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EFFECTS OF OPIOID NARCOTIC DRUGS ON ENERGY RESERVES OF SKELETAL MUSCLE—I

GLYCOGEN

DESMOND R. H. GOURLEY*

Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Va. 22903, U.S.A.

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Abstract—Morphine, at concentrations of 0·77 to 8 mM, did not significantly alter the rate of glucose uptake by diaphragm muscles from normal rats. At concentrations of 5 mM and higher, however, morphine reduced the glycogen content of diaphragm muscle. In isolated intact extensor digitorum longus and soleus (EDL + SOL) muscles of the rat, glucose uptake was likewise unaffected by morphine but the glycogen content was decreased. Of the narcotic drugs that were tested *in vitro* for glycogenolytic activity, methadone was the most potent, followed in order by meperidine, morphine and hydromorphone. After incubation *in vitro*, the glycogen content of diaphragm muscles from rats injected with a single dose of morphine was lower than that of muscles from corresponding control rats. Under identical experimental conditions, the glycogen content of diaphragm muscles from rats chronically treated with increasing doses of morphine was essentially the same as that of muscles from corresponding control rats, suggesting that the sensitivity of the muscle glycogen system to morphine decreased during chronic morphine treatment. Tolerance was not observed *in vitro* where methadone had a greater glycogenolytic effect in the diaphragm muscles from rats chronically treated with morphine than in muscles from the control rats. Propranolol, in a concentration sufficient to block the glycogenolytic action of epinephrine in EDL + SOL muscles, did not block the glycogenolytic action of methadone. The activities of both phosphorylase and glycogen synthase were greatly depressed, while phosphoglucomutase activity was unchanged in muscles incubated with methadone. It is concluded that the glycogenolytic effect of methadone in skeletal muscle is not mediated through the release of catecholamines in the muscles, nor can it be explained by a differential action on the activities of the enzymes involved in the last stage of synthesis and the first stage of breakdown of muscle glycogen.

A POSSIBLE approach to understanding the mechanism of drug tolerance and dependence is through study *in vitro* of a biochemical system that is influenced by drugs known to induce these phenomena in animals. Walsh *et al.*[1–7] have reported that morphine increases the uptake of glucose by diaphragm muscles isolated from normal rats, but not from rats chronically treated with morphine for 5–6 weeks. In the hope that the glucose uptake system of muscle tissue might be amenable to a more detailed study of the molecular changes associated with drug tolerance and dependence, we attempted to reproduce the reported effects of morphine in this system. Although we did not observe an increase in glucose uptake by diaphragm muscle in the presence of morphine, several narcotic drugs including morphine were found to greatly diminish the glycogen content of the diaphragm and other isolated muscles. These experiments are reported in this paper.

* Present address: Department of Pharmacology, Eastern Virginia Medical School, 358 Mowbray Arch, Norfolk, Va. 23507, U.S.A.

MATERIALS AND METHODS

Animals. Male rats of the Sprague–Dawley strain purchased from Flow Research Animals, Inc. were used throughout the experiments and allowed free access to food and water. Except where noted otherwise in the tables, the average weight of the rats (N = 443) was 74·3 ± 12·7 (S. D.) g at the time of tissue preparation.

Preparation of isolated muscles. Each rat was killed by decapitation. When the diaphragm muscle was to be dissected, the abdomen was opened and the tendon connections of the muscle were cut as close as possible to the rib cage. The muscle was rinsed in about 15 ml of Krebs–Ringer solution in a Petri culture dish at room temperature (about 25°) and was trimmed of excess connective tissue, fat, peripheral blood vessels and blood clots. The V-shaped ventral portion was discarded and each of the approximately equal sections was immersed in about 5 ml of fresh Krebs–Ringer solution (containing no glucose) and allowed to remain at room temperature for 50–60 min. Each section was then blotted lightly on filter paper, weighed quickly on a torsion balance, and placed into 1·5 ml of Krebs–Ringer solution containing 8 mM glucose without or with added drug in a 25 × 40 mm glass-stoppered weighing bottle. Thus each animal provided paired control and drug-treated sections of diaphragm muscle. The weighing bottle was flushed with 100% oxygen, stoppered and placed in a Dubnoff metabolic incubator at 37° and shaken at 70 oscillations/min for varying periods of time. Under these conditions but with no drug added, there was no significant difference in the glycogen content of the two sections of diaphragm muscle at the end of a 1-hr incubation period (average glycogen content of 12 sections randomly assigned odd numbers, 10·1 ± 1·1 μmoles/g wet wt; even numbers, 12·5 ± 1·7 μmoles/g wet wt; $P > 0·1$).

When the extensor digitorum longus and soleus (EDL + SOL) muscles were to be dissected, the skin was stripped from the hind limbs and the muscles were freed from the surrounding muscles with a glass hook moistened with Krebs–Ringer solution. The tendons at the origin and insertion of each muscle were cut and the muscles were handled only by the tendon tissue. The muscles were immersed in about 10 ml of Krebs–Ringer solution in a 50-ml beaker and paired in the following way: the EDL muscle from the right leg with the SOL muscle from the left leg, and vice versa. After 40–80 min at room temperature, the crossed pairs of muscles were removed, blotted lightly on filter paper, weighed quickly on a torsion balance, and placed into 1·5 ml of Krebs–Ringer solution containing 8 mM glucose without or with added drug in a weighing bottle. One pair of EDL + SOL muscles from each animal thus served as the control for the corresponding pair of muscles which were exposed to the drug. As in the diaphragm muscle experiments, the weighing bottle was flushed with oxygen and incubated at 37° for varying periods of time. Under these conditions but with no drug added, there was no significant difference in the glycogen content of the two crossed pairs of EDL + SOL muscles at the end of a 1-hr incubation period (average glycogen content of 12 pairs of EDL + SOL muscles randomly assigned odd numbers, 15·6 ± 1·7 μmoles/g wet wt; even numbers, 15·7 ± 1·0 μmoles/g wet wt; $P > 0·9$).

Chemicals and solutions. All inorganic salts and glucose were analytical grade. The Krebs–Ringer phosphate solution contained, in m-moles/l.: NaCl, 130; KCl, 5·0; $MgSO_4$-7 H_2O, 1·0; $CaCl_2$, 1·26; NaH_2PO_4-H_2O, 0·64; and Na_2HPO_4, 3·96 (pH = 7·4). This solution was used except where otherwise noted. Meperidine hydrochloride and L-epinephrine bitartrate were purchased from Winthrop Laboratories; pro-

pranolol hydrochloride from Ayerst Laboratories, Inc.; racemic methadone hydrochloride from Eli Lilly & Co.; hydromorphone hydrochloride from Bilhuber–Knoll Corp.; and morphine sulfate (used to inject into rats) and morphine hydrochloride used to add to muscle tissue *in vitro*) from Merck & Co., Inc. All dosages given in this paper are expressed as the base.

Analytical methods. Glycogen was isolated from the muscles by a modification of the method described by Walaas and Walaas.[8] After hydrolysis of the glycogen, glucose was determined by the Glucostat (Worthington Biochemical Corp.) procedure. To determine glucose in the Krebs–Ringer solution at the end of the muscle incubation period, a measured sample was removed immediately and protein was precipitated with $Ba(OH)_2$ and $ZnSO_4$, the precipitate washed once, and glucose determined by the Glucostat method. Glucose uptake by a muscle section or pair was taken as the difference between the glucose present in Krebs–Ringer solution incubated without muscle tissue (without and with the appropriate concentration of morphine) and the glucose present in the Krebs–Ringer solution incubated with muscle tissue. Protein was determined by the method of Lowry *et al.*[9]

Preparation of muscle extracts and measurement of enzyme activities. To measure the effect of methadone on the activity of muscle enzymes, 20 muscles (EDL + SOL) from 10 rats were incubated at 37° in 5·0 ml Krebs–Ringer phosphate solution containing 8 mM glucose (after the usual equilibration period) without or with methadone for 1 hr. The muscles were then blotted on filter paper, their tendons trimmed off, and they were frozen in liquid nitrogen. The frozen muscles were powdered in a mortar chilled in liquid nitrogen, and a sample of the powder was homogenized at 0° in 5 vol. of a solution containing 50 mM Tris, 5 mM EDTA and 50 mM potassium fluoride (pH 7·8) in a glass homogenizer with a motor-driven Teflon pestle. Each ml of the extraction solution also contained 0·5 mg of rabbit liver glycogen (which had been purified by passage through an ion exchange resin) to activate phosphorylase and glycogen synthase.* The homogenate was cleared by centrifugation at 35,000 *g* for 15 min and the supernatant was passed through Sephadex G-50 at 5° and monitored spectrophotometrically at 250 nm. The first peak, which contained the protein fraction, was retained for enzyme assays and the remainder was discarded.

Phosphorylase (α-1,4-glucan: orthophosphate glucosyltransferase, EC 2.4.1.1.) activity was measured by a modification of the method described by Villar-Palasi and Gazquez-Martinez.[10] To 100 μl of a solution containing 75 mM glucose 1-phosphate, 1% glycogen, 50 mM EDTA adjusted to pH 6·8 with HCl, without (phosphorylase a activity) or with (total phosphorylase activity) 1 mM 5′-AMP was added 100 μl of enzyme and the mixture was incubated at 37° for 15 min. To stop the reaction, 8·6 ml of 0·125 M acetate buffer (pH 4) was added, and 1·0 ml of 2% ammonium molybdate in 10 mM H_2SO_4 and 0·2 ml of Fiske–SubbaRow reducing agent were mixed with these solutions, incubated for 1 hr at 37°, and the absorbance at 640 nm was determined after remaining for 3 hr at room temperature. Phosphorylase activities were expressed as nmoles of Pi liberated/min per mg protein.

The activities of glycogen synthase I and total glycogen synthase (UDP-glucose: glycogen α-4-glucosyltransferase, EC 2.4.1.11.) were assayed by the method of Thomas *et al.*[11] The activities were expressed as nmoles of ^{14}C-glucose incorporated into glycogen/min per mg protein.

* D. R. H. Gourley and J. D. Schwarzmeier, *Life Sci.* **13**, 1353 (1973).

The activity of phosphoglucomutase (α-D-glucose 1,6-diphosphate: α-D-glucose 1-phosphate phosphotransferase, EC 2.7.5.1.) was assayed by a modification of the method of Bergmeyer and Klotzsch[12] for the determination of D-glucose 1-phosphate. One ml was prepared to contain 50 μl of 1 M Tris adjusted to pH 7·6 with HCl, 30 μl of 0·1 M magnesium chloride, 30 μl of 0·02 M EDTA, 50 μl of NADP (Sigma Chemical Co., 3·3 mg dissolved in 1 ml water and adjusted to pH 6 with NaOH), 20 μl of 50 mM α-D-glucose 1-phosphate (dipotassium salt, Sigma Chemical Co.), 20 μl of 2 mM glucose 1,6-diphosphate (tetracyclohexylammonium salt, Sigma Chemical Co.) and 20 μl of glucose 6-phosphate dehydrogenase (Boehringer Mannheim Corp., 1 mg/ml, diluted 10-fold). The enzyme (20 μl) was mixed with this solution and the appearance of NADPH was measured at 340 nm in a Gilford recording spectrophotometer. Phosphoglucomutase activity was expressed as n moles of glucose 6-phosphate formed/min per mg protein.

Preparation of morphine-tolerant rats. Rats were divided into two groups of approximately equal average weight (62–64 g). The animals in one group were injected with 15 mg/kg morphine as the sulfate salt on the first day. On the second day, 20 mg/kg morphine was administered at 8:30 a.m. and 3:30 p.m. On subsequent days, the dosage was increased rapidly at first and more slowly later until a dose of 220 mg/kg twice daily was reached on day 17. The last injection was given on day 18 1 hr before the rats were killed. Injections were given subcutaneously until the volume of solution exceeded 0·5 ml which was about day 10. Thereafter, injections were intraperitoneal. The second groups of rats served as controls and received comparable volumes of 0·9% NaCl.

Statistical procedures. The significance of the difference between means was tested by conventional methods.[13] The paired variate procedure was used when the two means represented data from muscles or muscle sections taken from the same animal. The unpaired variate procedure was used when the two means represented data from muscles taken from different animals. Values preceded by ± represent S. E. M. except after the mean weight of a group of rats where ± S. D. is given.

RESULTS

Glucose uptake and glycogen content of skeletal muscle in the presence of morphine. The first experiments were attempts to confirm the observation reported by Peng and Walsh[1,2] that morphine increased the rate of glucose uptake by diaphragm muscle from the normal rat. Attempts were made to reproduce as closely as possible the experimental conditions described by Peng and Walsh. We were unable, however, to duplicate exactly the Krebs–Ringer phosphate medium used by the earlier investigators. Peng and Walsh prepared their medium from a formula given by Herman and Ramey,[14] presumably formula 2 which specified a concentration of $CaCl_2$ of 3 mM. In a later paper from the same laboratory,[4] the Krebs–Ringer phosphate medium was reported to contain 2·8 mM calcium. The maximum concentration of calcium we were able to dissolve in formula 2 of Herman and Ramey was 1·5 mM. In preliminary experiments with this single modification of formula 2 of Herman and Ramey, morphine had no effect on glucose uptake by diaphragm muscle from the normal rat.

A more extensive series of experiments was performed with a further modification of the Krebs–Ringer phosphate medium to contain 1·26 mM calcium and 4·6 mM phosphate (vs 10 mM phosphate in the medium of Herman and Ramey). The results

TABLE 1. EFFECTS OF MORPHINE ON GLUCOSE UPTAKE AND GLYCOGEN CONTENT OF RAT SKELETAL MUSCLES*

Expt. No.	Muscle (No. of observations)	Treatment	Glucose uptake (μmoles/g per hr)	Glycogen content (μmoles/g per hr)
1	Diaphragm† (17)	Control	9·54 ± 0·38	8·34 ± 0·53
		Morphine (0·77 mM)	8·98 ± 0·28‡	7·88 ± 0·46‡
2	Diaphragm (14)	Control	10·27 ± 0·51	7·26 ± 0·50
		Morphine (8 mM)	10·83 ± 0·45‡	6·12 ± 0·28§
3	Diaphragm† (18)	Control	6·15 ± 0·22	5·62 ± 0·32
		Morphine (0·385 mM)	6·08 ± 0·18‡	5·61 ± 0·44‡
4	EDL + SOL (6)	Control	4·87 ± 0·41	7·72 ± 0·56
		Morphine (5 mM)	5·43 ± 0·44‡	5·09 ± 0·39‖

* Values given are the means ± S. E. M. of the number of observations in parentheses. All diaphragm muscles were incubated with or without morphine for 2 hr; the EDL + SOL muscles were incubated for 3 hr. In experiments 1, 2 and 4, the Krebs Ringer solution had the composition given in Materials and Methods. In experiment 3, the Krebs Ringer solution contained, in m-moles/l.: NaCl, 128; KCl, 5·12; $MgSO_4$·7 H_2O, 1·28; $CaCl_2$, 2·73; and Tris, 8·0 (adjusted with HCl to pH 7·4). In all experiments, the medium contained 8 mM glucose.

† From rats weighing 151 ± 15 (S. D.) g (N = 35).

‡ Not significantly different from corresponding control value ($P > 0·05$).

§ Significantly different from corresponding control value ($P < 0·05$).

‖ Significantly different from corresponding control value ($P < 0·01$).

of two experiments, which are typical of many others not reported, are given in Table 1 (Expts. 1 and 2). Morphine had no significant effect on the rate of glucose uptake by diaphragm muscle at either a concentration of 0·77 mM, which was the concentration reported by Peng and Walsh[2] to increase the rate of glucose uptake by 61 per cent, or 10 times this concentration.

As noted above, the calcium concentration of the Krebs-Ringer phosphate medium used in Expts. 1 and 2 was less than half that of the medium cited by Peng and Walsh.[2] Fong and Walsh[7] reported that 0·385 mM morphine also increased the rate of glucose uptake by diaphragm muscle from normal rats in a Tris-buffered medium containing 2·73 mM calcium. Therefore, the experiment was repeated with the Tris-buffered medium and the morphine concentration described by Fong and Walsh.[7] In the higher calcium medium, however, 0·385 mM morphine did not significantly affect the rate of glucose uptake under our experimental conditions (Expt. 3, Table 1).

We have also studied the effect of morphine on glucose uptake by EDL + SOL muscles from normal rats. The data shown as Expt. 4 in Table 1 are typical of several experiments with this intact muscle preparation. As in our diaphragm experiments, morphine had no effect on the rate of glucose uptake by EDL + SOL muscles during a 3-hr incubation period.

Thus, in two isolated muscle preparations from the normal rat, the diaphragm and the EDL + SOL muscles, we have never observed any alteration in the rate of glucose uptake in the presence of morphine in concentrations in the range of 0·385 to 8 mM. At the higher concentrations, however, morphine significantly reduced the concentration of glycogen which remained in the muscles at the end of the incubation *in vitro*. In other experiments, not reported here in detail, 2 mM morphine did not significantly affect the glycogen levels of EDL + SOL muscles.

Glycogenolytic effect of various opioid narcotic drugs in isolated skeletal muscle. Since relatively high concentrations of morphine were required to reduce the glycogen

content of skeletal muscles *in vitro*, other opioid narcotic drugs were also tested to determine their possible glycogenolytic action in the EDL + SOL muscles. It was found in preliminary experiments that the glycogen content of EDL + SOL muscles was significantly decreased as early as 1 hr after addition of morphine. Thus, a 1-hr period of exposure of incubation with the various drugs was used in the following experiments. The data are summarized in Fig. 1, in which the glycogen content of the drug-treated muscles, expressed as a percentage of the glycogen content of control muscles, is plotted as a function of drug concentration. In addition to morphine, meperidine and hydromorphone reduced the muscle glycogen content under these conditions, but the reductions were not statistically significant at concentrations below those plotted in Fig. 1 (solid squares). In contrast, the glycogen levels of the EDL + SOL muscles were much more sensitive to methadone (open circles, Fig. 1).

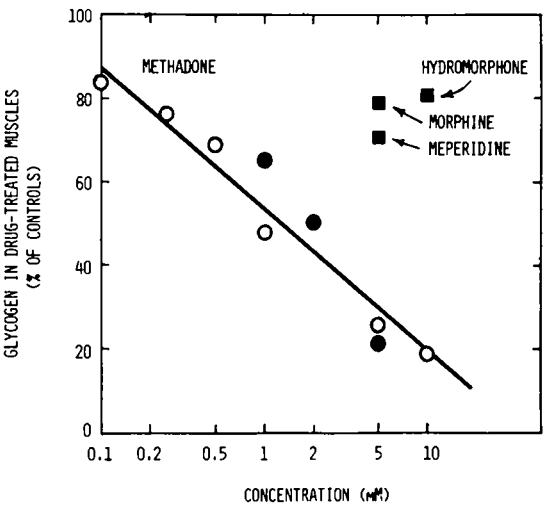

FIG. 1. Glycogen content of DL-methadone-treated EDL + SOL muscles (○) and diaphragm muscle (●) of the rat, expressed as a percentage of the glycogen content of the corresponding control muscles. The line was drawn by eye through the points representing the effect of methadone on the glycogen content of the EDL + SOL muscles. For comparison, the glycogen contents of EDL + SOL muscles incubated with hydromorphone, morphine or meperidine under the same conditions are shown (■). Concentrations of methadone, hydromorphone, morphine and meperidine lower than those shown did not significantly reduce the glycogen content of muscles. Muscles were incubated with drug for 1 hr. Each point in the figure represents the mean of six observations.

The data in Table 1 suggest that the glycogen levels of the diaphragm muscle may not be as sensitive to the effects of morphine as are those of the EDL + SOL muscles, and this is supported by the data summarized in Fig. 1. In the case of methadone, concentrations below 1 mM did not significantly affect the glycogen content of the diaphragm muscle, although the glycogenolytic effect of methadone at concentrations of 1 mM and greater was similar to that observed in the EDL + SOL muscles (data for the diaphragm muscle are shown as closed circles in Fig. 1). Since methadone appeared to be the most potent glycogenolytic agent of the opioid narcotic drugs tested, further studies were carried out with methadone rather than morphine.

Time course of the glycogenolytic effect of methadone in EDL + SOL muscles. To obtain some clue to the mechanism of the glycogenolytic action of methadone, the

time course of the fall in glycogen content after the addition of methadone was determined. The glycogen contents of paired EDL + SOL muscles were determined at various intervals of incubation ranging from 2 to 60 min in the presence or absence of 5 mM methadone. The data are shown in Fig. 2. The glycogen levels of both control and methadone-treated muscles fell over the 60-min incubation period, but the rate of glycogenolysis in the presence of methadone was greater. The glycogen content was significantly lower than that of the control muscles as early as 2 min after the addition of methadone and the difference in the glycogen contents of control and methadone-treated muscles was maximal within about 15 min. No further depletion of glycogen occurred in the methadone-treated muscles after about 45 min.

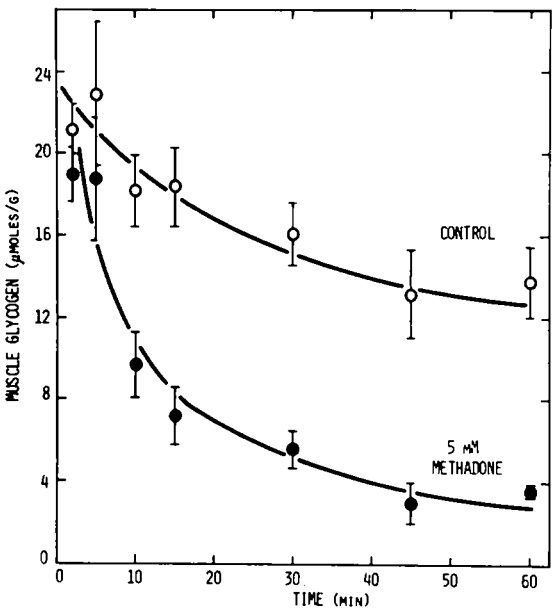

FIG. 2. Glycogen content of EDL + SOL muscles incubated without (○) and with (●) 5 mM DL-methadone for various periods of time. Each point is the mean of six observations and the vertical bars represent two S. E. M. At each time interval, the mean glycogen content of the methadone-treated muscles was significantly different from that of the corresponding control muscles at a level of 1 per cent or less.

Effect of methadone on glycogen content of muscles from rats chronically treated with morphine. Chronic treatment of animals with morphine results in the rapid development of tolerance to many effects of the opioid narcotic drugs. Akera and Brody[15] reported that tolerance to methadone was difficult to achieve in rats, and our experience in preliminary trials confirmed their observations. Therefore, to determine whether tolerance developed to the glycogenolytic action of methadone *in vitro*, the effect of the drug was studied in muscles from rats tolerant to morphine (see Materials and Methods) rather than to methadone. As has been known for many years,[16] rats injected with 0·9% NaCl (control group) gain weight much more rapidly than rats treated with increasing doses of morphine. At the end of the treatment period, the EDL + SOL muscles of the control rats (wt, 185 g) were too thick for adequate oxygenation *in vitro*, and it was necessary to use the diaphragm muscle. The diaphragm muscles from the

TABLE 2. EFFECT OF METHADONE ON GLYCOGEN CONTENT OF DIAPHRAGM MUSCLES FROM RATS PRETREATED CHRONICALLY OR ACUTELY WITH MORPHINE*

Pretreatment	Glycogen content (μmoles/g)		P
	Control	Methadone	
Chronic NaCl injection (14)†	6·96 ± 0·53	2·66 ± 0·27	< 0·001
Chronic morphine injection (13)‡	6·28 + 0·45 (P > 0·3)	1·40 ± 0·15 (P < 0·001)	< 0·001
Acute NaCl injection (7)§	9·92 ± 0·73	1·79 ± 0·25	< 0·001
Acute morphine injection (8)§	6·99 ± 0·58 (P < 0·01)	1·22 ± 0·10 (P = 0·05)	< 0·001

* Values given are the means ± S. E. M. of the number of observations in parentheses in the first column. The schedule for the chronic treatments is given in the Materials and Methods section. The last morphine dose of 220 mg/kg was given 60 min before rat was killed. The acute treatment was given 30 min before rat was killed. The single acute dose of morphine was 24 mg/kg. The concentration of methadone in all experiments was 2 mM. The incubation period was 1 hr.

† Average weight of rats was 178 ± 17 (S. D.) g.

‡ Average weight of rats was 110 ± 18 (S. D.) g.

§ Average weight of rats was 91 ± 6 (S. D.) g.

control rats (average weight of the two sections combined, 314 ± 7 mg) weighed significantly more than the muscles from the rats chronically treated with morphine (average weight of combined sections, 238 ± 11 mg; P < 0·001). It was assumed that diffusion of oxygen would not be limiting in the larger diaphragm muscles.

It was shown in Fig. 1 that methadone was glycogenolytic in diaphragm muscle at concentrations of 1 mM and greater. To avoid possible borderline effects in the diaphragm muscles of the chronically treated animals, a concentration of 2 mM methadone was used. The results are summarized in Table 2. The glycogen content of the muscles of the rats chronically treated with morphine, when incubated without methadone, was not significantly different from the glycogen content of the muscles of the rats chronically injected with NaCl. Methadone had a marked glycogenolytic effect in the diaphragm muscles of both control and chronic morphine-treated rats but the glycogen content of the muscles from rats chronically treated with morphine was significantly lower after methadone than that of the muscles of the control rats. Thus, after chronic treatment with morphine, the muscle glycogen level was apparently more sensitive to the effect of methadone *in vitro*.

Effect of methadone on glycogen content of muscles from rats acutely treated with morphine. The results of experiments with the diaphragm muscles from chronically treated rats may be compared with similar experiments with muscles from rats which received a single injection of either 0·9% NaCl or morphine (Table 2). The dose of morphine injected (24 mg/kg) was estimated from preliminary experiments to produce marked analgesia in 30 min (with the hot plate technique). As shown in Table 2, the glycogen content of the muscles of the rats acutely treated with morphine, when incubated without methadone, was significantly lower than the glycogen content of the muscles of the corresponding NaCl-injected rats. These data are consistent with the hypothesis that a single dose of morphine decreases the glycogen content of the diaphragm muscle *in vivo* as it does *in vitro*, although the effect *in vivo* on the glycogen content might be secondary to another primary action of morphine in the whole animal. Since the glycogen content of the diaphragm muscles of rats chronically

treated with morphine was essentially the same as that of the muscles of the corresponding control rats, the sensitivity of the muscle glycogen system to morphine *in vivo* apparently decreased during chronic morphine treatment.

As in the cases of muscles from untreated animals (Fig. 1) and from chronically treated animals (Table 2), methadone had a marked glycogenolytic effect in the muscles of rats acutely treated with either NaCl or morphine. After incubation with methadone, the glycogen content of muscles from the morphine-treated rats appeared to be lower than the glycogen content of muscles from the NaCl-treated rats. The difference, however, is of borderline statistical significance and, in fact, methadone reduced the glycogen content of the muscles from both NaCl- and morphine-treated rats to the same extent, namely 18 per cent of the corresponding control diaphragm sections. It is concluded, therefore, that a single injection of morphine did not alter the sensitivity of the glycogen system to methadone *in vitro*.

Failure of propranolol to block the glycogenolytic action of methadone. It is not clear from the data presented so far whether the glycogenolytic effect is a consequence of a direct action of opioid narcotic drugs or is secondary to some other action of the drugs. There is general agreement that in intact animals epinephrine is released by morphine, but there is less agreement about the source of the epinephrine. An obvious source is the chromaffin cells of the adrenal medulla, but in the rat the pressor response to morphine is not eliminated when the adrenal glands are removed.[17] Moreover, morphine in relatively high doses does not trigger the release of catecholamines from the isolated retrograde perfused adrenal gland of the cat.* There is reason to believe, therefore, that the release of catecholamine caused by morphine in intact animals is from sites other than the adrenal medulla, perhaps peripheral. Because of this possibility, it was desirable to determine whether the glycogenolytic action of the opioid narcotic drugs on isolated skeletal muscles is mediated through the release of catecholamines from nerve endings in the muscles.

The rationale of the experiments was to determine if the glycogenolytic action of methadone was blocked by the β-adrenergic blocking drug, propranolol. Data from experiments designed to establish the concentration of propranolol required to block the glycogenolytic effect of epinephrine in EDL + SOL muscles appear in Table 3. Epinephrine at both 1 μg/ml (5·5 μM) and 10 μg/ml (55 μM) significantly decreased the glycogen content of the EDL + SOL muscles. Propranolol, at 0·1 mM, was without

TABLE 3. GLYCOGEN CONTENT OF EDL + SOL MUSCLES EXPOSED TO EPINEPHRINE OR PROPRANOLOL*

Drug (concn)	Glycogen content (μmoles/g)		P
	Control	Drug-treated	
Epinephrine			
(1 μg/ml)	24·6 ± 2·1	20·1 ± 1·7	< 0·01
(10 μg/ml)	26·3 ± 2·0	19·6 ± 2·3	< 0·01
Propranolol			
(0·1 mM)	17·6 ± 1·7	18·8 ± 1·8	> 0·1
(1·0 mM)	20·0 ± 2·3	15·7 ± 2·2	< 0·01

* Values given are the means ± S. E. M. of six observations. The period of exposure to drug in each experiment was 30 min.

* M. J. Peach, personal communication.

effect on the glycogen content of the muscles during an exposure period of 30 min and, therefore, could be tested further for its blocking action. At 1 mM, however, propranolol itself significantly depressed the glycogen content of the muscles, and this concentration could not be used to block the glycogenolytic action of other drugs in skeletal muscles.

TABLE 4. EFFECT OF EPINEPHRINE OR METHADONE ON GLYCOGEN CONTENT OF EDL + SOL MUSCLES PRETREATED WITH PROPRANOLOL*

Drug (concn)	No. of observations	Glycogen content (μmoles/g) Untreated†	Propranolol-pretreated†	P
Epinephrine (3 μg/ml)	9	15·0 ± 1·4	18·6 ± 1·8	< 0·01
Methadone (0·5 mM)	6	19·5 ± 1·6	20·4 ± 1·5	> 0·3
Methadone (5 mM)	6	9·6 ± 1·2	8·7 ± 0·8	> 0·3

* Values given are the means ± S. E. M. of the number of observations in the second column.

† Cross-paired muscles were incubated at 37° for 10 min in 1·0 ml of Krebs Ringer solution without (untreated) or with 0·1 mM propranolol (pretreated). Then 0·5 ml drug (epinephrine or methadone) was added to both sets of muscles. The solution added to the propranolol-pretreated muscles contained propranolol so that its concentration in the final solution remained constant. Incubation continued for an additional 30 min. Total incubation time, 40 min.

To demonstrate that 0·1 mM propranolol blocks the glycogenolytic action of epinephrine, the EDL + SOL muscles were incubated initially for 10 min without or with propranolol. Epinephrine (3 μg/ml) was then added to both untreated and propranolol-pretreated muscles. The data in Table 4 show that the glycogen content of the muscles in the presence of propranolol was significantly greater than that of the untreated muscles, i.e. propranolol had blocked, at least partially, the glycogenolytic action of epinephrine. Similar experiments were carried out by adding methadone to untreated and propranolol-pretreated muscles. There was no significant difference in the glycogen contents of either untreated or propranolol-pretreated muscles after the addition of either 0·5 or 5 mM methadone. Thus the glycogenolytic action of methadone was not blocked by a concentration of propranolol which was sufficient to at least partially block the glycogenolytic action of epinephrine. It seems unlikely, therefore,

TABLE 5. EFFECT OF METHADONE ON THE ACTIVITIES OF SOME ENZYMES OF EDL + SOL MUSCLES*

Enzyme	Enzyme activity (nmoles/min/mg protein) Control	Methadone	P	Methadone-treated (% of control)
Phosphorylase a	32·3 ± 5·4	12·6 ± 3·2	< 0·05	39
Total phosphorylase (a + b)	471 ± 79	295 ± 82	< 0·001	63
Glycogen synthase I	22·5 ± 2·9	14·4 ± 2·4	< 0·02	64
Total glycogen synthase (I + D)	32·4 ± 6·1	17·8 ± 3·5	< 0·05	55
Phosphoglucomutase	1153 ± 218	1158 ± 301	> 0·05	100

* Values given are the means ± S. E. M. of five separate experiments except the phosphoglucomutase activities which were determined in only four of the experiments. The methadone concentration was 5 mM; the incubation time was 1 hr.

that methadone induces glycogenolysis through the release of catecholamines in the muscles themselves.

Effect of methadone on activity of enzymes of glycogen breakdown and synthesis. Another possible locus of action of the opioid narcotic drug effect on muscle glycogen content is one or more of the enzymes involved in glycogen synthesis and breakdown. To relate these experiments to the experiments on the glycogenolytic effect, a concentration of methadone that produced a very marked depression of glycogen levels was tested (5 mM). The results are summarized in Table 5. Methadone decreased the activity of both phosphorylase a and total phosphorylase, but was considerably more inhibitory to phosphorylase a. This result supports the conclusion above that catecholamine release is not involved in the glycogenolytic action of opioid narcotic drugs. If epinephrine were released in the muscles by methadone, the activity of phosphorylase a in the treated muscles would have been stimulated rather than inhibited.[18] Methadone also decreased the activity of both glycogen synthase I and total glycogen synthase, but phosphoglucomutase activity was not affected by the drug. Methadone, therefore, did not affect all glycolytic enzymes in isolated skeletal muscle but markedly inhibited the activity of the two enzymes directly involved in glycogen breakdown and synthesis. It seems unlikely, however, that the glycogenolytic action of methadone can be attributed to its effect on phosphorylase and synthase activities because both are inhibited by the narcotic drug, and the glycogenolytic enzyme, phosphorylase a, is considerably more sensitive to methadone than is the synthetic enzyme, glycogen synthase I.

DISCUSSION

This investigation began with the objective of confirming the observation of Peng and Walsh[1,2] that morphine increased the rate of glucose uptake by diaphragm muscle from normal rats. All of our attempts were unsuccessful. Reasonable care was taken in the present work to follow closely the experimental procedure of Peng and Walsh, but there were several differences which, although minor, should be mentioned. For example, Peng and Walsh used female rats (140–180 g), strain unspecified, fasted for 24 hr. We used male rats in two weight ranges [74 $\pm$ 13 and 151 $\pm$ 15 (S. D.) g], Sprague–Dawley strain, fed *ad lib.* up to time of use. In preparing the tissue, Peng and Walsh rinsed the freshly dissected diaphragm muscle in phosphate-buffered saline containing glucose at 0° for an additional 15 min. It was then bisected, blotted and weighed before incubation in 2·0 ml of medium without or with morphine. We rinsed the freshly dissected diaphragm muscle at room temperature, trimmed and bisected it and allowed it to soak at room temperature for 40–80 min in glucose-free medium before it was blotted, weighed and incubated in 1·5 ml of Krebs–Ringer solution containing glucose without or with drug. These differences in technique do not seem to be great enough to account for the difference in results.

Of possibly more significance is the difference in the calcium content of the two phosphate-buffered media. Fong and Walsh[7] reported that the morphine stimulation of glucose uptake, regularly observed in diaphragm muscles from normal rats when the concentration of extracellular calcium was 2·73 mM, disappeared when calcium was omitted from the incubation medium. It is possible, therefore, that stimulation of glucose uptake in diaphragm muscle by morphine requires a concentration of extracellular calcium even greater than that present in our Krebs–Ringer solution

(1·26 mM). However, we observed no increase in the rate of glucose uptake in morphine-treated diaphragm muscles from normal rats when they were incubated in a Tris-buffered medium prepared according to Fong and Walsh[7] to contain 2·73 mM calcium. This suggests that the difference in the calcium contents of the phosphate-buffered medium used by Walsh and his coworkers[1–7] and by us is not primarily responsible for our failure to confirm this observation that morphine increased the rate of glucose uptake in diaphragm muscle.

A major finding in the present work is the glycogenolytic effect of morphine and other opioid narcotic drugs. With morphine, however, significant glycogenolysis in the diaphragm muscle only occurred at concentrations of 5 mM or greater. This is, of course, a much higher concentration of morphine than would be attained *in vivo* even when high doses were administered. Methadone, on the other hand, had a glycogenolytic effect at concentrations as low as 0·1 mM, and the effect increased in proportion to the methadone concentration. A possible explanation for the difference in effectiveness of the two drugs is the marked difference in their lipid solubilities. Methadone has a partition coefficient (1-octanol/buffer, pH 7·4) of 55 whereas morphine has a partition coefficient of only 1.[19] Thus it would be expected that much less of the morphine added *in vitro* would penetrate the muscle fibers. The partition coefficients of meperidine and hydromorphone in the same system are not known, but because of the high concentrations of these drugs and morphine required to produce a significant glycogenolytic effect, further studies of possible relationships between concentration and effect were not made.

The mechanism by which the opioid narcotic drugs effect a net loss of glycogen in skeletal muscle is not known although it seems reasonably clear that methadone is not acting secondarily through the release of catecholamines in the muscle. It was expected that whatever feature of the control of glycogen metabolism is sensitive to narcotic drugs might develop tolerance to the high concentrations of morphine which the chronically treated animals were receiving in the latter part of the treatment period, and therefore cross-tolerance to methadone. If this had occurred, methadone should have produced little or no decrease in glycogen content in the diaphragm muscles from rats chronically treated with morphine. Instead, methadone produced a greater decrease in the glycogen level of the diaphragm muscles from tolerant rats than in muscles from normal rats. We have no explanation for the increased sensitivity of the glycogen content of muscles from tolerant rats to methadone *in vitro*, but it represents a change from rats either previously untreated with morphine or treated only once with morphine which may be worth further study.

In order of decreasing glycogenolytic potency, the drugs tested in these experiments *in vitro* rank: methadone > meperidine = morphine > hydromorphone. It is customary to rank the potency of the opioid narcotic drugs in terms of the dose required for analgesic action *in vivo*. In order of decreasing analgesic potency, the drugs are: hydromorphone > methadone = morphine > meperidine.[20] There is, therefore, no correlation between the analgesic potency of these drugs *in vivo* and their glycogenolytic potency *in vitro*.

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