again the effects of the derivatives parallel the results seen previously with cyclic AMP (*23*), and emphasize the necessity of identification of cell types for meaningful interpretation of iontophoresis studies.
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24. Electroosmosis was employed for two reasons: (i) In iontophoresis experiments the current used to eject drugs is of concern because it may alter cell firing directly; our study is prone to this artifact since cyclic AMP and the analogs used all carry a net negative charge (*11*) and require the same polarity of current for iontophoresis; (ii) the limited solubility of 8-PCPT cyclic AMP (0.02 to 0.04*M*) and its tendency to gel in the pipette produced excessive drug barrel resistances, resulting in electrical interference and failure of tip neutralization with current passage.
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26. Relative values of protein kinase activation (*14*) refer to ranges of K_a' values, defined as the ratio of K_a for cyclic AMP to K_a for the analog (from Lineweaver-Burke plots). The scale for relative protein kinase activation is as follows: Scale 0 = $K_a' < 0.01$; scale 1 = $0.01 \leq K_a' \leq 0.099$; scale 2 = $0.10 \leq K_a' \leq 0.49$; scale 3 = $0.50 \leq K_a' \leq 2.0$; scale 4 = $K_a' > 2.0$. Pearson moment correlation coefficients and regression lines were calculated on a digital PDP-12 computer, with two different correlation programs being used for comparison: STATIS 12 (DECUS No. 12-148) and 5CORR (DECUS No. 12-146).
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Erythrocyte Receptors for (*Plasmodium knowlesi*) Malaria: Duffy Blood Group Determinants

Abstract. *Duffy blood group negative human erythrocytes (*FyFy*) are resistant to infection by* Plasmodium knowlesi, *a simian malaria that infects Duffy positive human erythrocytes. The* P. knowlesi *resistance factor, Duffy negative erythrocytes, occurs in high frequency in West Africa, where the people are resistant to vivax malaria. This suggests that Duffy blood group determinants (*Fy^a *or* Fy^b*) may be erythrocyte receptors for* P. vivax.

West Africans (*1*) and approximately 70 percent of American blacks (*2*) are resistant to infection by *Plasmodium vivax*, although they are susceptible to the other three species of human malaria. The vivax resistance factor completely blocks infection; sickle cell trait which decreases mortality from *P. falciparum* does not block infection (*3*). The resistance to *P. vivax* is evident after sporozoite induced infection (*1*) or after inoculation of infected erythrocytes (*2*). Since parasitized erythrocytes initiate infection in the recipient without an exoerythrocytic cycle, the resistance factor must interfere with the merozoite's ability to invade erythrocytes or to develop once within them.

In a search for erythrocyte receptors for malaria parasites (merozoites) (*4*), we tested erythrocytes that lacked various antigenic determinants for susceptibility to invasion by a simian malaria (*P. knowlesi*). This species invades human erythrocytes in culture (*5*) and infects man (*6*). Of the erythrocytes tested, one type which lacked Duffy a and b antigens, Duffy negative (*FyFy*) (*7*), was resistant to invasion. This genotype is present in approximately 90 percent of West Africans (*8*); it is extremely rare in other racial groups (*9*) who are susceptible to *P. vivax*. This striking association suggested that the Duffy negative genotype (*FyFy*) might be the factor involved in resistance to *P. vivax* in West Africans and that invasion by *P. vivax* could require a Duffy positive erythrocyte (Fy^a or Fy^b).

To explore these questions, we studied the relation between Duffy blood group determinants and invasion by *P. knowlesi*. Human erythrocytes were obtained from five whites and five blacks, positive for either or both of the Duffy antigens (a and b) and from 11 blacks negative for these antigens. The erythrocytes were washed three times with culture medium (*10*), and mixed with *P. knowlesi* (*11*) infected rhesus erythrocytes that contained primarily schizonts. This suspension (5000 human erythrocytes and 500 parasitized erythrocytes per cubic millimeter) was added to 16-mm flat-bottom Linbro tissue culture wells (0.5 ml per well) and incubated for 3 hours at 35°C in an atmosphere containing 2 percent CO_2. The merozoite invasion frequency was determined from Giemsa-stained smears prepared from erythrocytes in the wells and counted (under code) (*5*). Duffy positive and negative erythrocytes were studied at the same time under identical conditions.

The average invasion frequency for Duffy positive erythrocytes was 80.3 parasitized erythrocytes per 1000 erythrocytes

Table 1. The effect of various proteolytic enzymes on Duffy blood group determinants and malaria invasion of Duffy positive human erythrocytes. Removal of Duffy blood group determinants is accompanied by reduced invasion by *Plasmodium knowlesi*. Abbreviation: RBC, erythrocytes.

RBC phenotype	Enzyme*	Duffy† typing	Titer after‡ adsorption	Infected§ RBC per 10,000 RBC
Fy(a+ b–)	None	+++	–	610
	Trypsin (1 mg/ml)	+++	–	870
	Chymotrypsin (0.01 mg/ml)	+++	1 : 1	380
	Chymotrypsin (0.1 mg/ml)	–	1 : 8	46
	Chymotrypsin (1 mg/ml)	–	1 : 8	17
Fy(a– b+)	None	++++	–	850
	Trypsin (1 mg/ml)	++++	–	1110
	Chymotrypsin (0.01 mg/ml)	++++	–	840
	Chymotrypsin (0.1 mg/ml)	++++	1 : 1	78
	Chymotrypsin (1 mg/ml)	–	1 : 8	25

*See (*5*) for details of enzymatic treatment. †Scoring: ++++, macroscopic clumps, clear supernatant; +++, macroscopic clumps, slightly reddish supernatant; –, no microscopic clumps. ‡ Antiserum to Duffy antigen was absorbed with RBC as follows: three drops of packed erythrocytes which had been washed with 0.85 percent NaCl were mixed with four drops of antiserum to Duffy antigen (titer = 1 : 16), and the suspension was incubated for 40 minutes at 37°C. The supernatant was used for Duffy typing with the appropriate Duffy positive erythrocytes. A titer of 1 : 8 indicates no adsorption of antibody, since mixture with cells caused a 1 : 2 dilution of the original antiserum. §The counts were the sums of duplicate chambers. At high rates of invasion (> 100 infected RBC per 10,000 RBC), less than 10,000 RBC were counted, and the numbers in the table were estimated.