Vol. 103, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 16, 1981 Pages 189-195

STUDIES ON BRAIN OPIATE RECEPTOR STABILITY *IN VITRO*: INHIBITION OF OPIATE RECEPTOR BINDING BY ADENOSINE-5'-DIPHOSPHATE

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Received September 28,1981

SUMMARY: Incubation of rat brain homogenates at 37° causes a time-dependent decrease in opiate receptor binding which does not occur with a washed membrane fraction. The supernatant fraction contains a heat-stable inhibitor which is partially destroyed by apyrase and completely removed by activated charcoal. ADP causes a similar inhibitory effect in homogenates, but not with washed membranes, which is characterized by a decrease in both opiate agonist and antagonist binding in the absence or presence of NaCl. The ADP inhibition is antagonized by ATP, α,β-methyleneADP, β-thioADP and EDTA. It is concluded that ADP, unlike the guanine nucleotides, facilitates the nonspecific degradation of opiate receptors by an endogenous soluble factor.

The exogenous addition of the guanine nucleotides, GDP and GTP, to incubations of washed rat brain membranes has been reported to inhibit opiate receptor binding (1-3). In the presence of 100 mM NaCl, this inhibition is specific for agonists of the opiate receptors and represents a possible means of regulating opiate receptor function (1,2). Thus, it is of interest to determine whether inhibition of opiate receptor binding by an endogenous component of rat brain homogenates can be demonstrated. The present report shows the presence of an endogenous inhibitor, but the characteristics of the inhibition are different from that caused by guanine nucleotides. Further experiments indicated that ADP also inhibited opiate receptor binding in a manner different from that reported for guanine nucleotides (1,2).

METHODS

Preparation of brain tissue:

Whole brains from male Sprague-Dawley rats weighing 200-350 g were each weighed and homogenized in 10 volumes (w/v) of ice-cold 50 mM Tris-HCl buffer (pH 7.7 at room temperature) using a motor-driven teflon/glass homogenizer. For some experiments, a once-washed particulate fraction was obtained by centrifuging the homogenate at 40,000 g for 20 minutes at 4°, rehomogenizing the pellet in 10 volumes of the buffer, recentrifuging as above and rehomogenizing the final pellet in another 10 volumes of Tris buffer.

0006-291X/81/210189-07$01.00/0

Preparation and treatment of boiled supernatant fraction:

The 40,000 g supernatant fraction from rat brain homogenized as described above was aliquoted into 13x100 mm test tubes which were inserted into boiling water for 3 minutes. After cooling on ice, the tubes were centrifuged in a table-top clinical centrifuge to remove the denatured protein.

Some boiled supernatant fractions were treated for 20 minutes at 37° with activated charcoal (Norit, 30 mg/ml), potato apyrase (1 mg/ml) or trypsin (0.3 mg/ml), and then reboiled and filtered through glass-fiber filters (Whatman GF/C). The powdered charcoal was obtained from Fisher Scientific Company. The apyrase (5.9 ATPase and 3.4 ADPase units/mg protein) and twice-crystalized bovine pancreas trypsin (12,000 BAEE units/mg protein) were obtained from Sigma Chemical Company.

Binding assay:

Rat brain homogenates or once-washed particulate fractions (equivalent to 10 mg wet weight of brain) were preincubated for various periods of time with the appropriate additions in a total volume of 2.0 ml of Tris-HCl buffer (pH 7.7 at room temperature) in a Dubnoff shaking incubator at 37°. Then 1.0 nM (^{3}H)etorphine or (^{3}H)diprenorphine was added, and the incubation was continued for another 20 minutes. The samples were cooled 5 minutes in crushed ice before filtration through GF/C filters, followed by two 8-ml washes with ice-cold 50 mM Tris buffer. After drying, the filters were each counted in 10 ml of ACS scintillation fluid (Amersham Corp.) using a Beckman LS-3133T liquid scintillation spectrometer. Each incubation was carried out in duplicate or triplicate, and additional incubations contained 10 μM naloxone to correct for nonspecific binding. Each experiment was repeated three times, and the data for specific binding are expressed as percentage of control, using the mean ± S.E.M. for the 3 experiments.

Chemicals:

The (^{3}H)etorphine (36 Ci/mmole) and (^{3}H)diprenorphine (10 Ci/mmole) were purchased from the Amersham Corporation. The monopotassium salt of ADP, the trilithium salt of β-thioADP, and NAD were obtained from Boehringer/Mannheim. All of the other chemicals were obtained from Sigma Chemical Company.

RESULTS

In preliminary experiments (data not shown), it was found that preincubation of whole rat brain homogenate in Tris buffer for up to 30 minutes before the addition of the opiate receptor agonist (^{3}H)etorphine resulted in a progressive loss of (^{3}H)etorphine binding, but no significant loss of binding occurred with the washed particulate fraction over the same period of preincubation time. As shown in Table 1, the supplementation of the washed particulate fraction with twice the equivalent volume of supernatant or boiled supernatant fraction resulted in a progressive decrease in (^{3}H)etorphine binding. Some inhibition of binding (∿20%) also occurred during the 20-minute incubation with (^{3}H)etorphine.

If the inhibition of (^{3}H)etorphine binding were due to the presence of endogenous guanine nucleotides, no inhibition of binding of the opiate recep-

Table 1: Effect of preincubation time on the binding of (^{3}H)etorphine to the washed particulate fraction alone, or supplemented with twice the equivalent volume of supernatant or boiled supernatant fractions

Preincubation Time (min)	% of 0-Preincubation Time Binding to the Particulate Fraction		
	Particulate Fraction	+Supernatant	+Boiled Supernatant
0	100 ± 1	80 ± 1	83 ± 2
5	105 ± 4	84 ± 1	68 ± 2
10	109 ± 4	78 ± 1	57 ± 1
20	105 ± 4	65 ± 2	39 ± 1
30	95 ± 6	52 ± 1	30 ± 2

tor antagonist (^{3}H)diprenorphine would occur in the presence of 100 mM NaCl (1). However, as shown in Table 2, the boiled supernatant fraction caused a concentration-dependent inhibition of both (^{3}H)etorphine and (^{3}H)diprenorphine in either the absence or presence of 100 mM NaCl.

In an attempt to further characterize the inhibitory factor(s) in the boiled supernatant fraction, the effect of treating this fraction with activated charcoal, trypsin or apyrase on the inhibition of opiate receptor binding was determined (Table 3). Treatment with trypsin had no effect on the inhibitory activity of the boiled supernatant fraction. The inhibitory activity, however, was completely removed by charcoal and was significantly diminished by apyrase treatment. Since apyrase has both ATPase and ADPase activity, the effects of ADP and ATP, as well as other adenosine-containing compounds, on the binding of (^{3}H)etorphine and (^{3}H)diprenorphine to the washed particulate fraction were determined. No inhibition of binding by these compounds at a concentration of 1 mM was observed (data not shown). When similar experiments were carried out with whole brain homogenates, however, both ADP and ATP inhibited the binding of (^{3}H)etorphine and (^{3}H)diprenorphine in the absence or presence of 100 mM NaCl (Table 4). ADP caused a greater inhibition than ATP. These experiments indicated that ADP and ATP required a soluble factor in order to cause inhibition of opiate receptor binding. Further experiments with ADP indicated that it caused a concentration- and time-dependent inhibition of

Table 2: Effect of various amounts of boiled supernatant on (^{3}H)etorphine and (^{3}H)diprenorphine binding to the washed particulate fraction in the absence or presence of 100 mM sodium chloride[1]

Amount of Boiled Supernatant (ml)	(^{3}H)Ligand Binding (% of control)			
	(^{3}H)Etorphine		(^{3}H)Diprenorphine	
	-NaCl	+NaCl	-NaCl	+NaCl
0	100 ± 3	100 ± 3	100 ± 7	100 ± 2
0.1	89 ± 6	85 ± 5	92 ± 3	90 ± 4
0.2	77 ± 5	65 ± 6	74 ± 5	71 ± 7
0.4	58 ± 6	50 ± 6	62 ± 4	60 ± 6

[1]Boiled supernatant fraction was preincubated with the washed particulate fraction for 5 minutes before addition of (^{3}H)ligand.

opiate receptor binding in homogenates (Table 5). Scatchard analysis of the inhibitory effect of a 5-minute preincubation of homogenates with 1 mM ADP on (^{3}H)diprenorphine binding indicated that ADP caused a decrease in the number of binding sites from 207±6 to 163±7 fmoles/10 mg tissue and an increase in the K_d from 0.25±0.04 to 0.44±0.06 nM.

To study the specificity of the inhibitory effect of ADP, and to attempt to find an antagonist of ADP, several compounds were tested for their ability to mimic or block the effect of ADP in brain homogenates (Table 6). The inhibitory effect of ADP was mimicked by 2'-deoxyADP and 1,N^6-ethenoADP. ATP partially blocked ADP, whereas α,β-methyleneADP, β-thioADP and EDTA completely blocked the inhibitory effect of ADP. The finding that (^{3}H)diprenorphine

Table 3: Effect of charcoal, trypsin or apyrase treatment on the inhibition of (^{3}H)diprenorphine binding to the washed particulate fraction by the boiled supernatant fraction[1]

Treatment of Boiled Supernatant	(^{3}H)Diprenorphine Binding (% of control)
None	60 ± 1
Charcoal	105 ± 3
Trypsin	62 ± 3
Apyrase	82 ± 2

[1]Treated boiled supernatant was preincubated 5 minutes with the washed particulate fraction before addition of (^{3}H)diprenorphine.

Table 4: Effect of adenine nucleotides on (^{3}H)etorphine and (^{3}H)diprenorphine binding to rat brain homogenates in the absence or presence of 100 mM sodium chloride[1]

	(^{3}H)-Ligand Binding (% of control)			
	(^{3}H)Etorphine		(^{3}H)Diprenorphine	
Compound	-NaCl	+NaCl	-NaCl	+NaCl
Control	100 ± 6	100 ± 6	100 ± 4	100 ± 5
Adenosine	101 ± 2	120 ± 10	112 ± 1	105 ± 5
AMP	94 ± 1	100 ± 9	98 ± 4	92 ± 1
Cyclic AMP	101 ± 1	104 ± 10	101 ± 3	96 ± 3
ADP	69 ± 3	66 ± 6	58 ± 1	65 ± 2
ATP	82 ± 4	79 ± 8	80 ± 1	77 ± 2

[1] Homogenates were preincubated with compound (1 mM) 5 minutes before the addition of (^{3}H)ligand. NaCl decreased control etorphine binding by 35% and increased control diprenorphine binding by 9%.

binding in the presence of the latter 3 compounds was greater than the control binding indicates the possibility that these 3 compounds also blocked an endogenous inhibitor in the brain homogenate.

DISCUSSION

While this investigation was in progress, other reports of supernatant factors inhibitory to opiate binding appeared (4,5). The possibility that opiate receptor binding and/or function may be regulated by an endogenous intracellular factor is attractive from the standpoint that such regulation could be involved in the mechanism of tolerance to narcotic drugs. There have

Table 5: Effect of preincubation time on the binding of (^{3}H)diprenorphine to rat brain homogenates alone, or supplemented with various concentrations of adenosine-5'-diphosphate

	% of 0-Preincubation Time Binding			
[ADP], mM	0 min	5 min	10 min	20 min
0	100 ± 6	101 ± 5	86 ± 3	80 ± 3
0.125	85 ± 2	90 ± 5	80 ± 8	58 ± 2
0.25	84 ± 3	81 ± 6	63 ± 5	48 ± 2
0.5	76 ± 3	69 ± 4	54 ± 3	39 ± 2
1.0	67 ± 4	59 ± 4	48 ± 3	36 ± 3

Table 6: Screening of ADP analogues for ADP-mimetic or blocking activity[1]

Expt. No.	Compound	% of Control (^{3}H)Diprenorphine Binding[2] -ADP	+ADP
1	Control	100 ± 2	56 ± 2
	ADP-ribose	86 ± 1	52 ± 1
	ADP-glucose	98 ± 6	62 ± 2
	P^1,P^2-diadenosine-pyrophosphate	95 ± 1	59 ± 2
2	Control	100 ± 3	59 ± 1
	2'-DeoxyADP	58 ± 1	64 ± 3
	$1,N^6$-EthenoADP	54 ± 1	59 ± 2
	α,β-MethyleneADP	115 ± 3	117 ± 2
3	Control	100 ± 2	57 ± 1
	NAD	93 ± 2	43 ± 7
	β-thioADP	123 ± 1	123 ± 1
	Na_2EDTA	124 ± 6	125 ± 1
4	Control	100 ± 4	59 ± 3
	Adenosine	108 ± 10	59 ± 3
	AMP	88 ± 8	56 ± 2
	ATP	89 ± 4	82 ± 3

[1]Rat brain homogenates were preincubated 5 minutes with 1 mM compound ± 1 mM ADP before addition of (^{3}H)diprenorphine.

[2]Control (^{3}H)diprenorphine binding in expts. 1, 2, 3 and 4, respectively, were 1078±25, 1060±35, 1004±25 and 1041±42 cpm (mean±SEM, N=3).

been several unsuccessful attempts to demonstrate changes in opiate receptor binding in homogenized brain tissue with the development of tolerance (6-8); but in all of these studies, a soluble regulatory component would have been greatly diluted or washed out during the preparation of membrane fractions. A few studies with brain slices indicate possible changes in opiate receptor binding during the development of tolerance (9,10). GTP is a candidate for such an intracellular regulator of opiate receptor binding, particularly since its inhibitory effect is specific for opiate agonists under certain conditions (1-3). This agonist specificity for GTP has also been observed in this laboratory (Brase, unpublished).

In the present study, the inhibition of opiate receptor binding by the boiled supernatant fraction and by ADP did not show agonist specificity. In

addition, attempts to reverse the ADP-induced inhibition of (^{3}H)diprenorphine binding by washing, or by the addition of α,β-methyleneADP after preincubation of homogenates with ADP, failed (11). Consequently, this inhibition was likely due to a nonspecific destruction of opiate receptors. A similar destruction of opiate receptors occurs with ascorbic acid-induced lipid peroxidation, which is also blocked by EDTA (12). The inhibitory effect of ADP appeared to require the presence of a soluble factor in the homogenate, because the inhibition did not occur when the washed particulate fraction was used. Although the identity of this factor is unknown, it is of interest to note that ADP-chelated iron has been reported to promote lipid peroxidation in liver microsomes (13). In addition, ferrous ions have been reported to inhibit opiate receptor binding (14). Future studies will be required to determine whether ADP facilitates the peroxidation of brain lipids under the incubation conditions of the present study, in which ADP inhibits opiate receptor binding. These studies indicate that protection of opiate receptors from endogenous destructive factors may be important for carrying out adequate *in vitro* binding studies.

ACKNOWLEDGEMENT: This research was supported in part by NIH Biomedical Research Development Grant RR09028-03.

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