Biochemical Pharmacology, Vol. 25, pp. 47–51. Pergamon Press, 1976. Printed in Great Britain.

ACCUMULATION OF ETORPHINE BY SLICES OF CEREBRAL CORTEX AND CORPUS STRIATUM OF RATS*

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(*Received* 1 *November* 1974; *accepted* 31*March* 1975)

Abstract—A system in slices of cerebral cortex and corpus striatum is described which concentrates etorphine at very low concentrations (<5 nM) but does not appear to require metabolic energy for this process. At higher concentrations of etorphine, the accumulation is by passive diffusion. The accumulation at low concentrations is saturable and relatively stereospecific. This saturable accumulation may be related to the stereospecific binding material (putative receptors) described by others.

Taking advantage of the fact that most active synthetic narcotic analgesics are the *l*-isomers while their *d*-isomers are either inactive or very much less active, Goldstein *et al.* [1] demonstrated that stereospecific binding of levorphanol to brain fractions and speculated that the binding material might be the opiate receptor. The availability of radiolabeled narcotics and antagonists with high specific activity has made possible the demonstration of stereospecific binding of these compounds to brain homogenates or fractions with very low dissociation constants [2–4]. These authors also believe that the binding represented the demonstration of the narcotic or opiate receptor. Numerous groups are now actively engaged in the study of stereospecific binding of narcotics to homogenates and fractions of brain [5–15].

In conjunction with our continued interest in the uptake and transport of narcotics into the central nervous system, we felt that the study of a possible stereospecific uptake or binding to putative receptors using preparations with relatively intact cells such as slices would be of interest. The active uptake of several narcotic drugs by cerebral cortical slices has been reported [16]. The acquisition of the potent narcotic, etorphine, in radioactive form with high specific activity made it possible in the present communication to study the narcotic uptake at the very low concentrations which one would expect to find *in vivo* after pharmacologic doses. The data indicate a system in slices of cerebral cortex and corpus striatum which accumulates etorphine stereospecifically but does not appear to require energy for this process.

METHODS

Animals. Male Holtzman (Madison, Wis.) rats weighing between 180 and 250 g were used in all experiments. Animals were made morphine dependent by implanting s.c. two morphine pellets, each containing 75 mg of the free base, for 72 hr [17].

Dissection of brain areas. After the animals were decapitated, brains were removed rapidly and various brain areas were separated and sliced by the technique described previously [18]. Slices of cerebral cortex were prepared from the dorsal and lateral surfaces of each hemisphere. The corpus striatum was isolated after removal of the corpus callosum as diagrammed by Marcucci *et al.* [19], and two slices were cut from the medial surfaces of each striatum. Slices were placed on a filter paper moistened with saline solution and put in an ice-cold chamber until they were ready for use. The slices were weighed just prior to incubation. The mean weight ± S. E. of 200 representative slices of cerebral cortex was 9·7 ± 0·2 mg and that of corpus striatum was 5·9 ± 0·1 mg.

Tissue incubation and estimation of etorphine accumulation. The incubation medium consisted of a modified Krebs–Ringer solution which contained 12 mM glucose and 0·05 M Tris [tris(hydroxymethyl)-aminomethane] buffer, pH 7·4 [20]. The medium was saturated with oxygen and the desired amount of radioactive etorphine was added just before the start of incubation. Twenty-ml beakers containing 5 ml medium with and without various drugs and inhibitors were placed in a metabolic shaker-incubator and warmed to 37° before the slices were put into the medium. After various times of incubation under an atmosphere of 100% oxygen, the slices were removed from the medium and washed twice by dipping the slices in two 30-ml portions of 0·32 M sucrose. The slices were then placed in counting vials and solubilized in 1 ml NCS (Nuclear Chicago Solubilizer, Amersham/Searle Corp.). The radioactive content of the resulting solution was determined by adding 10 ml PPO–POPOP solution (6 g/l. of 2,5-diphenyloxazole and 75 mg/l. of *p*-bis-[2-(5-phenyloxozolyl)]-benzene in toluene) and counting in a liquid scintillation spectrometer after the solution was kept in the dark for several hr. Since thin-layer chromatograms of the extracts of incubated slices revealed that etorphine was not metabolized in the tissue, the

* This investigation was supported by U.S. Public Health Service Grant DA 00289.

† U.S. Public Health Service Postdoctoral Trainee (GM 01117).

radioactivity represented only the parent compound under the described experimental conditions. Although radioactivity of an aliquot (0·1 ml) of the incubation medium was also determined in every experiment, the concentration remained constant since the volume of the medium was so much greater than that of the slices. Most of the results were calculated as (dis./min/g tissue)/dis./min/ml medium) and expressed as T/M (tissue/medium) concentration ratios.

In all experiments in which the effect of various compounds on etorphine accumulation was studied, each animal served as its own control and the slices were paired, i.e. slices from each animal were incubated with and without the compounds and the differences in the uptake were compared.

Statistics. The paired *t*-test was used to evaluate the effect of various compounds added *in vitro*. In the double-reciprocal plots [21], the regression lines were analyzed by the method of Wilkinson [22].

Chemicals. [^{3}H]etorphine (3·4 Ci/m-mole) was kindly supplied by Dr. Horace H. Loh, University of California, San Francisco Medical Center. Drugs which were received as gifts were etorphine hydrochloride from American Cyanamid Co., verapamil from Knoll Pharmaceutical Co., naloxone hydrochloride from Endo Laboratories, Inc., pentazocine from Sterling-Winthrop Research Institute and levorphanol and dextrorphan tartrates from Hoffmann-La Roche, Inc. All other chemicals were reagent grade obtained from commercial sources.

RESULTS

Etorphine accumulation by slices of cerebral cortex and corpus striatum. The two brain areas under study were chosen because an active uptake of various narcotics by cerebral cortical slices has been reported by Scrafani and Hug [16], and the highest density of stereospecific opiate binding material has been demonstrated in the striatum by Pert and Snyder [2].

Slices from both brain areas in the presence of $1{\cdot}5 \times 10^{-9}$ M etorphine were able to accumulate

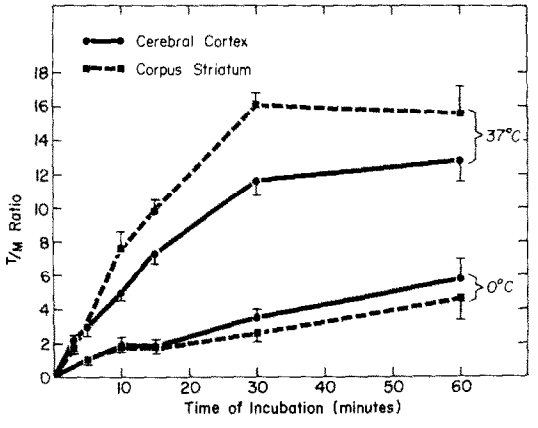

Fig. 1. Rate of accumulation of etorphine by slices of cerebral cortex and corpus striatum. Each point represents the mean ± S. E. of four animals. The concentrations of etorphine in the medium was $1{\cdot}5 \times 10^{-9}$ M.

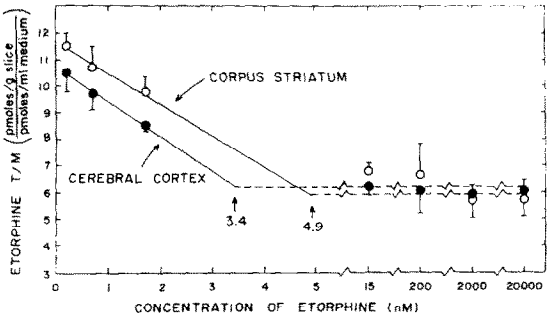

Fig. 2. Influence of etorphine concentration in the medium on its accumulation by cerebral and striatal slices. Accumulation of etorphine was determined after slices were incubated for 15 min. Each value represents the mean ± S. E. of four animals. Slices from morphine-dependent rats showed essentially the same accumulative properties.

etorphine, as seen by the T/M ratio for the accumulation which increased with increasing time of incubation and appeared to approach a maximum in about 30 min (Fig. 1). The maximum T/M attained was about 13 by cerebral slices and about 16 by slices of corpus striatum. When the slices were incubated at 0° instead of the usual 37°, the capacity to concentrate etorphine was inhibited by about 55 per cent in the cerebral slices and by about 70 per cent in the striatal slices. However, the slices still had considerable concentrative capacity at 0° as seen by the T/M of 5·8 by cerebral slices and 4·6 by striatal slices.

Influence of etorphine concentration on the accumulation. There appeared to be a biphasic accumulation by both cerebral and striatal slices as the medium concentration of etorphine was varied over a range of five orders of magnitude (Fig. 2). At very low concentrations up to about 3·4 nM for cerbral slices and 4·9 nM for striatal slices (extrapolated values), the accumulation appeared to be saturable. Above these concentrations, the T/M ratio remained constant as the concentration was raised by four orders of magnitude to 2×10^{-5} M. Thus, the latter accumulative process did not appear to be saturable. Slices from animals dependent on morphine did not show uptake characteristics different from those observed in slices from control rats. When the accumulation contributed by the non-saturable process, which was 40–70 per cent of the total accumulation in the low concentration range, was subtracted from the total accumulation and the kinetic constants of the saturable accumulation were estimated by double-reciprocal plots, there was no difference between cerebral and striatal slices from morphine-dependent and control rats with respect to the maximal accumulation of etorphine or the uptake affinity (Table 1). Although the affinity for etorphine was similar in cerebral and striatal slices, the striatal slices had a higher maximal capacity to take up etorphine.

Effect of metabolic inhibitors and organic basic compounds on the saturable etorphine accumulation. The results of these experiments using 5×10^{-10} M etorphine in the medium are tabulated in Table 2. Except for *N*-ethylmaleimide, none of the usual inhibitors of active transport systems inhibited the accumulation of etorphine. *N*-ethylmaleimide caused a 69 and 91

Table 1. Kinetics of the saturable accumulation of etorphine*

Slice	Treatment of animals	$K_a \pm$ S. E.† (nM)	$V_{max} \pm$ S. E.† (moles/g/15 min $\times 10^{10}$)
Cerebral cortex	Control	1·34 ± 0·03	0·07 ± 0·01
	M-dependent‡	1·25 ± 0·50	0·09 ± 0·03
Corpus striatum	Control	2·36 ± 0·82	0·20 ± 0·058
	M-dependent‡	1·69 ± 0·13	0·13 ± 0·01

* Kinetic constants were determined from the saturable accumulation of etorphine after the non-saturable portion had been subtracted. The non-saturable accumulation of etorphine by the cerebral slices was estimated to be 6·0 pmoles/g of tissue/nM etorphine in the medium and that by striatal slices was 5·1 pmoles/g of tissue/nM etorphine.

† K_a = concn of etorphine at half-maximal accumulation. V_{max} = maximal accumulation of etorphine by the slices. Values were estimated by double-reciprocal plots and represent mean ± S. E. of four animals for each group. Values for both K_a and V_{max} did not differ between control and morphine-dependent animals.

‡ Rats were implanted s.c. with two morphine pellets for 3 days.

§ Value for corpus striatum is significantly greater than that for cerebral cortex ($P < 0·05$, Student t-test).

per cent inhibition of the saturable accumulation in cerebral and striatal slices respectively. The usual basic transport competitors, hexamethonium and *N*-methylnicotinamide, also failed to affect the saturable accumulation of etorphine. Verapamil, a compound reported to specifically block calcium transport through cell membranes, completely inhibited the cerebral and striatal accumulation at a concentration of 2×10^{-3} M.

Since an active transport system for dihydromorphine had been reported by Scrafani and Hug [16] using much higher concentrations than those used in our study, the effect of the same inhibitors as above was tested with a higher concentration of 2×10^{-6} M etorphine. This concentration of etorphine is similar to the K_m value for dihydromorphine uptake described by Scrafani and Hug. The data revealed that none of the six inhibitors including *N*-ethylmaleimide and the basic amines had any influence on the accumulation of etorphine by both cerebral and striatal slices.

Effect of calcium ion on the saturable etorphine accumulation. Since calcium ions are involved in narcotic transport by choroid plexus [20] and inhibit both stereospecific narcotic binding to putative receptor material [2, 13, 23] and morphine analgesia [24], the effect of medium containing no calcium, no calcium plus the calcium chelator, EGTA (ethyleneglycol-bis-(β-aminoethyl ether)*N*,*N*'-tetraacetic acid), and high concentrations of calcium on etorphine accumulation was studied. It can be seen from the results in Table 3 that neither the omission of calcium nor 10- to 100-fold increases in the normal calcium concentration of the incubation medium inhibited the saturable accumulation of etorphine by either cerebral or striatal slices.

Effect of narcotics and narcotic antagonists on the saturable etorphine accumulation. Relatively high concentrations (2×10^{-6} M) of narcotic analgesics, morphine, methadone and levorphanol inhibited by varying degrees the saturable accumulation of etorphine by cerebral and striatal slices (Table 4). The narcotic-antagonist analgesic, pentazocine, and the narcotic antagonist, naloxone, were potent inhibitors of the etorphine accumulation by cerebral and striatal slices, and naloxone appeared to be the best inhibitor among the compounds employed. The use of dextrorphan, the inactive isomer of levorphanol, as an inhibitor revealed that the saturable accumulation of etorphine was relatively stereospecific. At a comparable degree of inhibition by both isomers, levorphanol was about 100 times more potent than dextrorphan as an inhibitor of etorphine accumulation.

Table 2. Effect of metabolic inhibitors and organic basic compounds on the saturable accumulation of etorphine

Concn of compound (M)	N*	Cerebral cortex† T/M (mean)‡ Control	Cerebral cortex† T/M (mean)‡ Experimental	Cerebral cortex† Mean difference ± S.E.	Cerebral cortex† Per cent of control	Corpus striatum† T/M (mean)‡ Control	Corpus striatum† T/M (mean)‡ Experimental	Corpus striatum† Mean difference ± S. E.	Corpus striatum† Per cent of control
2,4-Dinitrophenol (2×10^{-3})	7	3·87	3·15	+0·44 ± 0·71	81·4	6·30	5·02	−2·34 ± 1·35	78·9
N-ethylmaleimide (2×10^{-3})	5	4·07	1·24	−3·31 ± 0·638	30·5	6·44	0·58	−5·90 ± 1·028	9·0
Iodoacetamide (2×10^{-4})	5	3·32	3·84	+0·52 ± 0·74	115·7	5·47	6·38	+1·11 ± 0·97	116·6
Ouabain (2×10^{-4})	5	3·30	2·66	−0·96 ± 1·19	80·6	5·63	5·36	−0·26 ± 0·58	95·2
Sodium fluoride (2×10^{-3})	7	3·56	3·00	−0·56 ± 0·72	84·3	7·19	5·45	−2·88 ± 1·70	75·8
Sodium azide (2×10^{-3})	7	3·56	4·01	+0·45 ± 0·77	112·6	7·19	5·10	−2·10 ± 2·05	70·9
Hexamethonium (2×10^{-3})	5	2·89	2·51	−0·37 ± 1·04	86·9	5·54	5·16	−0·31 ± 1·21	93·2
N-methylnicotinamide (2×10^{-3})	5	2·96	3·26	+0·31 ± 0·47	110·1	5·79	6·66	+0·88 ± 1·74	115·0
Verapamil (2×10^{-3})	5	3·71	−0·40	−3·85 ± 0·538	< 0	5·15	−0·75	−5·90 ± 0·708	< 0

* Number of animals.

† Slices were incubated with 5×10^{-10} M etorphine for 15 min.

‡ These values represent the saturable accumulation of etorphine after the non-saturable portion of the total uptake was subtracted. Saturable accumulation of cerebral slices was 43 per cent of the total uptake and that of striatal slices was 57 per cent of the total uptake.

§ $P < 0·05$ (paired t-test).

Table 3. Effect of calcium ion on the saturable accumulation of etorphine

Condition	N*	Cerebral cortex† T/M (mean)‡ Control§	Experimental	Mean difference ± S.E.	Per cent of control	Corpus striatum† T/M (mean)‡ Control§	Experimental	Mean difference ± S.E.	Per cent of control
Calcium-free	4	2·79	2·95	+0·32 ± 0·27	105·7	4·31	3·58	−0·73 ± 0·88	83·1
Calcium-free + EGTA‖ (2 mM)	5	2·76	3·14	+0·38 ± 0·52	113·8	4·31	3·65	−0·65 ± 0·90	84·7
High calcium (13 mM)	5	2·80	2·85	+0·05 ± 1·04	101·8	4·23	4·28	+0·06 ± 1·04	101·2
High calcium (130 mM)	5	2·63	2·72	+0·85 ± 0·43	103·4	4·57	3·67	−0·90 ± 1·00	80·3

* Number of animals.

† Slices were incubated with 5×10^{-10} M etorphine for 15 min.

‡ These values represent the saturable uptake of etorphine after the non-saturable portion of the total uptake was subtracted. Saturable uptake of cerebral slices was 43 per cent of the total uptake and that of striatal slices was 57 per cent of the total uptake. None of the changes in the calcium concentration altered the accumulation of etorphine by either cerebral or striatal slices.

§ Control slices were incubated in medium containing the usual 1·3 mM of Ca^{2+}.

‖ EGTA = ethyleneglycol-bis-(β-aminoethyl ether)*N*,*N*′-tetraacetic acid.

DISCUSSION

The data in the present study indicate that there is a biphasic accumulation of etorphine by slices of cerebral cortex and corpus striatum. At very low concentrations, the slices take up etorphine against an apparent concentration gradient and the process is saturable. However, above a concentration of about 5 nM of etorphine in the medium, the uptake of etorphine by the slices appears to be non-saturable. Scrafani and Hug [16] also reported a similar biphasic uptake system in cerebral cortical slices using dihydromorphine, but they used much higher concentrations of the drug. At concentrations they employed of 5×10^{-7} to 1×10^{-4} M, we were unable to detect saturable uptake of either etorphine or dihydromorphine (unpublished data).

Scrafani and Hug [16, 25] also described their uptake system as an energy-requiring one which satisfied the usual criteria for an active transport system. Our results indicate that concentrative capacity and saturability of the slices satisfy some of the criteria of a carrier-mediated process, but except for one inhibitor which we shall discuss later, none of the usual metabolic inhibitors had any effect on the saturable accumulation of etorphine. The accumulation of etorphine was, however, inhibited by narcotics and narcotic antagonists which have structural similarities to etorphine. Additionally, by usage of levorphanol and dextrorphan as competitive inhibitors, saturable accumulation of etorphine was shown to be relatively stereospecific. Lowering the incubation bath temperature to 0° inhibited the accumulation of etorphine by the slices, but the slices still retained a substantial concentrative capacity. Although some of the criteria for an active transport system have been demonstrated by the accumulative process in cerebral and striatal slices, conclusion as to the active nature of the uptake should be drawn with caution since the important criterion of the requirement of metabolic energy is not totally met.

Other investigators have reported the non-saturable, passive uptake of narcotics such as dihydromorphine [26] and morphine [27, 28]. However, the concentrations used by these investigators were again in a high range where the saturable component of narcotic accumulation probably would have been

Table 4. Effect of various narcotics and narcotic antagonists on the saturable accumulation of etorphine

Concn of compound (M)	N*	Cerebral cortex† T/M (mean)‡ Control	Experimental	Mean difference ± S. E.	Per cent of control	Corpus striatum† T/M (mean)‡ Control	Experimental	Mean difference ± S. E.	Per cent of control
Morphine (2×10^{-6})	6	3·54	1·10	−2·44 ± 0·69§	31·1	6·64	2·15	−4·48 ± 0·65§	32·4
Methadone (2×10^{-6})	6	4·31	1·27	−3·04 ± 0·61§	29·5	7·49	2·13	−5·40 ± 1·15§	28·4
Naloxone (2×10^{-6})	4	3·11	−0·40	−3·14 ± 0·28§	< 0	6·03	0·39	−5·64 ± 1·31§	6·5
Pentazocine (2×10^{-6})	5	4·11	0·19	−3·92 ± 0·55§	4·6	7·29	1·12	−6·16 ± 1·40§	15·4
Levorphanol (2×10^{-6})	5	4·51	0·70	−3·81 ± 0·69§	15·5	6·90	0·69	−6·21 ± 1·19§	10·0
(2×10^{-5})	5	2·95	−1·03	−3·98 ± 0·41§	< 0	4·63	−0·94	−5·57 ± 0·86§	< 0
Dextrorphan (2×10^{-6})	5	4·51	3·55	−0·96 ± 1·22	78·7	6·90	7·18	+0·28 ± 0·85	104·1
(2×10^{-5})	5	2·95	1·98	−0·96 ± 1·68	67·1	4·63	1·54	−3·08 ± 1·07§	33·3
(2×10^{-4})	5	2·97	0·49	−2·47 ± 0·74§	16·5	5·07	−0·49	−5·56 ± 0·88§	< 0

* Number of animals.

† Slices were incubated with 5×10^{-10} M etorphine for 15 min with and without various compounds.

‡ These values represent the saturable accumulation of etorphine after the non-saturable portion of the total uptake was subtracted. Saturable accumulation of cerebral slices was 43 per cent of the total uptake and that of striatal slices was 57 per cent of the total uptake.

§ $P < 0.05$ (paired *t*-test.

masked. Our data agree with those of the above authors as to the passive nature of the etorphine uptake system in cerebral and striatal slices at high narcotic concentrations, but at low concentrations, there is a separate, saturable accumulative system which apparently requires no metabolic energy.

The question arises as to whether or not the saturable etorphine accumulation is related to the stereospecific narcotic binding to putative receptor material described by others [1–15]. There are several similarities between the narcotic binding and uptake systems which point positively toward such a relationship. The high accumulative capacity is associated with the brain area which has been reported to contain the highest density of binding material [2]. A biphasic accumulation of etorphine was observed with a saturable and non-saturable component. A similar biphasic stereospecific binding of dihydromorphine to membranes of corpus striatum and brainstem homogenates has also been demonstrated [8, 9]. Both the binding and accumulative systems are saturable without the requirement of metabolic energy and the kinetic constants of both systems are in similar ranges, i.e. binding constant [2, 6, 9, 13] (cf. uptake constant) and maximal binding capacity [2, 6, 9, 13] (cf. maximal accumulative capacity). Etorphine binding [6] and accumulation of etorphine are stereospecific in that the pharmacologically active isomer, levorphanol, inhibits etorphine binding and accumulation relatively more than the inactive isomer, dextrorphan. Both accumulation and binding of etorphine are inhibited by other narcotics and narcotic antagonists, and the accumulation is not inhibited by the usual basic transport competitors.

Although the above comparisons suggest a relationship between narcotic binding to putative receptor material and narcotic accumulation by cerebral and striatal slices, there are some differences between the two systems. The degree of inhibition of narcotic binding by various narcotics has been shown to parallel the pharmacologic potencies of the drugs [2, 6, 12], but such a correlation between inhibition of etorphine accumulation and pharmacologic potency of the inhibitors (other narcotics) was not apparent. The number of binding sites (V_{max} for binding) for narcotics and narcotic antagonists has also been reported to be greater in morphine-dependent animals than in control animals [5, 13], whereas such an increase in the maximal accumulative capacity of etorphine in morphine-dependent animals was not observed. One laboratory has reported that neither the binding affinity nor the maximal binding capacity is altered as a result of the development of narcotic dependence [10]. However, there appears to be general agreement among the investigators that changes in binding affinity or capacity are not related or correlated with the development of tolerance or dependence.

Several laboratories have reported that calcium ions inhibit the binding of narcotics to putative receptor material [2, 13, 23]. Yet, neither large excess of calcium ions nor calcium deficit affected the accumulation of etorphine. Interestingly, verapamil, a specific calcium transport inhibitor, had a substantial inhibitory effect on the accumulation of etorphine. We have suggested that verapamil may act as a tertiary amine competitor of methadone uptake by the choroid plexus [29], and there is a possibility that verapamil may act on etorphine accumulation in a similar manner.

The differences between the binding and accumulative systems indicate that further experimentation is necessary to firmly establish a possible relationship between binding and accumulation of narcotics. Even if the accumulation process was distinctly separate from that of binding, an accumulative system with no energy requirements and high concentrative capacity would not be expected to limit the access of narcotics to their receptors. The accumulative system would represent a means for concentrating narcotics near the receptor sites; however, whether the binding system represents association with pharmacologic receptors remains to be proven.

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