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Presynaptic Neuromuscular Inhibition by Porphobilinogen and Porphobilin

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ABSTRACT **The rat hemidiaphragm was studied *in vitro* as a test system to evaluate the effects of heme precursors and the uroporphyrins upon neuromuscular excitability. Porphobilinogen and porphobilin had no effect on the *resting* miniature end-plate potential frequency, but the K^+-augmented frequency was significantly reduced. Porphobilin and porphobilinogen gave 50% of their maximal effect at concentrations of 0.008 and 0.6 μg/ml, respectively; the effect increased with concentration. Uroporphyrin I at 0.05–1.0 μg/ml caused a 25% decrease in frequency, but the effect did not increase with concentration. At similar concentrations, uroporphyrin III was without effect.**

The concentrations of porphobilin and porphobilinogen effective in inhibiting the K^+ stimulation, which are several orders of magnitude lower than the effective concentrations of simple amino acids, are those which might reasonably be expected in the sera of patients with acute intermittent porphyria.

As is now well known, the control of heme biosynthesis depends upon the initial and rate-limiting enzyme, δ-aminolevulinic acid synthetase (ALA synthetase). In hepatic acute intermittent porphyria, the content of this enzyme in the liver has been consistently found to be increased, in keeping with the characteristic over-production and urinary excretion of δ-aminolevulinic acid and porphobilinogen (PBG), the latter in largest quantity. The nature of this abnormal induction of ALA synthetase is as yet unexplained, as is its relationship to the occurrence of the neurological disturbances that characterize the disease.

It has been suggested (1, 2) that a constitutive operator mutation affecting the operon for ALA synthetase might represent the underlying genetic abnormality or (3, 4) that the induction might be related to a secondary disturbance of feedback inhibition, possibly even a defect outside the heme synthetic pathway. In the former case, a "toxic" effect of one or more of the substances produced in excess would be expected; in the latter, additional possibilities include the effect of unknown substances or the deficiency of a substance required to protect nerve function. Goldberg (5) favored such a deficiency, supporting his belief by establishing the lack of toxicity of ALA or PBG given intravenously in rats.

The primacy now attached to the concept of over-production of porphyrin precursors in this disease clearly indicated the need for further information as to possible adverse effects of one or more of these precursors, such as ALA or PBG, on the nervous system. We have used an *in vitro* vertebrate neuromuscular junctional system to evaluate the capacity of porphyrins and their precursors to influence junctional transmission. Rat phrenic nerve-hemidiaphragm junctions have been employed to assay the effects of PBG and of uroporphyrins on the excitatory postsynaptic potentials called miniature end-plate (mep) potentials induced by increasing the K^+ concentration in the banking solution. PBG and porphobilin (PB) have been found to have a significant presynaptic inhibitory effect, similar in character to but more marked than that reported for ALA (6). Uroporphyrins I and III were found not to be clearly effective.

Abbreviations: ALA, δ-aminolevulinic acid synthetase; PB, porphobilin; PBG, porphobilinogen; mep potential, miniature end-plate potential.

METHODS

The Liley rat hemidiaphragm preparation (7), removed from ether-anesthetized 125–150 g Wistar rats, was mounted on lucite lenses in a water-jacketed chamber of small volume on a Zeiss micromanipulator. Glass capillary microelectrodes filled with KCl and a conventional high-impedance combination of probe, amplifier, and oscilloscope were used for microelectrode studies of potentials in single muscle fibers (8). Transients were detected and counted with a signal conditioner and conventional electronic counter. At least 100 counts at a single junction were made to reduce the sampling error due to the Poisson distribution of mep potentials released by the motor nerve terminal (9). Most counts at single junctions exceeded 1000. Prejunctional release of the chemical transmitter acetylcholine was estimated by the frequency of mep potentials (10). Junctions were sampled throughout the muscle and appeared to be representative of the range of activity for nerve terminals in the specimen studied. Microelectrodes were inserted under visual control near visible junctions; correct placement was indicated when mep potentials with rise-times of less than 1 msec were observed. Normal bathing solutions contained 151 mM Na^+, 5 mM K^+, 2 mM Ca^{2+}, 1 mM Mg^{2+}, 0.5×10^{-6} M choline, and 200 mg/100 ml glucose in a biphosphate–bicarbonate buffer. The predominant anion was chloride. [Na^+] was not adjusted, since we had found in preliminary experiments that addition of K^+ above the "normal" concentration did not affect results. Studies were carried out at 31°C (11) and pH 7.35.

PBG was crystallized from the urine of human cases of acute intermittent porphyria by the method of Cookson and Rimington (12). To remove porphyrin or other trace impurities, we applied the PBG to an anion-exchange resin eluted in

the usual way (13), recrystallized it, and stored it *in vacuo* at 4°C, under which conditions it keeps well. PB was prepared by allowing a solution of PBG, adjusted to pH 5.0, to stand in the dark at 37°C until the Ehrlich reaction had become negative. Thin-layer chromatography on talc plates, according to With's method (14), permitted separation of uroporphyrins, after which the pH was adjusted to 4.0, and the precipitated PB was filtered, washed with water, and dried under reduced pressure over P_2O_5 at 22°C. These methods are to be described in detail elsewhere.

Synthetic crystalline uroporphyrin III methyl ester was kindly supplied by Dr. S. F. MacDonald of the National Research Council of Canada. Uroporphyrin I was crystallized as the methyl ester from bovine porphyric urine, after purification by thin-layer chromatography (15). The recrystallized esters were hydrolyzed on standing overnight in 7.5 N HCl. The free porphyrin was precipitated at pH 4.0 (acetic acid) and dissolved in isotonic phosphate buffer, pH 7.4.

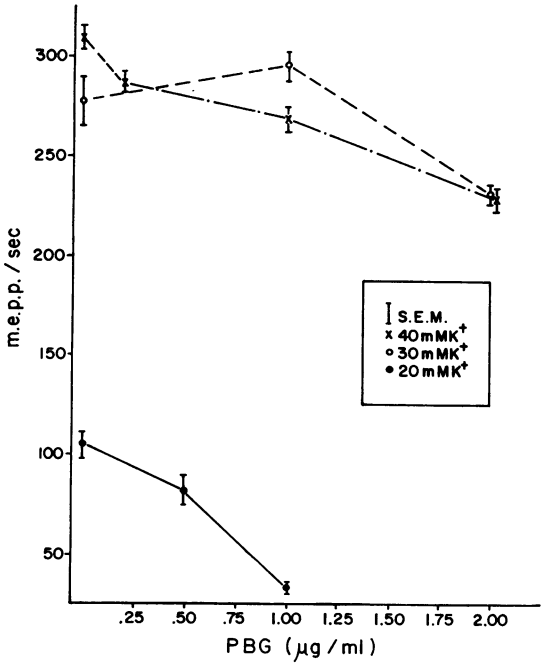
Because porphyrins can chelate divalent ions, the uroporphyrin solutions were adjusted to "normal" ionized calcium activity by means of a membrane-type ion-specific electrode (Orion Industries).

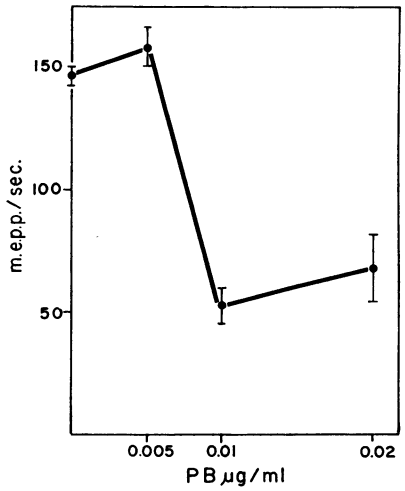
In all studies, control observations were made in a population of fibers (at least 10); the PBG or uroporphyrin was then added, and its effect was evaluated serially in a single fiber. A second population was then sampled. The control solution was replaced, a series of "recovery" observations were made, and a second "control" population was again sampled.

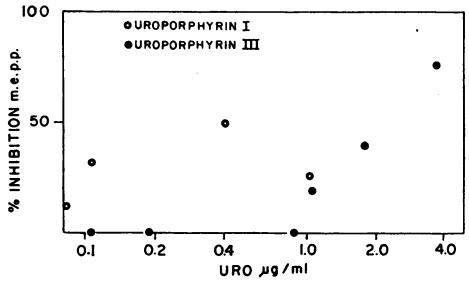
Isometric twitches were observed in rat hemidiaphragm upon supermaximal square-wave stimulation of the phrenic nerve. Muscle strips were mounted in a thermostated 2.0-ml chamber and connected to a strain gauge transducer to permit recordings in the customary fashion.

RESULTS

Addition of PBG to "resting" junctions in concentrations up to 2.0 μg/ml (8.8 μM) caused no change in frequency of mep potentials. In addition, no change in amplitude of the muscle fiber response (post-synaptic effects) was grossly evident. However, stimulation of the motor nerve terminals by depolarization with 20 and 30 mM K^+ was inhibited by PBG (Fig. 1). At a concentration of 1.0 μg/ml, the frequency of mep potentials was reduced from 84.2 ± 9.5 per sec (mean ± SE, n = 10 in all cases) to 32.2 ± 3.3 in the presence of 20







(*Left*) FIG. 1. Miniature end-plate potential frequency as a function of the concentration of porphobilinogen (PBG). Three concentrations of potassium (×40, O 30, and ● 20 mM) were utilized to augment the resting discharge of acetylcholine from the motor nerve terminals in the rat hemidiaphragm. Each point represents the mean ± SE of 10 or 11 fibers.

(*Upper right*) FIG. 2. Dose–response function of porphobilin (PB) on frequency of mep potentials in motor nerve terminals depolarized by exposure to 20 mM K^+. Each point represents mean values (control 44 fibers, other points 11 fibers); vertical bars represent SE.

(*Lower right*) FIG. 3. Uroporphyrin effects upon potassium-augmented acetylcholine release at the motor nerve terminals in rat diaphragm. Frequency of mep potentials was augmented by exposure to 20 mM K^+. Percent inhibition = [1-(uroporphyrin frequency)/(control frequency)] × 100. Each point represents the mean of 11 fibers under each condition.

mM K^+, but with 30 mM K^+ 2.0 μg/ml PBG was required to bring about a reduction from 278.3 ± 9.2 to 231.5 ± 5.4 per sec. With 40 mM K^+, as little as 0.2 μg/ml (0.88 μM) PBG reduced the frequency from 308.7 ± 7.3 per sec (mean ± SE, n = 11) to 288.8 ± 6.9 per sec ($P = 0.05$). Effects were manifest within 2 min. Increasing amounts of PBG were more effective and the median effective dose for significant ($P < 0.001$) inhibition of acetylcholine release (as judged by decrease in mep potential frequency) was about 0.7 μg/ml.

PB was approximately ten times as potent as PBG in reducing augmented mep potential frequency (Fig. 2). Either 0.01 or 0.02 μg/ml in 20 mM K^+ reduced the frequency markedly, with $0.001 < P < 0.005$. The duration of action of PB was considerably longer than for PBG, ALA, or either of the uroporphyrins studied. Effects (reduction of the frequency in 20 mM K^+) did not dissipate until the preparations were washed for more than 60 min, whereas the effects disappeared almost immediately when the porphyrin precursors were removed.

Concentrations of uroporphyrin III between 0.18 and 0.87 μg/ml had no effect on the response to 20 mM K^+ (Fig. 3); concentrations of 1.25 μg/ml and higher gave a linear dose–response curve. Uroporphyrin I caused observable inhibition at 0.1 μg/ml, but it gave no greater inhibition at 1.1 μg/ml than did uroporphyrin III (Fig. 3), and no consistent dose–response pattern was observed.

When the normal bathing solution was replaced with one containing 2.0 μg/ml of PBG, a 15% reduction occurred in the tension of twitches indirectly induced at 2/sec and the tetanus observed on nerve stimulation at 50/sec. These effects were uniformly reversible. Twitch/tetanus ratios were not altered and no curariform pattern of response was found with rapid, nontetanizing, nerve stimuli in the presence of PBG.

DISCUSSION

Miniature end-plate potentials represent the postsynaptic activity of units ("quanta" or "packets") of the chemical transmitter, acetylcholine, released by the motor nerve terminal (10). In all vertebrate species studied, including man (16), the action potential of the motor nerve results in the approximately simultaneous release of a large number of these quantal units, leading to the greater end-plate potential that effects neuromuscular transmission. The presynaptic (neural) effects of experimental manipulation similarly influence mep frequency and mep amplitude. The frequency of mep potentials can be taken as a measure of transmitter release, and experimentally induced changes indicate presynaptic influences upon transmission.

The present study indicates that PBG does not influence acetylcholine release at rest or the amplitude of the muscle fiber response to the transmitter. However, when the nerve terminal was made to release large numbers of transmitter units by ionic depolarization, PBG and PB caused a profound reduction in transmitter release and a decline in the twitch tension. Previous studies (6) have shown that similar effects on twitch tension and mep potential frequency are observed with effective (7.5 mM) concentrations of ALA, the immediate metabolic precursor of PBG. Reduction in the amount of transmitter release under physiologic stimulus (nerve action potential) of neuromuscular function can be inferred from these observations. Although this relationship is not linear, increasing depolarization has been shown to result in increasing release of acetylcholine (17). The present findings do not exclude an effect of PBG or PB on nerve or the response of muscle to direct stimulation. The former would be more difficult to determine, but a study of mep potentials evoked by nerve stimulus is planned.

Effective concentrations of PBG were what might reasonably be expected to be present in the sera of patients with clinical manifestations of acute intermittent porphyria, although as yet no actual data of this type have been reported. In a recent unpublished study (C. J. W. and R. A. C.) of serum PBG concentrations, values ranging from 70 to 140 μg/100 ml have been observed in several asymptomatic subjects who were excreting large excesses of PBG in the urine; in normal individuals the range was 0–5 μg/100 ml. In a fatal case the serum PBG was 340 μg/100 ml, this being by far the highest value yet observed. The urine PBG was 350 mg/24 hr. This patient had quadriplegia, bulbar signs, and respiratory failure.†

In contrast to PBG and PB, neither uroporphyrin was significantly effective in this model system, at concentrations much greater than those of the total uroporphyrin observed in the blood serum in this disease (in our experience, 0–10 μg/100 ml). It is perhaps of greater importance that in cutaneous porphyria, whether hepatic (porphyria cutanea tarda) or erythropoietic (uroporphyrin type) the serum uroporphyrin concentration is usually much higher than in acute porphyria, yet these patients lack any neurologic disturbance.

Goldberg *et al.* (5) failed to find evidence of any adverse effect of PBG, when injected intravenously in rabbits and cats, in amounts up to 100 mg/kg body weight. In one rabbit having an external biliary fistula 10 mg was injected. The animal showed no abnormal symptoms during the next 3 days. None of the PBG was detected in the urine‡, nor was any increase found other than a "slight rise in the level of bile protoporphyrins". In one experiment PB produced a histamine-like contraction of guinea pig intestine. These authors believed, in fact, that this was due to contaminating histamine, as the PB had not been prepared from crystalline PBG. The possiblity was not mentioned that the normal rabbit might metabolize or dispose of PBG at a rapid rate as compared with a human case of acute porphyria.

The large excesses of PBG in acute intermittent porphyria suggested that a deficiency of hepatic deaminase (uroporphyrinogen synthetase) might be found in this disease (18, 19). Such a deficiency has in fact been demonstrated by Heilmeyer and Clotten (20) and has been confirmed by Strand *et al.* (21), and by Miyagi and Watson (to be published). In the fatal case mentioned above Miyagi and Watson were unable to detect hepatic deaminase activity. If PBG is related to disturbance of the nervous system in acute porphyria, an efficient renal clearance might explain asymptomatic cases (clinical

† We are indebted to Prof. Burton Coombs, Dept. of Medicine, Southwestern University Medical School and Parkland Hospital, Dallas, Texas, for providing the data in this case and transmitting samples of blood serum, urine, and liver.

‡ The failure to detect PBG in the urine in Goldberg's experiment is unusual. Several similar experiments have been carried out by Dr. Ken Miyagi and Mr. Dennis Miller, Medical Research Unit, Northwestern Hospital, Minneapolis, to whom we are indebted for the typical result that after intravenous injection in a normal rabbit of 17 mg of crystalline PBG dissolved in isotonic phosphate buffer, pH 7.4, 8.7 mg was recovered in the urine in 8 hr, essentially none thereafter.

remission) in which large amounts of PBG are excreted in the urine[§]. If renal clearance were to become impaired, the blood and tissue content of PBG might become significant in respect to effects of the type reported here. Goldberg and Rimington (22) failed to detect PBG in tissues other than liver and kidneys in a fatal case of acute intermittent porphyria. They did not, however, mention any attempt to detect it in the blood serum, but it is noteworthy that the values for kidney were as high as or higher than those for the liver.

It is evident that detailed quantitative data on serum, urine, and tissue PBG and PB concentrations are needed. Determination of PB offers great difficulty, as it does not exhibit an Ehrlich reaction and its spectral absorption in the blue region is broad and diffuse. One of us (C. J. W.) has noted from time to time in the past that darkening of the freshly passed urine in the early phase of relapse of acute intermittent porphyria is often due to PB rather than porphyrin, at least as judged by color and absorption spectrum. Of course, the presence of PB in freshly passed urine provides no information as to its possible presence in the blood or tissues; no evidence is available in this respect.

The effects of PBG and PB reported here are more pronounced than those previously reported for ALA. The epileptogenic effect of the porphyrin precursors noted by others (23) may reflect central synaptic effects similar to the influence on excitation in the peripheral functional system. Recent studies (24) have shown that some inhibitory central transmitters are amines and amino acids. ALA and PBG, both in this general category, might act directly upon central functional postsynaptic receptor sites and thus account for other clinical phenomena. The cellular mechanisms of action of PBG and ALA require further study. An influence of ALA upon cation transport (sodium movement) was suggested (6) by observations with dual electrodes in muscle. A more efficient movement of sodium across the membrane could result in stabilization of the nerve, leading to impaired conduction.

§ In A. K., male of 63 years, who has been studied for years after a well-established diagnosis of acute intermittent porphyria, the urine in remission consistently has a PBG content of 150–250 mg/24 hr. In one 24-hr period, the amount was 209 mg (5.1 mg/100 ml), urine volume 4100 ml, and the serum PBG concentration determined in the middle of the 24 hr period was 0.19 mg/100 ml. From these data, a renal PBG clearance of 76.2 ml/min may be calculated.

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