Biochemical Pharmacology, Vol. 28, pp. 2961–2966

0006-2952/79/1001–2961 $02.00/0

EFFECT OF MORPHINE *IN VIVO* ON UPTAKE OF [^{3}H]CHOLINE AND RELEASE OF [^{3}H]ACETYLCHOLINE FROM RAT STRIATAL SYNAPTOSOMES

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(*Received* 31 *July* 1978; *accepted* 17 *April* 1979)

Abstract—The effect of morphine on the rat striatal cholinergic system was investigated *in vitro* by measuring the rates of [^{3}H]choline uptake and [^{3}H]acetylcholine release in striatal synaptosomes after *in vivo* injections of morphine sulfate. Morphine caused a 50 per cent increase in the V_{max} of [^{3}H]choline uptake. Although a concomitant increase was also measured in the amount of [^{3}H]acetylcholine released, it could be explained by the previous increase in uptake. It is suggested that morphine had an overall stimulatory effect on the striatal cholinergic system which may be a transynaptic phenomenon rather than a direct effect on the cholinergic cell.

The major mechanisms of action of narcotic and analgesic drugs, such as morphine, are still largely unknown. In spite of a large body of literature on the subject, controversy still exists as to a possible link between morphine actions and cholinergic transmission [1]. It had been suggested that the confusion may be due, in part, to multiple sites of action of morphine within the complex framework of neuronal tissue. It was the purpose of this study to investigate the effects of morphine on the cholinergic system of the striatum in a system which may be less complex than those studied previously. Specifically, the rates of choline uptake and acetylcholine release from striatal synaptosomal preparations were determined after *in vivo* exposure to morphine.

EXPERIMENTAL

Male Sprague–Dawley rats (TIMCO, Houston, TX) (weight 100–200 g) were injected s.c. with various concentrations of morphine sulfate (Merck Chemical Co, Rakway, NJ) and/or naloxone hydrochloride (Edo Labs. Inc., Garden City, NY) (1 mg/kg), or saline. At various times post-injection, as stated in the results, the rats were decapitated, and the striata were removed and homogenized in ice-cold 0.32 M sucrose solution using a glass–teflon tissue grinder. The homogenate was centrifuged for 5 min at 1,000 *g*. The resultant supernatant solution was re-centrifuged at 12,000 *g* for 15 min to sediment a crude, nuclei-free synaptosomal fraction. The synaptosome pellet was resuspended using a Dounce homogenizer in a volume of ice-cold 0.32 M sucrose equivalent to that used in the initial homogenization. Two hundred μl of the suspension was removed and analyzed for protein content [2]. The suspension was again centrifuged at 12,000 *g* for 15 min. The pellet was resuspended in a Ca^{2+}-free modified Ringer's solution consisting of 10 mM *d*-glucose, 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES), 150 mM NaC, 6.2 mM KC, 1.2 mM Na_2PO_4, and 1.2 mM $MgSO_4 7H_2O$), pH 7.4 (with NaOH). (This buffer will be referred to as standard buffer.) Following centrigugation at 15,000 rev/min for 15 min, the pellet was again resuspended in standard buffer and diluted to approximately 0.5 mg/ml of protein (two striata/10 ml). The synaptosomal suspensions were then analyzed for [^{3}H]acetylcholine release.

[^{3}H]Choline uptake studies. Synaptosomal suspensions were incubated at 37° or on ice for 4 min in medium containing about 0.5 mg protein/ml, 0.25 μM [^{3}H]choline (methyl-[^{3}H])chloride (New England Nuclear, Boston, MA) and 10 μM neostigmine methylsulfate (Sigma Chemical Co., St. Louis, MO) in standard buffer. The synaptosomal suspension was rapidly filtered onto GF/A glass–fiber filters (Whatman, Clifton, NJ) at (−)5 psi. The tubes and the filter were rinsed with an additional 6.0 ml of standard buffer. The filters were vacuum dried for a few seconds, placed in liquid scintillation counting vials containing 1.0 ml of 1% sodium dodecyl sulfate in 20 mM EDTA, and left overnight at room temperature. Ten ml of counting solution [3] was added and the vials were counted in a Searle (Des Plaines, II) Mark II liquid scintillation system; counting efficiency was 29.2 per cent.

Ca^{2+}-free buffer was used for these experiments in order to minimize any effects of the tested compounds on release systems. Since neuronal release had been shown to be Ca^{2+}-dependent, release activity in the synaptosomes during the assay period should be minimal, thus allowing a full measurement of labeled compounds taken up. Uptake systems for neurotransmitters have been shown to be Ca^{2+}-independent under the conditions employed here [4]. Neostigmine was included for the purpose of inhibiting formation of choline from released acetylcholine, both labeled and unlabeled, which could theoretically interfere with uptake measurements.

[^{3}H]Acetylcholine release study. Synaptosomal suspensions (about 0.5 mg protein/ml) were incubated in the presence of 0.25 μM [^{3}H]choline and 10 μM neostigmine methyl sulfate in standard buffer for 10 min at 27°. Synaptosomes were collected on (GF/A) glass–

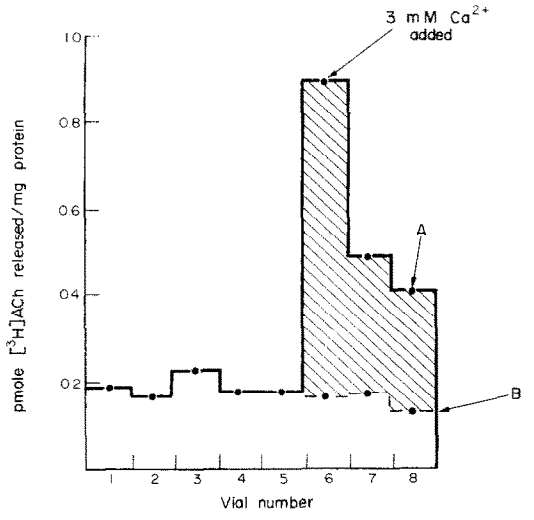

Fig. 1. Ca^{2+}-dependent, K^+-stimulated release of [^{3}H]ACh from control synaptosomes. Control synaptosomal suspensions (0.5 mg protein) were preincubated with 0.25 μM [^{3}H]choline (10 min, 37°), collected on a filter and superfused with Ca^{2+}-free buffer containing depolarizing levels of K^+ (56.2 mM). Superfusates were continuously collected; each collection period lasted for 15 sec. During the collection of vial 6, superfusion medium was either changed to one containing 3 mM $CaCl_2$ (A) or maintained unchanged (B). The amount of calcium-dependent, potassium-stimulated release was determined by subtracting B from A and is shown as shaded bars. Data are from a representative experiment.

fiber filters and rinsed with 5 ml of standard buffer. The filters were then placed in a modified Swinnex filtering unit (Millipore Co., Bedford, MA) and superfused with buffers using a peristaltic pump (Harvard Apparatus, Millis, MA).

To determine the amount of acetylcholine released by calcium-dependent, potassium-induced stimulation, the synaptosomes on the filter were first depolarized by superfusion with standard buffer containing a depolarizing level of potassium (56.2 mM total concentration) in the absence of calcium. The superfusion medium was quickly changed to one containing 3 mM calcium; potassium concentration was maintained at 56.2 mM. Superfusates (1 ml) were collected every 15 sec and counted. At the conclusion of each superfusion sequence, filters were removed and counted as described above.

A representative 'release profile' from control synaptosomes is shown in Fig. 1. A depolarizing level of potassium (56.2 mM) was maintained throughout the superfusion. The B portion of the figure represents the cpm released in the absence of calcium. Portion A of the figure represents the cpm released when calcium is added to the superfusion medium. The amount of calcium-dependent, potassium-stimulated release was determined by subtracting B from A, and is shown in Fig. 1 as shaded bars. In cases where the B portion of the curve was not actually measured, values for B were extrapolated. The addition of calcium in the absence of elevated potassium had little effect on release rates (data not shown).

Under all conditions used in this assay, including addition of morphine and/or naloxone, more than 95 per cent of the released radioactivity was associated with [^{3}H]acetylcholine ([^{3}H]ACh) as determined by thin-layer chromatography after ethanol extraction and using a solvent system of *n*-butanol–H_2O–formic acid (60:35:15 v/v).

RESULTS

Control experiments. In a series of control experiments, preparations of striatal synaptosomes were analyzed to determine the parameters of the high affinity choline uptake system and the calcium-dependent, potassium-stimulated release of acetylcholine. The effects of various incubation times, temperature and protein concentration were examined; the results are shown in Fig. 2. The amount of [^{3}H]choline taken up at 4° was always less than 10 per cent of the total taken up at 37°. In subsequent calculations, the amount taken up at 4°

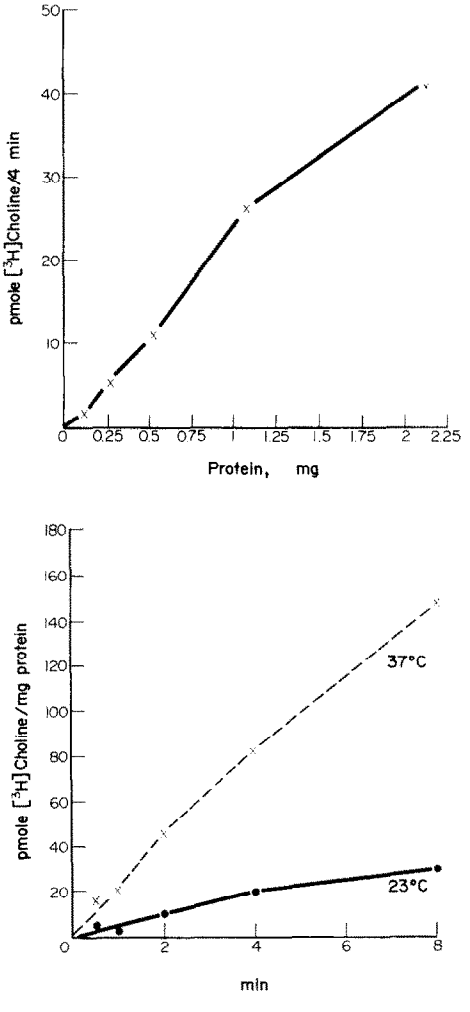

Fig. 2. Uptake of [^{3}H]choline by control striatal synaptosomes. Panel A: control synaptosomal suspensions (0.1 to 2.1 mg protein) were incubated at 37° or on ice (4°) for 4 min, rapidly filtered, and washed. Filters were counted; binding at 4° was subtracted from binding at 37°. Panel B: control synaptosomal suspensions (0.5 mg protein) were incubated at 37°, 23°, or on ice for 1–10 min and processed as described in panel A. Data are means from four to six experiments. S.E.M. < 10 per cent.

Fig. 3. Effects of morphine *in vivo* on synaptosomal [³H]choline uptake *in vitro*. Synaptosomes were prepared from animals receiving injections of either 15 mg/kg of morphine sulfate or saline 30 min prior to decapitation. Synaptosomal suspensions were incubated in [³H]choline (0.05 to 2 μM) for 4 min at 37° and on ice (4°). Incubation was terminated by rapid filtration and washing. Uptake at 4° was substracted from uptake at 37°. Values are means from six experiments, S.E.M. < 10 per cent.

under each condition studied was used as a blank value and subtracted from the amount of [³H]choline taken up under comparable conditions at 37°. High affinity uptake of [³H]choline was linear up to 8 min of incubation, 0.25 to 2 mg/ml of synaptosomal protein, and from 0.01 to 0.5 μM [³H]choline (Figs. 2 and 3).

Unless otherwise noted, all subsequent incubation media contained approximately 0.5 mg of synaptosomal protein ml and 0.25 μM [³H]choline for 4 min at 37°.

The release of [³H]acetylcholine from striatal synaptosomes was found to be stimulated by potassium, and dependent upon the presence of extracellular calcium ions. The amount of [³H]acetylcholine released was linear in the range of calcium concentrations tested (from 0.1 to 1.0 mM) (Fig. 4).

Effect of morphine on [³H]*choline uptake.* Injection of morphine sulfate (15 mg/kg), s.c., 30 min prior to decapitation, increased the rate of [³H]choline uptake by the striatal synaptosomes. The binding curve shown in Fig. 3 indicates an upward shift in the maximal rate of choline uptake. Analysis of these data via a Lineweaver–Burk plot (Fig. 5) revealed a V_{max} for saline control samples of 60.8 pmoles/mg of protein for 4 min. compared to 100 pmoles/mg of protein for 4 min for morphine-treated animals. The K_m, however, was not significantly altered (0.507 μM for controls, 0.629 μM for morphine-treated).

The stimulation of [³H]choline uptake increased as the dose of morphine was increased from 5 to 50 mg/kg (Fig. 6). However, the increase in uptake was statistically significant only with 30 and 50 mg/kg injections.

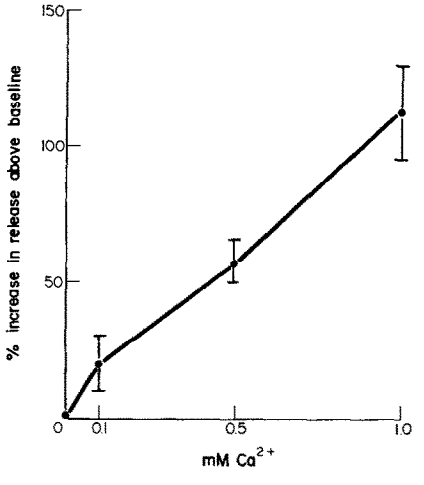

Fig. 4. Release of [³H]acetylcholine from control synaptosomes as a function of calcium concentration. Assay conditions are the same as those described in the legend of Fig. 1 except that the concentration of CaC_2 added during the sixth collection period was varied from 0.1 to 1.0 mM. The amount of Ca^{2+}-dependent, K^+-stimulated release determined in this manner is plotted as a function of calcium concentration. Data are means of four experiments ± S.E.M.

	K_m, μM	V_{max}, pmoles/4 sec/mg protein
Saline	0.507	60.8
Morphine	0.629	100

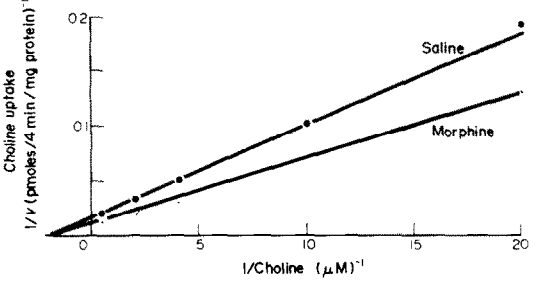

Fig. 5. Kinetic analysis of [³H]choline uptake by synaptosomes from control and morphine-treated rats. Values were obtained by replotting data from Fig. 3. K_m and V_{max} were determined by linear regression analysis (r = 0.94).

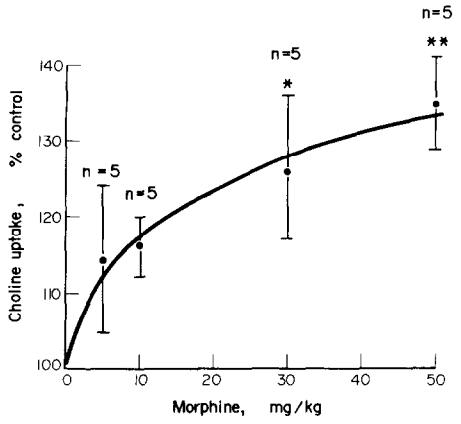

Fig. 6. Dose–response of synaptosomal uptake of [^{3}H]choline to morphine sulfate treatment *in vivo*. Assay and injection conditions are the same as those described in the legend of Fig. 3 except that choline concentrations were maintained at 0.25 μM and morphine sulfate concentrations were varied between 5 and 50 mg/kg body weight. The single asterisk (*) indicates significantly different from controls at the P = 0.05 level; and the double asterisk (**) indicates significantly different from controls at the P = 0.01 level.

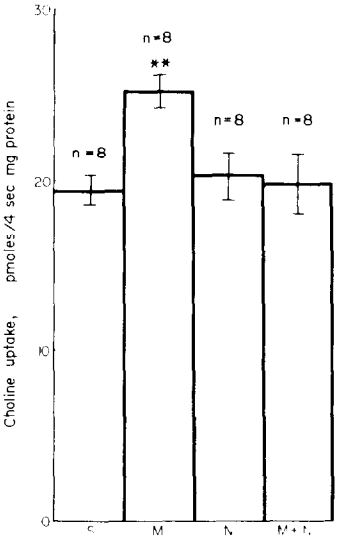

Fig. 8. Effect of naloxone on the morphine-induced increase in synaptosomal uptake of [^{3}H]choline. Naxoxone (1 mg/kg) was injected simultaneously with morphine (50 mg/kg) into one group of animals (M + N) 1 hr prior to decapitation and preparation of striatal synaptosomes. Other groups of animals received injections of saline (S), morphine (M), or naloxone (N) alone. Assay conditions are the same as those described in the legend of Fig. 3 except that [^{3}H]choline concentrations were maintained at 0.5 μM. Data are means ± S.E.M.; n = 8. The double asterisk (**) indicates significantly different from saline controls at the P = 0.01 level.

[^{3}H]Choline uptake was analyzed in synaptosomes prepared 15–240 min after injection of morphine at 50 mg/kg, s.c. The response to morphine was apparent as early as 15 min post-injection (Fig. 7). The choline uptake remained significantly higher up to 2 hr post-injection, and then declined to control values at 3–4 hr after the injection.

Naloxone hydrochloride (1 mg/kg), when injected simultaneously with morphine (50 mg/kg, s.c., 1 hr), blocked the morphine effect on [^{3}H]choline uptake. Naloxone alone did not change [^{3}H]choline uptake significantly from control values (Fig. 8).

In order to determine if morphine inhibited ACh synthesis from exogenously added [^{3}H]choline, synaptosomal lysates from control and morphine-treated animals were analyzed after 10 min of incubation with 0.5 μM [^{3}H]choline. The [^{3}H]ACh was separated from [^{3}H]choline by precipitation with gold chloride [5]. The percentage of total label associated with precipitated [^{3}H]ACh was unchanged by morphine treatment (C = 73 ± 8 per cent; M = 70 ± 10 per cent; n = 3).

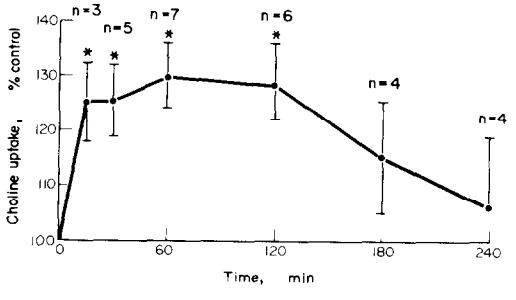

Fig. 7. Effect of morphine sulfate treatment *in vivo* on synaptosomal uptake of [^{3}H]choline *in vitro* as a function of post-injection time. Assay conditions are the same as those described in the legend of Fig. 3 except that [^{3}H]choline concentrations were maintained at 0.5 μM and the time between injection and preparation of synaptosomes was varied between 15 and 240 min. Data are means ± S.E.M.; for each time point is shown in the figure. The asterisk (*) indicates significantly different from control at the P = 0.05 level.

Effect of morphine on [^{3}H]acetylcholine release. The calcium-dependent, potassium-stimulated release of [^{3}H]acetylcholine from striatal synaptosomes was increased by morphine treatment *in vivo* (50 mg/kg, s.c., 1 hr before decapitation) (Fig. 9A). The morphine effect was inhibited by naloxone (1 mg/kg) injected simultaneously. As noted in earlier experiments (see above), synaptosomes from morphine-treated animals take up more [^{3}H]choline than controls and, therefore, have a larger pool of [^{3}H]acetylcholine from which release can take place. An attempt was made to take into account the difference in pool sizes between control and morphine-treated samples by calculating the percent of the labeled pool which was released. Thus, the percent of [^{3}H]acetylcholine released was calculated by dividing the pmoles of [^{3}H]acetylcholine released during stimulation by the size of the total labeled pool (i.e. the total radioactivity on the filter just prior to release). The percentages of [^{3}H]acetylcholine released from morphine and control animals were not significantly different when calculated in this manner (Fig. 9B). In addition, no change was observed in the time course of release after morphine or naloxone treatments. Results were similar for all concentrations of calcium tested (0.1 to 3 mM). In addition, the ratio of

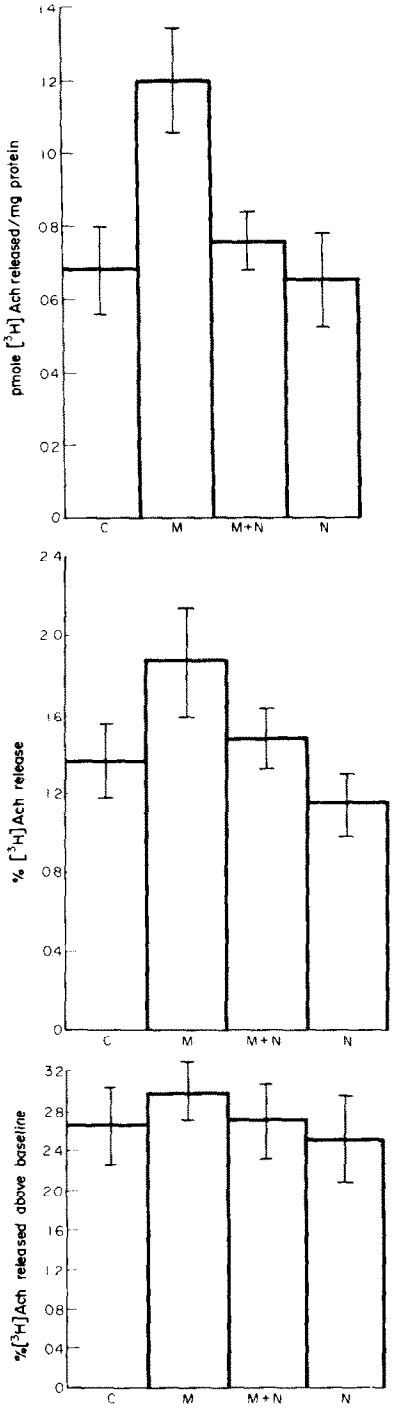

Fig. 9. Effects of morphine and naloxone *in vivo* on Ca^{2+}-dependent, K^+stimulated release of [^{3}H]ACh from synaptosomes *in vitro*. Naloxone (1 mg/kg) was injected simultaneously with morphine (50 mg/kg) into one group of thirteen animals (M + N) 1 hr prior to decapitation and preparation of striatal synaptosomes. Other groups of animals received injections of saline (S), morphine (M), or naloxone (N) alone. Assay conditions are the same as those described in the legend of Fig. 1. Only the amount of Ca^{2+}-dependent, K^+-stimulated release above baseline is shown as means ± S.E.M.; n = 13. Data are plotted as panel A: pmoles [^{3}H]ACh released/mg of protein; only (M) is significantly different from controls at the P = 0.05 level; panel B: percent of total [^{3}H]ACh taken up which was released; and panel C: percent released above baseline.

the evoked release to the baseline release was not significantly changed by morphine and/or naloxone injection (Fig. 9C).

DISCUSSION

The striatum had an abundance of cholinergic interneurons [6]. As previously reported by others [7], synatosomal preparations of rat striatum clearly demonstrate the presence of an active cholinergic system. High affinity uptake of choline was demonstrated in striatal synaptosomal fractions in that K_m and V_{max}, values were similar to those reported from hippocampal synaptosomes by Simon and Kuhar [8]. The [^{3}H]choline taken up by the striatal synaptosomes was rapidly synthesized into ACh and made available for release by potassium-stimulated, calcium-dependent mechanisms. The effect of morphine on these two aspects of the cholinergic system was evidenced by an approximate 50 per cent increase in the V_{max} of uptake. Although a concomitant increase was also measured in the amount of [^{3}H]acetylcholine released, this increase could be explained by the previously increased uptake. Thus, there was no evidence that morphine had a direct effect on the release mechanism.

Atweh *et al.* [7] also showed an increase in choline uptake in striatal synaptosomes after *in vivo* morphine administration; however, the increase was small and statistically insignificant. It is not entirely clear at to the reason for the small increase (10 per cent) reported by this group as compared to the larger increase (30 per cent) reported here; however, uptake methodologies used were somewhat different (centrifugation vs filtration) and, in addition, only a single concentration of morphine and a single concentration of [^{3}H]choline were used for their measurements rather than complete dose–response curves and kinetic analysis of uptake as presented here.

Increased choline uptake had been reported in hippocampus and other structures in the brain subsequent to stimulation of the cholinergic system in the intact animal [8]; thus, the level of cholinergic activity within a given brain are may be reflected in the rate of [^{3}H]choline uptake into synaptosomes prepared from that area. If such is the case, the increase in [^{3}H]choline uptake observed after morphine treatment may be indicative of morphine-stimulated cholinergic activity rather than a direct effect of morphine on the choline uptake system. This interpretation is supported by the finding that morphine *in vitro* has little effect on synaptosomal [^{3}H]choline uptake (D. Redburn., unpublished observations). Likewise, morphine *in vitro* has little effect on [^{3}H]acetylcholine release. These data suggest that the intact neuron is necessary for the effects of morphine to be realized and that the actions of morphine are mediated through receptor sites that are not functionally present in synaptosomal fractions, i.e. they may be located on non-synaptic regions of the neuron. The time course of the morphine effect on synaptosomal uptake is similar to the time of the changes in morphine concentration in whole brain after morphine injection [9].

Our results are limited to studies in the the striatum and may not apply to other brain areas. The cholinergic system in the striatum differs from that in the occipital

cortex in its sensitivity to chlorpromazine and haloperidol [10]. Thus, reports of decreases in acetylcholine release from the cerebral cortex *in vivo* may not be particularly relevant to the acetylcholine system in the striatum [11,12].

The data presented here offer no evidence as to the mechanism by which morphine might increase cholinergic activity. Additional experiments are needed to determine if morphine influences the striatal cholinergic system via a direct effect on other known neurotransmitter systems of the striatum. Of particular interest would be the possible influence of morphine on the reported dopamine-induced inhibition of striatal cholinergic neurons [13].

Acknowledgements—This research was supported in part by USPH Grant EYO 1655-02; Research Career Development Award 1K04 EYO 0088-02 (D.A.R.); and a Rockefeller Foundation Fellowship (T.C.).

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