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Influence of chronic pentobarbital or morphine treatment on the incorporation of $^{32}P_i$ and [^{3}H]choline into rat synaptic plasma membranes

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The search for changes in brain membrane components that can be related to the development of functional drug tolerance and dependence has not been successful. For example, although protein synthesis inhibitors block the development of functional morphine or pentobarbital tolerance [1–4], to date there has been no demonstration of specific changes in the quantity and/or nature of a brain protein that can be related to tolerance development. Considerable evidence indicates that barbiturates act mainly at synapses, inhibiting excitatory synaptic transmission [5–7] and either enhancing or preserving inhibitory transmission [8–10]. Morphine or related narcotics, through an interaction with a specific receptor, inhibit both the central and peripheral release of acetylcholine (ACh) [11–13]. In the isolated guinea pig myenteric plexus-gut preparation, the decrease in ACh release appears to be the specific mechanism by which narcotics block low frequency electrical stimulus-induced contractions [12, 13]. Thus, while the cause of tolerance development to either narcotics or barbiturates is unknown, it is not unreasonable to propose that tolerance development for both drug classes will involve a modification of a synaptic constituent. In the present study, we have examined the effects of chronic pentobarbital or morphine treatment on the incorporation of ^{32}P-phosphoric acid ($^{32}P_i$) and [^{3}H]choline into the phospholipids isolated from two unique populations of rat subcortical synaptic plasma membranes (SPM). The SPM were derived from a light (L) and heavy (H) population of nerve ending particles (NEP); the NEP-L preferentially transport labeled γ-amino-butyric acid while the NEP-H preferentially transport labeled norepinephrine [1].

Male Sprague–Dawley rats (Simonsen Laboratories, Gilroy, CA) weighing 180–200 g were implanted with a needle guide in the skull above the lateral ventricle. Four to 5 days after surgery, the animals were implanted s.c. with either two placebo, 75 mg morphine or 200 mg pentobarbital pellets. Twenty-four hr after pellet implantation, all groups of animals were given 100 μCi $^{32}P_i$ (carrier free) and 20 μCi [^{3}H]choline (sp. act. 10 Ci/m-mole, New England Nuclear Corp., Boston, MA). The isotopes were dissolved in 20 μl of a freshly prepared Krebs–Ringer bicarbonate buffer. Three and 24 hr after isotope administration, the animals were sacrificed and the subcortex was isolated by dissection. Microsomes, SPM-L and SPM-H, were prepared as described elsewhere [1]. Phospholipids were extracted and isolated, and specific activities were determined as described by Abdel-Latif and Smith [14]. Data were analyzed by means of Student's t-test (two-tailed).

By using the pellet implantation technique, it is possible to maintain a constant rate of tolerance development for at least 2 days [15, 16] during which time it is appropriate to measure changes in the incorporation of labeled precursors into membrane components. The data in Table 1 show that chronic pentobarbital treatment significantly increased the incorporation of $^{32}P_i$ into the SPM-H acid phospholipid fraction, which contained phosphatidylserine (PS) and phosphatidylinositol (PI). Due to technical difficulties in consistently separating PS from PI by the thin-layer chromatography (t.l.c.) techniques employed, the PS and PI data have been combined. However, in some experiments, it was possible to determine that more than 95 per cent of the label in this fraction was ^{32}P-PI. The increased incorporation of $^{32}P_i$ into the SPM-H acidic phospholipids

Table 1. Effect of chronic morphine or pentobarbital treatment on the incorporation of $^{32}P_i$ and [^{3}H]choline into synaptic plasma membrane-H phospholipids*

Group	Time (hr)	Activity (cpm/μmole lipid P) $\times 10^{-3}$ $^{32}P_i$ PS + PI	$^{32}P_i$ PE	$^{32}P_i$ PC	[^{3}H]choline PC
Control	3	67 ± 7	65 ± 8	82 ± 11	162 ± 14
Pentobarbital	3	95 ± 9 (141)	35 ± 4 (54)	86 ± 7	168 ± 16
Morphine	3	86 ± 8 (128)	65 ± 7	68 ± 9	139 ± 17
Control	24	384 ± 31	117 ± 14	154 ± 13	433 ± 36
Pentobarbital	24	484 ± 26 (126)	111 ± 16	126 ± 9	336 ± 24 (78)
Morphine	24	369 ± 24	103 ± 10	133 ± 14	318 ± 17 (73)

* Animals were implanted with two placebo, 75 mg morphine or 200 mg pentobarbital pellets, s.c. Twenty-four hr later, the animals were given 100 μCi $^{32}P_i$ (phosphoric acid) and 20 μCi [^{3}H]choline intraventricularly. The animals were sacrificed 3 and 24 hr later and synaptic plasma membranes were prepared from the subcortex as described elsewhere [1]. Phospholipids were extracted from the membranes, separated and their specific activities determined by conventional techniques. Data are the mean ± S.E. of four to six experiments; a pooled sample of three rat brains per experiment was used. The per cent change from control when significant differences ($P < 0.05$) were found is given in parentheses. Abbreviations used are: PS + PI, phosphatidylserine plus phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; and H, heavy, referring to synaptic plasma membranes derived from a heavy population of nerve ending particles.

Table 2. Effect of chronic morphine or pentobarbital treatment on the incorporation of $^{32}P_i$ and [^{3}H]choline into synaptic plasma membrane-L phospholipids*

Group	Time (hr)	Activity (cpm/μmole lipid P) × 10^{-3} $^{32}P_i$ PS + PI	$^{32}P_i$ PE	$^{32}P_i$ PC	[^{3}H]choline PC
Control	3	132 ± 15	65 ± 7	61 ± 8	129 ± 15
Pentobarbital	3	76 ± 7 (58)	77 ± 8	63 ± 10	103 ± 10
Morphine	3	82 ± 9 (62)	61 ± 9	44 ± 6 (72)	134 ± 14
Control	24	697 ± 31	153 ± 9	167 ± 11	376 ± 22
Pentobarbital	24	528 ± 27 (76)	138 ± 12	147 ± 21	359 ± 36
Morphine	24	557 ± 24 (80)	164 ± 17	173 ± 18	345 ± 41

* See Table 1 legend for details; L, light, referring to synaptic plasma membranes derived from a light population of nerve ending particles.

was a specific response in the sense that it did not occur in the SPM-L fraction (Table 2) or in the microsomes (data not shown). In fact, in the SPM-L fraction, chronic pentobarbital treatment significantly inhibited $^{32}P_i$ incorporation into the acidic phospholipids. This differential effect of chronic pentobarbital treatment on the turnover of SPM components has also been observed in regard to protein turnover [1]. Chronic pentobarbital treatment has been found to significantly increase the incorporation of [^{3}H]lysine into SPM-L but not SPM-H proteins [1]. In addition to the effects on the acidic phospholipids, chronic pentobarbital treatment significantly decreased $^{32}P_i$ incorporation into SPM-H phosphatidylethanolamine (PE) at 3 hr and decreased [^{3}H]choline incorporation into SPM-L phosphatidylcholine (PC) at 24 hr after isotope administration.

The effects of morphine on the incorporation of $^{32}P_i$ and [^{3}H]choline into SPM phospholipids were for the most part similar to those observed with chronic pentobarbital treatment. Chronic morphine treatment, however, did not significantly increase $^{32}P_i$ incorporation into the SPM-H acidic phospholipid fraction at 24 hr nor did chronic morphinization inhibit $^{32}P_i$ incorporation into PE at 3 hr. In a previous study [17], it was observed that chronic morphine injections increased the turnover of ([^{14}C]choline)-PC in the microsomal and mitochondrial fractions obtained from the diencephalon. The data in the present study, however, show that chronic morphinization either inhibited or had no effect on the incorporation of [^{3}H]choline and $^{32}P_i$ into PC.

At first, it was considered that this difference was related to the change in subcellular fractions examined. However, we have found that morphine pellet implantation either decreased or had no effect on the rates of $^{32}P_i$ and [^{3}H]choline incorporation into microsomal PC (data not shown). Thus, it would appear that the differences between the present results and those of our previous study are related to either the difference in the route and schedule of morphine administration and/or the technique for assessing changes in turnover (initial incorporation vs decay). It is well known that acute morphine treatment enhances $^{32}P_i$ incorporation into brain phospholipids [18–21]. Mulé [19] has further observed that tolerance develops to this stimulatory effect. The data in the present study appear to complement this earlier work of Mulé [19]. Chronic morphinization produced only a transient small increase in the incorporation of $^{32}P_i$ into the SPM-H acidic phospholipids; otherwise morphine inhibited or had no effect on SPM phospholipid turnover. Thus, the presumed, but not demonstrated, stimulation of SPM phospholipid turnover that would occur initially after morphine pellet implantation has disappeared. More importantly, the present experiments indicate that, during a phase of rapid morphine tolerance and dependence development, there are no simultaneous large increases in SPM phospholipid turnover. We have, then, been unable to find a change in SPM phospholipid turnover which would be analogous to the hypothetical increase in brain protein synthesis that is related to tolerance and dependence development [2].

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Stimulation of renal gluconeogenesis by verapamil and D-600*

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At least three "on-off" mechanisms are present in renal cortex that regulate the rate of glucose formation. Experiments *in vitro* with slices, tubules, and isolated perfused kidneys reveal that gluconeogenesis is stimulated by: (1) an increase in extracellular hydrogen ion concentration [1–6], (2) the presence of specific hormones, i.e. parathormone [7–10], catecholamines [11–13] and glucagon [11], and (3) calcium ion [1–3, 14, 15]. The mode of stimulation and the interaction of each of these mechanisms remain controversial [16], although there is substantial evidence to implicate cAMP in the endocrine and calcium-mediated effects. Beyond its role in renal cortex, Ca^{2+} has also been shown to mediate important metabolic and physiologic functions in heart [17], myometrium [18, 19], and in certain secretory tissues [20–23]. Ca^{2+}-associated activities in these tissues are inhibited by verapamil and D-600 [24–26]; presumably these agents block Ca^{2+} channels in the plasma membrane. In contrast to this seemingly generalized phenomenon, gluconeogenesis in renal tubules, a Ca^{2+}-stimulated function, is not blocked by verapamil and D-600. In fact, the gluconeogenic rate is accelerated in the presence of verapamil and D-600 and this response is independent of added Ca^{2+}.

Male, adult Sprague–Dawley rats (250–350 g) that had been fasted for 24 hr were used in all experiments. After the rats were stunned by cervical fracture, the kidneys were rapidly removed and placed in ice-cold saline or phosphate buffer. The detailed procedure for isolating tubules and the ultra-structural and biochemical characteristics of the preparation have been described [27]. Slices of cortex were made with the Stadie–Riggs microtome and then incubated in a buffered medium with collagenase, albumin and Ca^{2+}. After approximately 45 min, the suspension was filtered through three layers of gauze and the tubules were sedimented by centrifugation at 50 *g* for 2 min. The supernatant solution containing red blood cells and kidney cell debris was discarded and the tubules were resuspended in the Ca^{2+}-containing medium. After centrifugation and removal of the supernatant solution, the tubules were then suspended in a Ca^{2+}-free medium, spun down, washed once again in a Ca^{2+}-free medium, and then suspended in 2 vol. of Ca^{2+}-free medium.

All incubations were carried out in 25-ml Erlenmeyer flasks containing 2.5 ml of tubular suspension (approximately 12 mg protein) in a phosphate buffer (pH 7.4) at 37°. The incubation was started by addition of tubules to the medium which had been equilibrated at 37° under 100% O_2 for 5 min. Ethanolic solutions of verapamil and D-600 (kindly provided by Knoll Pharmaceutical Co.) were added in 10-μl volumes; 10 μl ethanol was added to control vessels. Substrates were added as neutral solutions so that the final concentration was 5×10^{-3} M. The incubation was terminated by immersion of the suspension in a boiling water bath for 30 sec. In those experiments where tubular calcium content was measured, the heating step was omitted and the tubules were rapidly separated from the incubation medium by centrifugation and then washed twice in Ca^{2+}-free medium before preparing a trichloroacetic acid filtrate of the tubules.

The glucose content of the supernatant solution prepared from the heat-treated tubular suspension was determined by the glucose oxidase method (Glucostat-Worthington Biochemical). Net glucose production by the tubules was taken as the difference in glucose content of the incubated suspensions in the presence of substrate and in the absence of added substrate. Oxygen consumption was measured at 37° in a closed system of 3 ml volume with the Clark electrode and continuously monitored on a Varian G-1000 recorder. A Techtron-AA5 atomic absorption spectrometer was used for determinations of the calcium content of the trichloroacetic acid extract of tubules.

In vitro, gluconeogenesis by rat renal cortex is limited by substrate availability [27–29]. In isolated tubules incubated in phosphate buffer, succinate and α-ketoglutarate appeared to be among the most effective precursors for new glucose formation [28, 29]. Exposure of tubules to 10^{-4} M verapamil or D-600 in short-term incubations (15 min) resulted in an increased rate of gluconeogenesis when either 5×10^{-3} M succinate or α-ketoglutarate was present as substrate (Table 1). Lowering the concentration of verapamil and D-600 to 10^{-5} M resulted in a lesser, but still significant stimulation of gluconeogenesis, while 10^{-6} M was without effect. Neither of these agents influenced the respiratory rate of the tubule preparation (control 30.9 nmoles/mg of protein/min, 10^{-4} M verapamil 30.7, 10^{-4} M D-600 31.0) in the presence or absence of added Ca^{2+} to the incubation.

Table 1. Effect of verapamil and D-600 on gluconeogenesis*

	Substrate	
	Succinate	α-Ketoglutarate
Control	24.2 ± 2.8	29.8 ± 5.6
Verapamil (10^{-4} M)	45.0 ± 8.3†	43.8 ± 14.5
D-600 (10^{-4} M)	32.6 ± 2.7†	44.6 ± 4.3†

Ca^{2+} (2.5 mM) was present. The values represent the mean ± S.E. for net glucose production (nmoles/mg of protein/15 min).

† These levels are statistically different ($P < 0.05$) from the corresponding controls.

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