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Cholinergic Actions of Metrizamide

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In a study of the possible mechanisms of the clinical side effects of metrizamide, it was applied to several in vitro model preparations. It was shown that, although high metrizamide concentrations are without noticeable effect on many basic neuronal functions, metrizamide does interfere with cholinergic mechanisms. At concentrations equivalent to, or significantly lower than, those probably achieved during clinical procedures, metrizamide is both an inhibitor of the enzyme acetylcholinesterase and an antagonist of cholinergic transmission. These data suggest the possibility that some of the side effects resulting from clinical procedures employing metrizamide may be explained by its actions on cholinergic synapses.

Metrizamide is a water-soluble contrast material now used in many neuroradiologic procedures, particularly myelography and cisternography. It is today the agent of choice for these procedures because of its superiority to previously used contrast materials with respect to imaging quality and patient tolerance [1–4]. Significant side effects, however, do occur. These include headache, nausea, vomiting, and, occasionally, seizures [5]. The cause of these side effects is entirely unknown. To better understand the mechanisms producing these side effects, we tested metrizamide for specific effects on neuronal functions. We found that metrizamide does have apparently specific actions on cholinergic mechanisms, although relatively high metrizamide concentrations are without noticeable effect on many basic neuronal functions. We show that metrizamide is both an acetylcholinesterase (AChE) inhibitor, as well as a cholinergic antagonist. These effects occur at concentrations equivalent to, or significantly lower than, those probably achieved during clinical procedures. We suggest that some of the side effects resulting from the clinical procedures using metrizamide are explained by its actions on cholinergic synapses.

Invertebrate preparations often provide convenient model systems with which to study the mechanisms of actions of pharmacologic agents. We used several crustacean neuromuscular preparations that are ideally suited for cellular pharmacology. Most of the experiments were carried out using the gm1 muscle, which is innervated by four excitatory cholinergic motor neurons, which branch to innervate each muscle fiber at a number of synaptic areas [6–9]. Other experiments were performed using the gm6 muscle, which is innervated by a single motor neuron that releases glutamate as its excitatory transmitter [9, 10]. Some of the stomatogastric muscles [11] also have inhibitory receptors for glutamate [12] and gamma-aminobutyric acid (GABA) (E. Marder, unpublished observations) that, when activated, cause the conductance to Cl^- ions to increase. These are all striated muscles, with the only known physiologic difference between them being the type of neurotransmitter receptors. Therefore, they provide an ideal opportunity to study the effects of a test substance on several simple synapses, mediated by different transmitters.

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Materials and Methods

The experiments were conducted on isolated nerve-muscle preparations from the stomatogastric region of the crab, *Cancer irroratus* (ref. [11] describes the anatomy and nomenclature of the muscles), using conventional electrophysiologic techniques and equipment [6–10]. The experimental setup is diagrammatically represented in figure 1. Muscles were removed from the animal and pinned into a 1–3 ml Sylgard- (Dow Chemical Co.) lined chamber. These preparations thus consist of a muscle, with insertions intact, and the cut end of the nerve innervating it. The muscle fibers are about 1 cm long and 100 μm in diameter. The gm1 muscle [11] is innervated by four motor axons, each of which branches to make a number of synaptic contacts with each of the muscle fibers in the muscle. The gm6 muscle is innervated by a single motor neuron.

Preparations were continuously bathed (see inflow and outflow marked in figure 1) with chilled (10°–12°C) physiologic saline [7]. Excitatory junctional potentials (EJPs) were elicited by stimulating the cut end of the nerve innervating the muscle with a suction electrode, using pulses of 0.5 msec in duration and 10–50 V in amplitude. Trains of EJPs were evoked by stimulating at frequencies of 1–10/sec for 2–5 sec every 30 sec.

Glass microelectrodes filled with 2.5 *M* KCl (10–20 MΩ) were used both to record the EJPs intracellularly and to pass current into the muscle fibers. In order to measure the input-impedance of a muscle fiber, two microelectrodes were inserted within 100 μm of each other, and current was passed through the first while the second was used to record membrane potential. In these experiments a Dagan 8500 two electrode voltage-clamp amplifier was used, in the constant-current (current-clamp) mode. Current pulses of varying amplitude and about 500 msec in duration were used to generate current-voltage plots (steady-state measurements were used). The input-impedance was calculated as the slope of the current-voltage plot.

Acetylcholine (ACh) was iontophoretically applied to a surface of a muscle fiber from a glass microelectrode filled with a solution of 1 *M* AChCl by passing positive constant currents (100–500 nA) for 2–1,000 msec with a WP Instruments iontophoresis unit. ACh responses were found by carefully moving the ACh-filled pipette over the surface of the fiber until a stable response was found. In some experiments the ACh responses were studied using the voltage-clamp mode of the Dagan voltage clamp. In these cases the ACh electrode was placed within a few hundred microns of the two intracellular microelectrodes. The adequacy of the voltage clamp was assessed by continuously monitoring the membrane potential of the fiber at high gain.

Experiments were done with either commercially available analytical-grade metrizamide (purchased from Gallard-Schlesinger Manufacturing Co.) or clinical-grade metrizamide (Amipaque). No obvious difference was seen. The metrizamide from either source was dissolved directly into physiologic crab saline directly before use and added to the bath by means of a switching port on the inflow.

Purified eel AChE was purchased from Sigma Chemical Co. and assayed colorimetrically by the method of Ellman et al. [13], using a Beckman spectrometer with attached recorder.

Results

The effects of metrizamide on excitatory neuromuscular junctions mediated by ACh and glutamate are contrasted in figure 2. In the experiment shown in figure 2A, the nerve innervating a cholinergic gm1 muscle was stimulated at 9/sec for 3 sec every 30 sec. Each stimulus caused an

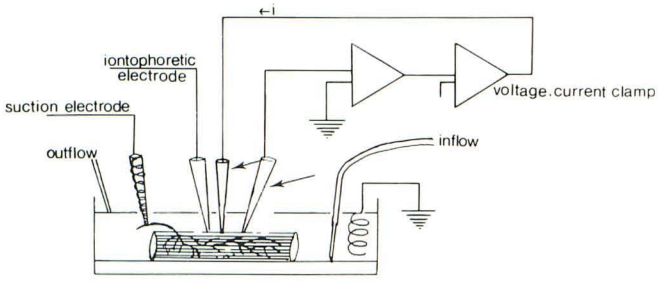

Fig. 1.—Experimental preparation. Shown are two intracellular microelectrodes (*arrows*), iontophoretic electrode used to apply ACh locally to surface of muscle fiber, suction electrode used to stimulate nerve, isolated nerve-muscle preparation, and simplified schematic of electronics.

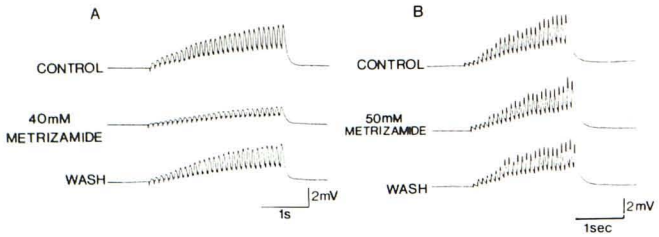

Fig. 2.—Intracellular recording of EJPs. **A**, Stimulation with suction electrode at nine/sec for 3 sec every 30 sec in cholinergic gm1 muscle; 5 min application of 40 m*M* metrizamide reversibly blocked EJP amplitude by 59%. Resting membrane potential −60 mV in all traces. **B**, Stimulation with suction electrode at 11/sec for 2 sec every 30 sec in glutamatergic gm6 muscle; 10 min application of 50 m*M* metrizamide had no effect. Resting membrane potential −51 mV in all traces.

individual depolarizing EJP. Due to facilitation, the last EJPs in the train were about five times the amplitude of the first EJP of the train (see top trace of fig. 2A). The middle trace of figure 2A shows that 40 m*M* metrizamide reduced the amplitude of the EJPs. The bottom trace shows that after the metrizamide was washed from the bath surrounding the muscle, the EJPs returned to their control amplitudes.

In contrast, 50 m*M* metrizamide had no effect on glutamate-mediated EJPs in muscle gm6 [9, 10] from the same animal (fig. 2B). This latter result indicates that metrizamide concentrations less than 50 m*M* do not nonspecifically interfere with action potential propagation or presynaptic release mechanisms, although they do not rule out some presynaptic effect at the cholinergic synapses of the gm1 muscle. To further determine if the effects of metrizamide are specific to cholinergic, excitatory responses, we examined the effects of metrizamide on two different inhibitory transmitter-activated conductances, and found in experiments (not shown) that metrizamide had no effect on GABA-mediated or glutamate-mediated increases in Cl^- conductance. These data suggest that the actions of metrizamide on the cholinergic excitatory synapses of the gm1 muscle are likely attributable to interactions with the ACh receptor-channel complex.

In order to determine if the reduction in EJP amplitude illustrated in figure 2 was dose-dependent, we measured the percentage decrease in EJP amplitude produced by different metrizamide concentrations. In all cases we cal-

culated the percentage decrease in amplitude using the last EJP of the train. However, since metrizamide had no apparent effect on the rate of facilitation, similar results would obtain for any stimulation paradigm. The reduction in cholinergic EJP amplitude by metrizamide was dose-dependent, as is indicated in table 1. Small blocks (20% ± 5% [n = 4]) were seen at 10 mM metrizamide; 25 mM metrizamide reduced the amplitude 40% ± 5% (n = 8); 50 mM metrizamide reduced the amplitude 65% ± 6% (n = 3). After applications of 100 mM metrizamide, the amplitude of the EJPs frequently did not recover to control levels, so it was impossible to assess accurately the percentage block.

The dose-dependent decrease in the amplitude of the EJPs (table 1) could be due to a decrease in the amount of transmitter released from the presynaptic terminal, a decrease in the number of channels in the membrane opened after ACh release, or a decrease in the muscle fiber input impedance. In order to determine if these metrizamide concentrations had a direct effect on muscle fiber input resistance, muscle fibers were penetrated with two 2.5 M KCl-filled microelectrodes. Current-voltage curves were constructed by passing current pulses of varying amplitude through one electrode while recording with the second electrode. The results of one such experiment are shown in figure 3, where it can be seen that 25 mM metrizamide, a concentration sufficient to reduce the amplitude of nerve-evoked EJPs 40%, had no measurable effect on the muscle input impedance. In five fibers tested from five muscles, the current-voltage relation was unaffected by concentrations of metrizamide as high as 50 mM. These results demonstrate that the decrease in EJP amplitude with increasing concentrations of metrizamide is not a trivial consequence of a decrease in the muscle fiber input impedance.

In order to assess directly the effects of metrizamide on postsynaptic ACh responses, a number of experiments were done in which gm1 muscle fibers were impaled with two microelectrodes in the presence of 20 mM $MnCl_2$, which blocks synaptic transmission at this junction (E. Marder and C. J. Lingle, unpublished observations), while ACh was iontophoretically applied to the surface of the fiber. Metrizamide reversibly reduced the amplitude of ACh iontophoretic responses (fig. 4). The block of the postsynaptic ACh response was also dose-dependent at similar concentration ranges to the block of the EJPs (table 1). A 10 mM application of metrizamide blocked the ACh response 26% (n = 2); 25 mM 49% ± 3% (n = 4); 50 mM metrizamide blocked 66% ± 7% (n = 4). In one successful trial 100 mM metrizamide reversibly blocked an ACh response 87%. The close agreement between the percentage block of ACh iontophoretic responses and the EJPs is consistent with the interpretation that the decrease in EJP amplitude is largely due to its effects on postsynaptic cholinergic mechanisms.

Since many cholinergic antagonists have anticholinesterase activity and many anticholinesterases show cholinergic antagonist activity [8, 14, 15] it was interesting to determine if metrizamide had anticholinesterase activity. We first determined that metrizamide inhibited AChE activity in crude homogenates from crab gm1 muscles, by assaying the enzyme using acetylthiocholine as substrate, as described by Ellman et al. [13] (data not shown). Then, to further quantitate these findings, and to remove the intrinsic difficulties in working with a crude enzyme preparation, we chose to use purified eel AChE (E.C. 3.1.1.7), which is well characterized and similar to mammalian enzymes in catalytic functions [16]. Figure 5 illustrates that metrizamide inhibits AChE activity in a dose-dependent manner, with an IC_{50}

TABLE 1: **Dose-Related Effects of Metrizamide**

Metrizamide Concentration (mM)	% reduction in: EJP Amplitude	% reduction in: ACh Response Amplitude
10	20 ± 5* (n = 4)	26 (n = 2)
25	40 ± 5 (n = 8)	49 ± 3 (n = 4)
50	65 ± 6 (n = 3)	66 ± 7 (n = 4)

* Indicates standard errors of the mean.

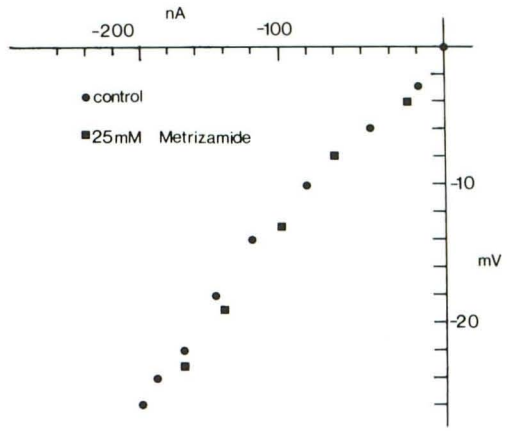

Fig. 3.—Effect of metrizamide on muscle input impedance. Muscle fiber penetrated with two intracellular electrodes. Current pulses 500 msec and 10–500 nA were passed into fiber with one electrode, resulting in voltage deflections recorded with the other. Metrizamide was added to bath and the process repeated. Current-voltage relation was unaffected by metrizamide application. Input impedance of this fiber was 100 kΩ (10^5 Ω), a value typical of crustacean muscle fiber.

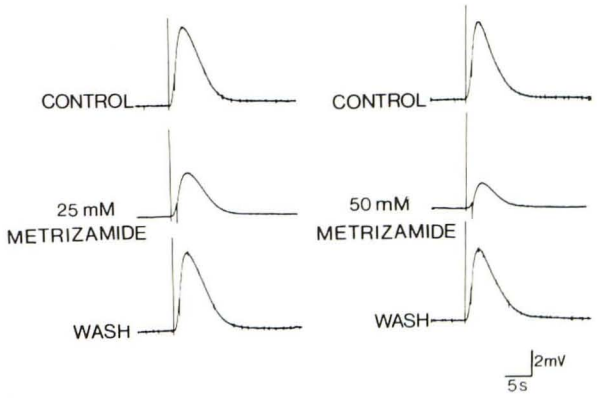

Fig. 4.—Metrizamide block of ACh response. Intracellular recording from gm1 muscle fiber, ACh iontophoretically applied at time shown by artifact (vertical lines). A 6 min application of 25 mM metrizamide reversibly blocked ACh response 43%; 6 min of 50 mM metrizamide reversibly blocked 68%. Resting membrane potential −65 mV.

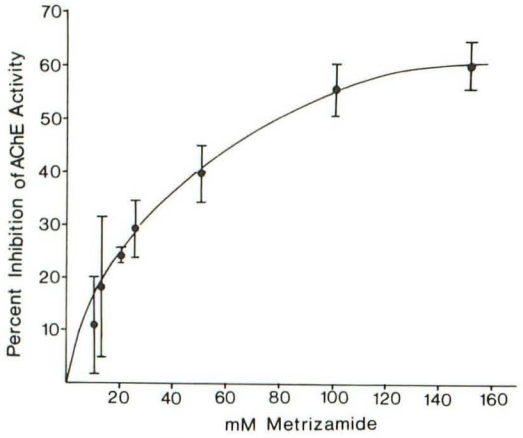

Fig. 5.—Metrizamide inhibition of AChE activity. Enzyme was assayed as in ref. [13], except that shorter times (6–12 min) were used. Points are means of four to 10 determinations; error bars indicate SEM. Each tube contained .08 U of enzyme.

about 85 m*M*. When we assayed AChE at varying substrate concentrations in the presence of 50 m*M* metrizamide (table 2) we found that the enzyme activity was decreased about 35%–40% at all substrate concentrations, which suggests that metrizamide is not a competitive inhibitor for AChE.

TABLE 2: **Inhibition of AChE Activity at Varying Substrate Concentrations by 50 m*M* Metrizamide**

Acetylthiocholine Concentration (m*M*)	Normalized Rate		Inhibition by Metrizamide (%)
	Control	50 m*M* Metrizamide	
0.06	.61 ± .08 (n = 4)	.39 ± .08 (n = 4)	36
0.12	.73 ± .08 (n = 5)	.45 ± .05 (n = 5)	38
0.24	1.0 (n = 6)	.61 ± .06 (n = 5)	39
0.47	1.09 ± .09 (n = 6)	.71 ± .08 (n = 4)	35
0.94	1.19 ± .14 (n = 5)	.75 ± .1 (n = 5)	37

Note.—Assays were done by the method of Ellman et al. [13] at room temperature, which was 19°–23°C. Eight to ten assays (10 min each) were done with each preparation of diluted enzyme. To control for variations in enzyme activity from day to day due to temperature or slight variations in enzyme dilution, rates were normalized to that obtained with 0.24 m*M* substrate in each enzyme preparation. Each final assay mixture contained .08 U of enzyme, which, assayed at .24 m*M* substrate under our conditions, yielded rates of 2.2–7.0 × 10^{-4} mol hydrolyzed/g/min.

Discussion

We have shown that: (1) metrizamide inhibited purified eel AChE with a IC_{50} of about 85 m*M* and (2) a 50% block of an ACh response was produced by 30–40 m*M* metrizamide. To evaluate these effects as possible mechanisms of clinical effects, we attempted to estimate the probable metrizamide concentrations in clinical procedures to compare them with the concentrations we have shown to be physiologically active.

When used in myelography or cisternography, 2–6.75 g of Amipaque are dissolved in diluent and introduced into the subarachnoid space. Metrizamide is denser than cerebrospinal fluid (CSF), and the injected substance moves as a bolus. Assuming the volume of the CSF to be 140 ml, this gives a concentration of about 60 m*M* (48 mg/ml) in the total (CSF), and one can assume that the concentration in the bolus as it arrives at the bottom of the fourth ventricle may be over 100 m*M* (80 mg/ml). At later times the metrizamide is more extensively distributed throughout the extracellular space [17]. If one takes the CSF volume as 140 ml and the extracellular cerebral fluid volume as 280 ml, and assumes complete equilibration through this volume, 6.75 g of Amipaque used in clinical procedures could result in a distributed concentration of 10–20 m*M* (8–16 mg/ml) in the total brain extracellular space. Since metrizamide is slowly cleared from the subarachnoid space over 24–48 hr [18] it is likely that for several hours neuronal tissues are exposed to metrizamide concentrations sufficiently high to influence cholinergic transmission.

The most common side effects after subarachnoid instillation of metrizamide are nausea and vomiting. The postulated emetic center is the area postrema of the medulla, which would be bathed in relatively high metrizamide concentrations before the drug became diluted with the CSF of the ventricles, upper brain cisterns, and the convexities of the subarachnoid spaces. The area subpostrema, just adjacent to the area postrema, contains AChE-staining neurons [19].

Metrizamide-induced seizures represent the most dramatic side effect of this drug. Although they are uncommon in normal individuals, they have an increased incidence in seizure-prone individuals or those treated with phenothiazines or other antipsychotic drugs [3, 20]. For this reason metrizamide is not suggested for those patients by the manufacturer. It is possible to suggest several mechanisms by which metrizamide might be seizure-producing.

Very high local concentrations of metrizamide may result as the bolus of metrizamide reaches brain tissue, and may be viewed as similar to a topical application of the drug. It is known that topical applications of ACh, other cholinergic agonists, cholinergic antagonists, and cholinesterase inhibitors have convulsant actions [21–23]. Therefore, it seems possible that either of the effects we describe could be seizure-producing. Other indications of the importance of cholinergic systems in seizure activity come from recent studies in the mechanism of epileptic kindling (in which electrical stimulation of the brain results in permanent seizure-prone individuals) [24, 25].

Metrizamide is currently advocated for use in angiography. In this application, the recommended dose (280 mgI/ml) [1] results in a plasma concentration of 5–10 m*M* (4–8 mg/ml). Although in normal individuals this dose may be easily tolerated, it is possible that patients treated for myasthenia gravis with anticholinesterase drugs could be at potential risk for cholinergic crisis.

In conclusion, our data suggest that, even at the very high concentrations attained during clinical procedures employing metrizamide, many important neuronal functions remain unaltered, as one would hope with an important clinically used contrast agent. Surprisingly, we also found relatively specific effects on cholinergic mechanisms, which may partially explain some of the transient complications associated with metrizamide use. Our findings in no way argue against the usefulness of metrizamide in the clinical setting, but are a clear reminder that high concentrations of any foreign

substance introduced into the nervous system are likely to have effects on important cellular functions.

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