MOLECULAR PHARMACOLOGY, 14, 448–453

Effects of Morphine on the Incorporation of [^{14}C]Serine into Phospholipid via the Base-Exchange Reaction

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(Received August 8, 1977)
(Accepted December 12, 1977)

SUMMARY

NATSUKI, REIKO, HITZEMANN, ROBERT & LOH, HORACE (1978) Effects of morphine on the incorporation of [^{14}C]serine into phospholipid via the base-exchange reaction. *Mol. Pharmacol.*, 14, 448–453.

The effects of morphine on the incorporation of [^{14}C]serine into phospholipid via the base-exchange reaction were examined using washed brain microsomal membranes as the enzyme source. The addition of morphine *in vitro* increased the basal rate (no added Ca^{++}) of [^{14}C]serine exchange, with the maximum increase occurring at 10 μM. This concentration of morphine also increased the Ca^{++}-stimulated exchange at Ca^{++} concentrations of 10, 15, and 20 mM but not 5, 25, or 30 mM. These morphine effects *in vitro* were blocked by naloxone. Both acute and chronic prior treatment with morphine decreased the basal rate of [^{14}C]serine exchange. However, chronic but not acute morphine treatment significantly increased the Ca^{++}-stimulated exchange of [^{14}C]serine at all Ca^{++} concentrations tested (2.5–30 mM). In contrast, chronic morphine treatment slightly but significantly stimulated [^{14}C]ethanolamine exchange at one Ca^{++} concentration (2.5 mM) and decreased [^{14}C]choline exchange at all Ca^{++} concentrations tested. Possible mechanisms underlying these various changes are discussed.

INTRODUCTION

The calcium-stimulated, energy-independent phospholipid base-exchange reaction has been demonstrated to occur both *in vitro* and *in vivo* in nervous tissue (1–5). The finding that the rate of base exchange is greater in neuronal than in glial cells (6) suggests that the reaction may have some significance in neuronal function (5). Our interest in the base-exchange reaction stems from observations suggesting that a relationship exists between brain calcium and the mechanism(s) of narcotic action. It is well known that morphine antinociception is blocked by calcium (7). Conversely, lanthanum, a calcium antagonist, can induce a morphine-like analgesia which is blocked by naloxone and which shows cross-tolerance with morphine (8). On the basis of these data, we postulate that acute and/or chronic morphine treatment may influence the calcium-dependent exchange reaction, causing a change in the turnover and/or composition of membrane phospholipids. For example, since phosphatidylserine is synthesized in the brain entirely via base exchange (9), a change in the rate of serine incorporation could alter the acidic phospholipid composition of brain membranes.

This work was supported in part by Grants DA-00564 and DA-01583 from the United States Public Health Service. This is Manuscript No. 78-13 from the Department of Pharmacology, University of California, San Francisco.

[1] Recipient of Research Scientist Career Development Award K2-DA-70554 from the National Institute of Mental Health.

0026-895X/78/0143-0448$02.00/0

In the present study we have examined the effects of various morphine treatments on the incorporation of serine into microsomal phospholipids via the base-exchange reaction. The data indicate that chronic morphine treatment specifically increases calcium-stimulated [^{14}C]serine incorporation into phospholipids. Under identical conditions the incorporation of [^{14}C]ethanolamine is only slightly increased and the incorporation of [^{14}C]choline is significantly decreased.

MATERIALS AND METHODS

Materials. L-[3-^{14}C]Serine (specific activity, 40 mCi/mmole), [1,2-^{14}C]ethanolamine (specific activity, 5 mCi/mmole) and [1,2-^{14}C]choline (specific activity, 10 mCi/mmole) were purchased from New England Nuclear. All other reagents were obtained from local suppliers. The organic solvents used were spectrophotometric grade and were not purified further.

Experimental protocol. Male Sprague-Dawley rats, weighing 180–220 g, were used in all experiments. For acute drug studies, the animals were administered morphine sulfate, 20 mg/kg subcutaneously, or an equal volume of 0.9% NaCl subcutaneously 45 min prior to death. For the chronic studies, the animals were injected subcutaneously with 10 mg/kg of morphine sulfate between 4 and 5 p.m. The next day the animals were implanted with one 75-mg morphine pellet at 8 a.m., one pellet at 12 p.m., and two pellets at 4 p.m. Control animals were injected with 0.9% NaCl and implanted with placebo pellets. Forty-eight hours after the first pellet implantation the animals were killed by decapitation, and their brains were removed and processed immediately.

Preparation of brain microsomes. The methods used for the preparation of brain microsomes are described by Gaiti *et al.* (5). After the final centrifugation, the microsomes were resuspended in sufficient 0.32 M sucrose plus 2 mM dithiothreitol by hand homogenization to give a microsomal protein concentration of 8 mg/ml.

Assay of base-exchange activity. Base-exchange activity was assayed essentially as described by Porcellati and colleagues (1–3, 5, 10). The reaction mixture contained 40 mM *N*-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid buffer, pH 8.1, 600 μg of protein, various concentrations of $CaCl_2$, and saturating concentrations of the labeled substrates. The reaction was started by simultaneously adding the $CaCl_2$ and/or microsomal protein. The incubation was continued for 15 min at 37° in a Dubnoff water bath. The incubation was terminated by diluting the reaction mixture with 8 ml of ice-cold buffer and immediately centrifuging the sample for 30 min at 100,000 × *g*. The pellet was then extracted twice with chloroform–methanol (1:1, v/v). The extracts were combined, the chloroform–methanol concentration was adjusted to 2:1 (v/v), and 0.2 volume of 0.9% NaCl was added. The phospholipid extract was partitioned twice with the NaCl solution. After partitioning, the phospholipid extract was evaporated under vacuum and redissolved in a small volume of chloroform, and an aliquot was applied to a 250-μm silica gel G thin-layer chromatography plate (Merck). The plate was developed in a solvent system of chloroform, methanol, concentrated acetic acid, and water (25:15:4:2 by volume). After development, the various phospholipids were identified by iodine vapor. Since phosphatidylinositol and phosphatidylserine were not well resolved with this procedure, an additional technique was employed. Phosphatidylserine and phosphatidylinositol were eluted from the silica gel using chloroform–methanol (1:1, v/v), and the extract was evaporated to dryness in a 13 × 100 mm test tube. After evaporation, 50 μl each of dry pyridine and acetic anhydride were added. Acetylation was allowed to continue at room temperature for 30 min. The reaction mixture was then evaporated under vacuum at 40°. The acetylated derivatives of phosphatidylserine and phosphatidylinositol were then redissolved in a small volume of chloroform, and an aliquot was applied to the silica gel G thin-layer plates. The chromatogram was developed in a solvent system of chloroform, methanol, and concentrated ammonium hydroxide (65:25:5 by volume). The acetylated derivatives were identified with iodine vapor, the spots were marked, scraped, and eluted,

and phospholipid specific activity was determined as described elsewhere (11). Protein was determined as described by Lowry *et al.* (12), using bovine serum albumin as the reference standard. Phospholipid phosphorus levels were determined as described by Bartlett (13).

The data in Figs. 1–5 are expressed in terms of phospholipid specific activity rather than as nanomoles per milligram of protein for the following reasons. Despite washing of the lipid extract, a significant portion (20–40%) of the label within the extract was not associated with the appropriate phospholipid when the basal rate of exchange was measured. Thus it was necessary to isolate the various phospholipids via thin-layer chromatography. Given the variable loss of lipid that occurs during this manipulation and the absence of internal standards, e.g., [^{3}H]phosphatidylserine, the only accurate and reproducible way to express the data was as nanomoles of base incorporated per micromole of lipid phosphorus. This approach to expressing the data seems permissible since none of the drug treatments used affected the amount of phospholipid (phosphatidylserine, ethanolamine phospholipids, and phosphatidylcholine) per milligram of protein (data not shown).

RESULTS

Effects of morphine on incorporation of [^{14}C]serine via base exchange. In the absence of added Ca^{++}, 10 μM morphine significantly stimulated (30%) the basal rate of [^{14}C]serine exchange into microsomal phospholipid (Fig. 1). Naloxone alone did not significantly affect exchange, but 1 μM naloxone completely blocked the stimulatory effect of 10 μM morphine. In addition to its effects on the basal rate of exchange, 10 μM morphine produced small but significant increases in the Ca^{++}-stimulated exchange of [^{14}C]serine at Ca^{++} concentrations of 10, 15, and 20 mM (Fig. 2). These effects were blocked by 1 μM naloxone (data not shown). Acute morphine administration significantly decreased the basal rate of [^{14}C]serine exchange from 1.72 ± 0.05 to 1.38 ± 0.07 nmoles of serine per micromole of lipid phosphorus per 15 min, but had no effect on the Ca^{++}-stimulated exchange.

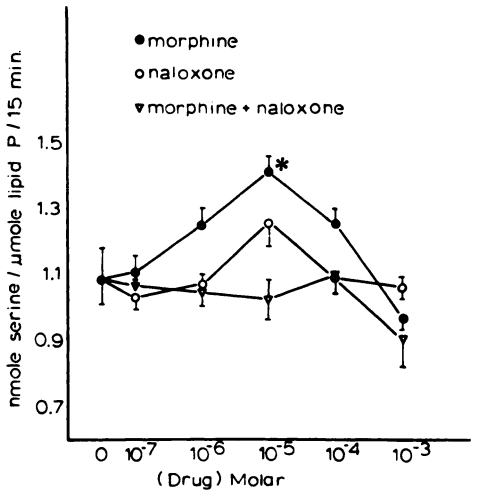

FIG. 1. *Effects of morphine and naloxone added in vitro on basal rate of [^{14}C]serine incorporation into brain microsomal phospholipid via base exchange*

The incorporation of [^{14}C]serine (1.5 mM) into microsomal phospholipid via the base-exchange reaction was measured as described in MATERIALS AND METHODS. The data illustrate the effects of the addition of various amounts of morphine or naloxone on the basal rate (no added Ca^{++}) of exchange. When morphine and naloxone were added simultaneously, the naloxone concentration was 1/10 the concentration of morphine. Data are the means ± standard errors of three experiments and are expressed as nanomoles of serine per micromole of lipid phosphorus per 15 min rather than as nanomoles of lipid serine per milligram of protein per 15 min, since the former measurement gave more accurate and reproducible results; expressing the data in this form is possible since none of the drug manipulations *in vitro* or *in vivo* affected lipid specific activity (micromoles of lipid phosphorus per milligram of protein).

* Significantly different from control; $p < 0.05$.

Effect of chronic morphine treatment on incorporation of [^{14}C]serine, [^{14}C]ethanolamine, and [^{14}C]choline into phospholipid via base exchange. Chronic morphine treatment significantly stimulated the Ca^{++}-dependent exchange of [^{14}C]serine into phospholipid (Fig. 3). Significant increases were observed at all Ca^{++} concentrations. As was observed with acute drug treatment, chronic morphine administration significantly decreased (25%) the basal rate of exchange.

While the chronic morphine-induced in-

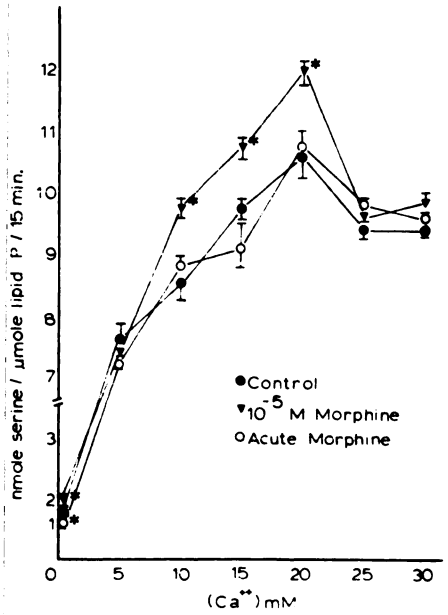

FIG. 2. *Comparison of effects of morphine administered in vitro and in vivo on Ca^{++}-stimulated incorporation of [^{14}C]serine into brain microsomal phospholipid via base exchange*

The data illustrate the effects of adding 10 µM morphine or treating the animals with 20 mg/kg of morphine sulfate for 45 min on the rate of the Ca^{++}-stimulated incorporation of [^{14}C]serine (1.5 mM) into microsomal phospholipid. Data are the means ± standard errors of three experiments, each performed in triplicate. Additional experimental details are described in the legend to Fig. 1.

* Significantly different from control; $p < 0.05$.

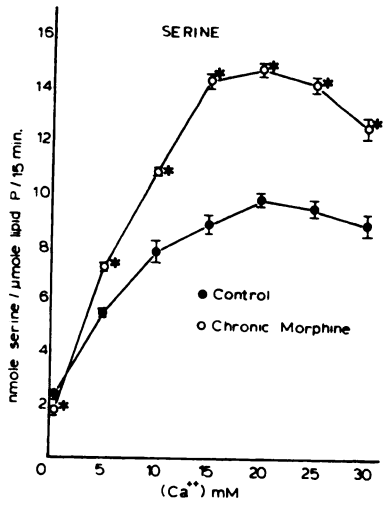

FIG. 3. *Effects of chronic morphine treatment on incorporation of [^{14}C]serine into brain microsomal phospholipids via base exchange*

Animals were administered morphine chronically for 48 hr by multiple pellet implantation (see MATERIALS AND METHODS). Control animals were implanted with placebo pellets. Forty-eight hours after the implantation of the first pellet, the animals were killed and brain microsomes were prepared and assayed for Ca^{++}-stimulated base-exchange activity. The data show the effects of chronic morphine administration on [^{14}C]serine (1.5 mM) incorporation and are the means ± standard errors of four to six experiments, each performed in triplicate. Additional experimental details are described in the legend to Fig. 1.

* Significantly different from control; $p < 0.05$.

creases in the Ca^{++}-stimulated exchange of [^{14}C]serine ranged from 32% to 60%, a smaller (11%) increase in the rate of [^{14}C]ethanolamine exchange was observed only at 2.5 mM Ca^{++} (Fig. 4). As with [^{14}C]serine exchange, chronic morphine treatment significantly decreased (24%) the basal rate of [^{14}C]ethanolamine exchange. In contrast to the effects on [^{14}C]serine and [^{14}C]ethanolamine exchange, both the basal and the Ca^{++}-stimulated exchange of [^{14}C]choline was inhibited in the chronically treated group (Fig. 5).

In some experiments we examined [^{14}C]-serine exchange in discrete brain regions of chronically morphinized animals. No significant regional differences were observed in regard to the chronic morphine-induced increase in the exchange of [^{14}C]serine into microsomal phospholipid (data not shown). Similarly, neither acute morphine treatment nor the addition of morphine *in vitro* produced any regionally specific effects on [^{14}C]serine exchange.

DISCUSSION

The results of the present study, demonstrating that chronic morphine administration has distinct effects on the exchange of serine, ethanolamine, and choline, suggest that three base-exchange systems are present. In this regard, Kafner *et al.* (4, 14) found that the exchange of the bases differs in terms of pH optimum, inhibition by structural analogues, sensitivity to detergents, stability on storage, sensitivity to phospholipases, heat lability, and the effect of glutathione. However, it should also be noted that some of these apparent differ-

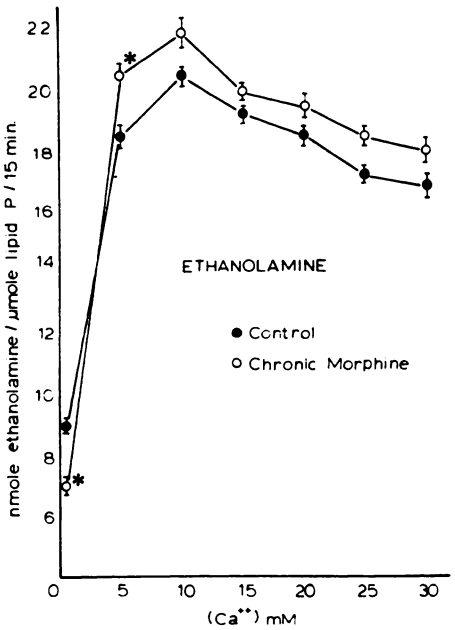

FIG. 4. *Effects of chronic morphine treatment on incorporation of [^{14}C]ethanolamine into microsomal phospholipids via base exchange*

The details were the same as in the legend to Fig. 3, except that [^{14}C]ethanolamine (final concentration, 1.7 mM) exchange was measured.

* Significantly different from control; $p < 0.05$.

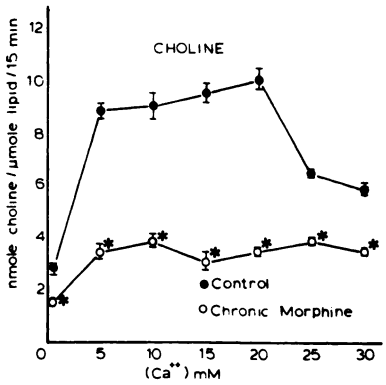

FIG. 5. *Effects of chronic morphine treatment on incorporation of [^{14}C]choline into microsomal phospholipids via base exchange*

Details were the same as in the legend to Fig. 3, except that [^{14}C]choline (final concentration, 4.5 mM) exchange was measured.

* Significantly different from control; $p < 0.05$.

ences between the exchange systems may be related to the incubation conditions employed (3, 5, 9). Thus it may be premature to conclude that chronic morphine treatment has a specific stimulatory effect on a specific Ca^{++}-stimulated serine exchange enzyme. However, in our opinion, the chronic morphine data are most simply explained by such a hypothesis.

Regardless of the precise enzymatic mechanisms involved, the data superficially predict that the ratio of phosphatidylserine to phosphatidylcholine within the membrane will increase during chronic morphine administration. However, to date we have been unable to detect a change in this ratio. There are several possible reasons for this phenomenon. (a) The amount of phospholipid available for exchange at any given time is quite small (1, 15–17). Thus there might be an increase in the amount of phosphatidylserine that is not detectable by our techniques. (b) A change in the ratio of phosphatidylserine to phosphatidylcholine may occur only in a specific population of membranes that have not been examined. (c) It is not possible to determine from the present study whether or not the increase in [^{14}C]serine incorporation into microsomal phospholipid occurs via a homologous or heterologous exchange mechanism (1). If the exchange were primarily of the former type, there would be no net phosphatidylserine levels. The functional consequence(s) of an increase in homologous serine exchange is unknown. However, the specific nature by which chronic morphine administration increases serine exchange suggests that the increased rate of exchange may play an important role in central adaptive mechanisms.

In addition to the effects of chronic morphine on Ca^{++}-stimulated exchange, the basal rate (no added Ca^{++}) of exchange was affected. Chronic morphine treatment significantly inhibited the basal rate of exchange for all three bases, although the exchange of choline was inhibited (38%) to a somewhat greater extent than the exchange of serine and ethanolamine (25% and 24% inhibition, respectively). These effects do not appear to be peculiar to chronic morphine treatment, since acute morphine administration was also found to inhibit the basal rate of serine exchange. This inhibition of the basal rate of exchange apparently is not caused by small amounts of

residual morphine present in the washed microsomes, since the addition of morphine *in vitro* was found to stimulate rather than inhibit the basal exchange of serine. It is of interest that although a high concentration (10 μM) of morphine was required, this effect showed narcotic specificity in that it was blocked by naloxone (1 μM). Of further interest is the observation that the addition of morphine *in vitro* also produced small increases in the Ca^{++}-stimulated exchange of serine at some Ca^{++} concentrations. However, differences in the magnitude of the effect and differences in the profile of Ca^{++} stimulation suggest that the mechanism(s) responsible for the effect *in vitro* differ from those responsible for the effect of chronic morphine treatment.

Previous studies have amply demonstrated that morphine can markedly affect the incorporation of $^{32}P_i$, [^{3}H]- or [^{14}C]glycerol, and [^{14}C]choline into brain phospholipids (17-22). In general, tolerance develops to these acute drug effects on phospholipid turnover (18, 23). The present study indicates additional effects of morphine on brain phospholipid synthesis. Significantly, and in contrast to previous studies, the most marked changes were found in the chronically morphine-treated animals. To our knowledge, this is the first demonstration of specific mechanisms by which neuronal membrane composition can be altered during narcotic tolerance and dependence development.

ACKNOWLEDGMENTS

The authors acknowledge the editorial and typing assistance of Barbara Halperin and Kaye Welch.

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