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Effect of pentobarbital on cyanide-induced tremors in mice and calcium accumulation in PC12 cells

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Tremors, seizures and central lesions are common manifestations of acute cyanide poisonings [1]. Our laboratory recently reported that cyanide-induced tremors may be related to alterations in neuronal regulation of intracellular calcium [2, 3]. Cyanide produces accumulation of cytosolic Ca^{2+} as a result of decreased availability of ATP which is necessary for maintenance of energy-dependent calcium homeostatic processes. Elevated cytosolic Ca^{2+} can induce the release of neurotransmitters, leading to generalized CNS excitation manifested as convulsions or tremors.

The inhibitory actions of barbiturates on neurotransmitter release [4], as well as other calcium-dependent neuronal processes, have been established [5, 6]. The ability of pentobarbital to decrease the incidence of cyanide-induced convulsions has been reported [7], but a more detailed study has not been conducted. The objective of this study was to characterize the effect of pentobarbital on cyanide-induced tremors in mice and to correlate these efforts with cyanide accumulation of cytosolic Ca^{2+} in PC12 cells.

Materials and methods

Male, Swiss-Webster mice (Laboratory Supply, Indianapolis, IN), weighing 27 ± 1 g, were used for the tremor studies. The effect of pentobarbital on KCN-induced tremors was studied using the following treatment regimens: (1) saline was administered, i.p., 15 min before KCN (12 mg/kg, s.c.); (2) pentobarbital (60 mg/kg) was administered, i.p., 15 min before saline, s.c.; and (3) pentobarbital (60 mg/kg) was administered, i.p., 15 min before KCN (12 mg/kg, s.c.). Tremors were measured as previously described [8, 9]. Briefly, motor activity produced mechanical displacement of a free-floating platform which caused resistance changes across a wheatstone bridge. The fluctuations in voltage over time were converted to an intensity–frequency profile by Fourier transformation equations. A useful parameter for characterizing animal activity is the peak frequency which is defined as that frequency with the greatest intensity.

PC12 cells were obtained from Dr. William Tank (University of Colorado, Denver, CO). Cell maintenance has been described previously [10, 11]. Cytosolic Ca^{2+} levels were measured using Quin II/AM as previously reported [12].

All treatment groups were composed of four or more animals or, in the experiments with PC12 cells, four or more different cell samples. Analysis of variance was used to determine statistical differences between treatment groups at a significance level of 0.05. If multiple comparisons were not a factor, a Student's *t*-test (two-tail) was employed, and the means were ranked using a Neuman–Keuls multiple range test.

Results and discussion

The effect of KCN and/or pentobarbital on the intensity-frequency profile is illustrated in Fig. 1 and the change in peak frequency over time in Fig. 2. Mice pretreated with saline and 15 min later administered KCN (12 mg/kg, s.c.) developed severe whole body tremors. The onset of the tremors occurred 15 min after administration of KCN and were most severe at this time period with a peak frequency of 20.95 ± 0.95 Hz. The peak frequency steadily declined after 30 and 45 min. The intensity–frequency profile revealed that the tremors at 30 min were composed of high-frequency peaks ranging from 11 to 18 Hz (Fig. 2). Prominent frequencies were measured at 11, 15 and 18 Hz. After 2–3 hr, normal exploratory and grooming behavior was observed.

Administration of pentobarbital (60 mg/kg, i.p.) followed 15 min later with saline produced intermittent and sporadic shivers in the mice (Fig. 1). The shivers began after 15 min and lasted approximately 45 min. The peak frequency of pentobarbital-induced shivers varied considerably over a range of 3–16 Hz. The intensity–frequency profile of the most severe shivers at 30 min revealed multiple medium-ranged frequencies up to 16 Hz in which all exhibited similar degrees of intensity (Fig. 2). When mice were pretreated with pentobarbital, (60 mg/kg, i.p.), 15 min before KCN (12 mg/kg, s.c.), no movement or motor activity was observed. The combination of KCN and pentobarbital produced no motor activity, other than a small respirator peak at 3 Hz. Frequencies of 2–3 Hz reflect

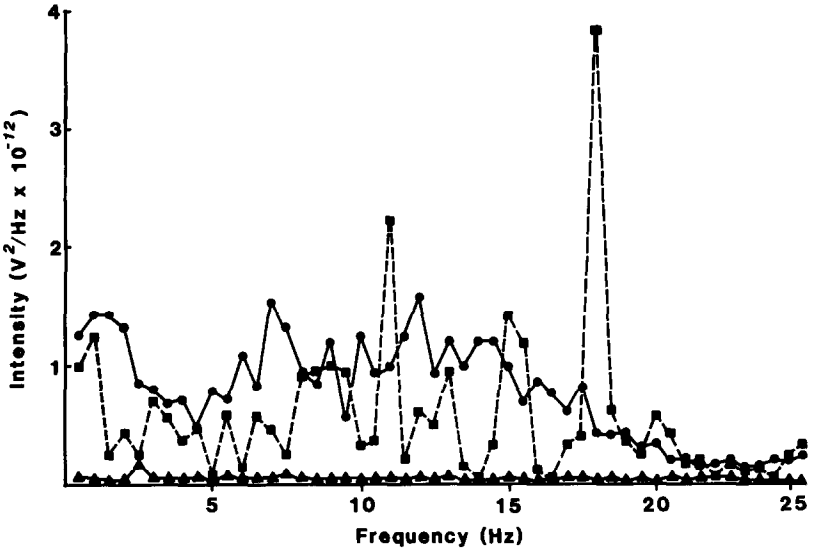

Fig. 1. Representative tremor intensity–frequency profiles observed in mice 30 min after KCN treatment. Experimental groups were pretreated with saline, i.p., followed 15 min later with KCN (12 mg/kg, s.c.) (■——■); pretreated with pentobarbital (60 mg/kg, i.p.) followed 15 min later with saline, s.c. (●——●); and pretreated with pentobarbital (60 mg/kg, i.p.) followed 15 min later with KCN (12 mg/kg, s.c.) (▲——▲).

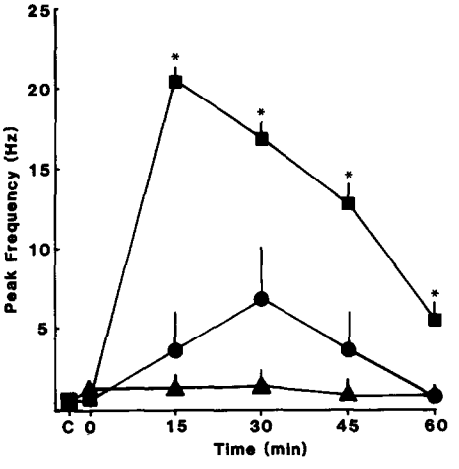

Fig. 2. Peak frequencies of intensity–frequency profiles of mice pretreated with saline, i.p., followed 15 min later with KCN (12 mg/kg, s.c.) (■); pretreated with pentobarbital (60 mg/kg, i.p.) followed 15 min later with saline, s.c. (●); and pretreated with pentobarbital (60 mg/kg, i.p.) followed 15 min later with KCN (12 mg/kg, s.c.) (▲). Control data (C) were obtained prior to injection and at zero time when KCN or saline was administered. Each point reflects the mean ± SE of six mice, and an asterisk indicates a significant difference ($P < 0.05$) between the cyanide–saline group and the other treatment groups at the same time period.

the respiratory rate of the mice (100–150 breaths/min). Control mice administered saline exhibited peak frequency at 1–4 Hz, which represents spontaneous motor activity and respiration (not shown).

Typical fluorometric tracings that reflect the change in cytosolic Ca^{2+} levels in PC12 cells treated with KCN and/or pentobarbital are illustrated in Fig. 3. The resting free cytosolic Ca^{2+} concentration of untreated cells was 115.0 ± 4.9 nM (N = 12). KCN, at a concentration of 10^{-3} M (the approximate cyanide concentration in the circulation following a KCN dose of 12 mg/kg [3]), produced a gradual, steady rise in free cytosolic Ca^{2+} to concentrations greater than 650 mM. Pentobarbital (10^{-6} M, 15-min preincubation) completely blocked the KCN-evoked calcium accumulation. The results obtained from multiple testing are summarized in Fig. 4. Pentobarbital significantly prevented ($P < 0.05$) the cyanide-induced accumulation of Ca^{2+} in the cytosol over the 30-min test period. Untreated, saline-treated, or pentobarbital-treated cells were found to maintain constant resting cytosolic Ca^{2+} levels over a 45-min period (data not shown).

The results from this study clearly indicate that pentobarbital blocks the tremors observed following a nonlethal dose of cyanide in mice. Further characterization of the mechanism, using PC12 cells as a neuronal cell model, revealed that pentobarbital blocked the cyanide-induced accumulation of ionic calcium in the cytosolic pool.

Previous studies by our laboratory indicated that cyanide-induced tremors have a calcium-dependent component. The onset of tremors corresponds with a significant elevation of whole brain total calcium [3]. Furthermore, the tremors and rise in total brain calcium exhibit similar dose–response curves, and both effects are blocked by diltiazem, a calcium channel blocker. PC12 cells have been used previously to characterize the effect of cyanide on the cytosolic Ca^{2+} or the active calcium pool of the cell. Cyanide, at concentrations typically observed following administration of 10–15 mg/kg, elevated cytosolic Ca^{2+} to levels sufficient to stimulate calcium-dependent pathways.

Cytosolic Ca^{2+} levels are maintained within narrow limits by numerous energy-dependent processes. Cyanide markedly decreases ATP production [7], which results in rapid accumulation of Ca^{2+} in the cytosol [13, 14]. Elevation of ionized cytosolic calcium can activate intracellular events [15], including calcium-dependent action potentials and release of neurotransmitters [4, 5, 15]. Consequently, extensive CNS excitation and discharge may produce tremors, seizures and convulsions.

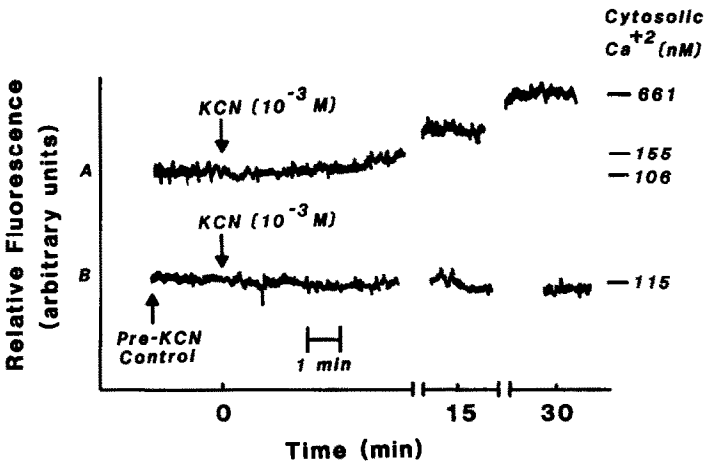

Fig. 3. Representative fluorometric recordings of pentobarbital-induced modification of KCN-induced accumulation of cytosolic calcium in PC12 cells. PC12 cells, loaded with Quin II, were (A) treated with KCN (10^{-3} M) or (B) pretreated with pentobarbital (10^{-6} M) followed 15 min later with KCN (10^{-3} M).

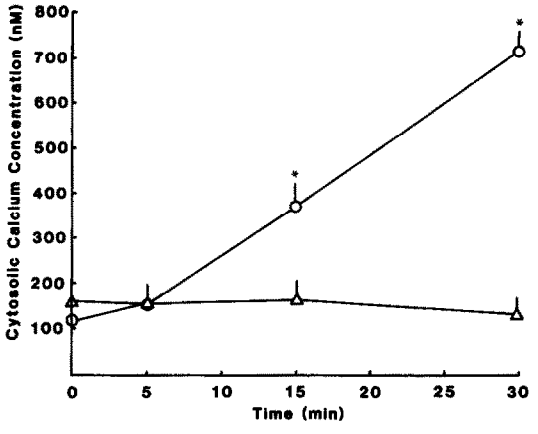

Fig. 4. Intracellular cytosolic calcium concentrations measured after incubation of PC12 cells with KCN, 10^{-3} M (○), or pretreated with pentobarbital, 10^{-6} M, followed 15 min later with KCN, 10^{-3} M (△). Zero time reflects the pre-cyanide cytosolic calcium levels in cells pretreated with saline or pentobarbital. Each point is the mean ± SE of four experiments, and an asterisk indicates a significant difference ($P < 0.05$) between the two different treatment groups at the same time point.

The inhibitory effects of pentobarbital on calcium-dependent stimulus–secretion coupling events have been characterized [16]. Pentobarbital acts at the presynaptic nerve terminal to significantly depress depolarization-dependent calcium influx by enhancing calcium channel inactivation or blocking the open calcium channel [6]. In the present study, pentobarbital effectively blocked the cyanide-induced accumulation of cytosolic calcium in PC12 cells, as well as the cyanide-induced tremors which appear to be a calcium-dependent event [2, 3]. In conclusion, this study demonstrates that sodium pentobarbital exhibits calcium-blocking properties and suggests a possible therapeutic approach for treating the tremors and seizures that accompany cyanide poisonings.

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