

The myotonia-inducing effects of furosemide and clofibrate, two widely used pharmaceutical agents, were investigated in excised human external intercostal muscle. The effects of anthracene-9-carboxylic acid (9-AC), a well-known myotonia-producing chemical, were also tested for comparison. In the presence of these drugs the electrical threshold was lowered, and a constant current pulse produced multiple spiking. Short trains of direct stimuli were often followed by after-activity, and this caused a myotonia-like prolongation of muscle contraction. Voltage-clamp experiments showed that 0.05 mM anthracene-9-carboxylic acid, 1 mM furosemide, and 1 mM clofibrate decreased the chloride conductance of the muscle fiber membrane to 14, 18, and 40%, respectively, of the normal value, and the myotonia-inducing potency of the 3 drugs was correlated with the decreased chloride conductance. The potassium currents were not affected by these compounds.

MUSCLE & NERVE 11:576-581 1988

## DRUG-INDUCED MYOTONIA IN HUMAN INTERCOSTAL MUSCLE

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Symptoms resembling in many respects the myotonia observed in various hereditary muscle diseases of man<sup>21</sup> may be produced in laboratory animals by several classes of chemical agents. For instance, myotonia produced by monocarboxylic aromatic acids is similar to that in myotonia congenita, and myotonia produced by blockers of cholesterol synthesis is similar to that of myotonic dystrophy.<sup>13</sup> As to the effect of these drugs on humans, there is a report of an accidental intake of the herbicide 2,4-dichlorophenoxyacetic acid resulting in transient myotonia.<sup>5</sup> Clinical observations of myotonic symptoms appearing in hypercholesterolemic patients at the time of their being treated with 20,25-diazacholesterol were also reported.<sup>23</sup> A systematic investigation of the effect of myotonia-inducing agents directly applied to human muscle fibers has not been made. There-

fore, we decided to study the effects of furosemide and clofibrate as well as of anthracene-9-carboxylic acid, the most potent among the myotonia-generating agents belonging to the class of monocarboxylic aromatic acids,<sup>11,12,20</sup> on excised human intercostal muscle. Furosemide<sup>7</sup> and clofibrate<sup>19</sup> have been shown to induce myotonia in laboratory animals, but their myotonia-inducing potency in humans has not yet been evidenced. Furosemide is widely used as a diuretic agent, and clofibrate is used in the therapy of hyperlipidemia. Part of the results have been presented to the 13th World Congress of Neurology.<sup>16</sup>

### MATERIAL AND METHODS

The project was approved by the Ethics Commission of the Technical University of Munich. Specimens of external intercostal muscle were obtained from eight patients undergoing thoracic surgery. The patients gave informed consent to the operation. None of the patients had clinical or electrophysiological signs of a neuromuscular disorder. For transportation and dissection, and in control experiments, the muscles were bathed in Bretag's<sup>6</sup> solution containing (in mM) NaCl, 107.7; KCl, 3.48; CaCl<sub>2</sub>, 1.53; MgSO<sub>4</sub>, 0.69; NaHCO<sub>3</sub>, 26.2; NaH<sub>2</sub>PO<sub>4</sub>, 1.67; sodium gluconate, 9.64; glucose, 5.5; and sucrose, 7.6. The solution was bubbled with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> to set the pH to 7.4. A chloride-free solu-

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Acknowledgments: This work was performed while Dr. Kwieciński was absent on leave from the Department of Neurology, Medical Academy, Warsaw, Poland, as a Visiting Fellow of the Alexander von Humboldt-Stiftung. We are grateful to Dr. H.W. Präuer for help with surgery. The work was supported by the Deutsche Forschungsgemeinschaft (Le 481/1).

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Accepted for publication June 29, 1987.

0148-639X/1106/0576 \$04.00/6  
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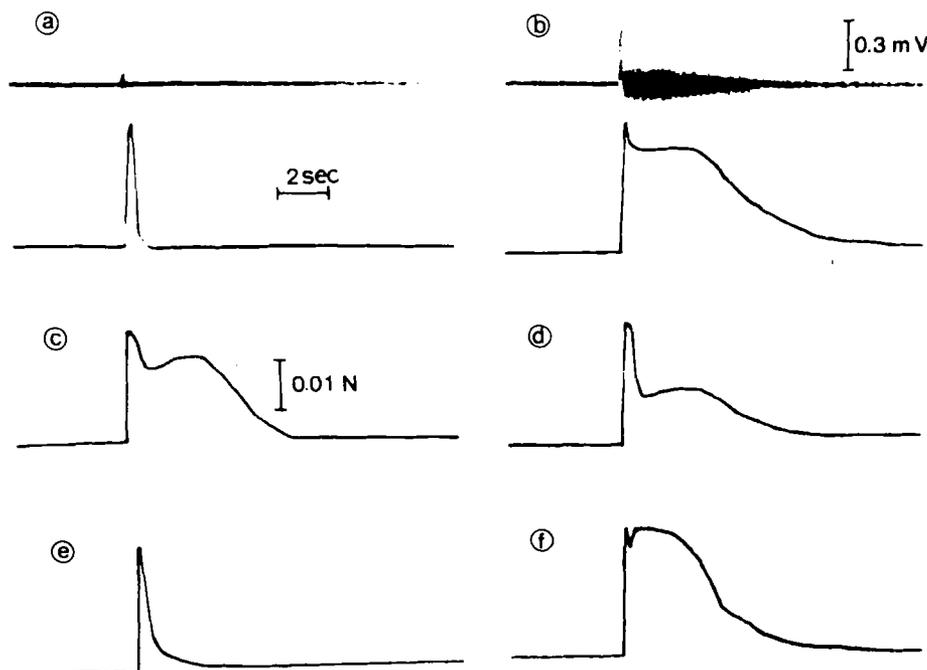
tion was made by replacing NaCl and KCl with the respective methane sulfonate salts and CaCl<sub>2</sub> with calcium gluconate. Tetrodotoxin (Roth, Karlsruhe, FRG) and dantrolene sodium (Norwich Pharmacal Company, Norwich, NY), when added, were at 0.3 and 4 mg/l, respectively. Experimental myotonia was produced by the addition of 0.05 mM anthracene-9-carboxylic acid (9-AC, Aldrich Chemicals Inc., Milwaukee, WI), 1 mM furosemide (Hoechst, Frankfurt/Main, FRG), or 1 mM clofibrac acid (ICI-Pharma, Plankstadt, FRG) to the bathing fluid. These concentrations of furosemide and of clofibrac are somewhat higher than the usual therapeutic concentrations.

Muscle bundles containing about 500 fibers were placed in a shallow chamber kept at 37°C. For contraction experiments, one tendon of the bundle was connected to a quasiisometric semiconductor force transducer (Akers, Horton, Norway). The fibers were stimulated via two chlorided silver wires running along the sides of the bundle. Supramaximal current pulses of 0.2 msec duration were delivered as 200 msec trains at 50 Hz at intervals of at least 5 minutes (to avoid the warm-up phenomenon). The electrical activity of the muscle (EMG) was recorded extracellularly via two platinum wires placed on the surface of the

muscle bundle.<sup>22</sup> The excitability parameters were determined with the 2-microelectrode technique using 120 msec DC pulses;<sup>14</sup> the passive membrane properties and current-voltage relations were measured with the 3-microelectrode voltage-clamp method.<sup>2,17</sup> The results obtained in several fibers from several patients were pooled and expressed as means  $\pm$  SD. The significance of the difference between means was determined with Student's *t*-test.

## RESULTS

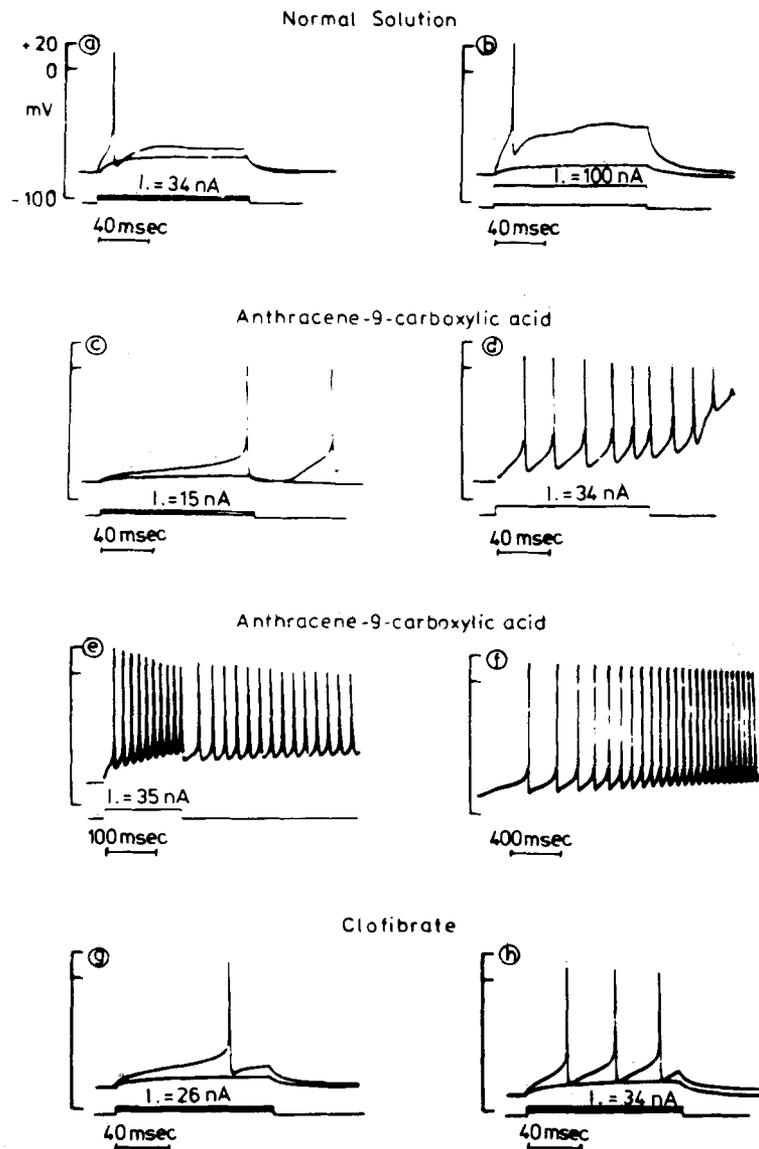
**Induction of Myotonia.** Anthracene-9-carboxylic acid, furosemide, and clofibrac provoked, each in its own manner, myotonia-like activity in the excised human muscle. Isometric contractions recorded from muscle bundles bathed in the standard solution in the absence and in the presence of either of the three drugs, and in the chloride-free solution, are shown in Fig. 1. In each solution the bundles were allowed to equilibrate for at least 15 minutes. Since the time course of a contraction may depend on the amplitude and on the duration of the pulses in a tetanic train, the stimulation parameters were kept constant.<sup>22</sup> In the chloride-free solution (Fig. 1b), in the presence of 0.05 mM anthracene-9-carboxylic acid (Fig. 1c), and in the



**FIGURE 1.** Isometric contractions recorded from different muscle bundles in normal Bretag's solution (a), in chloride-free Bretag's solution (b), and in Bretag's solution containing 0.05 mM anthracene-9-carboxylic acid (c), 1 mM furosemide (d), and 1 mM clofibrac (e). In f 0.05 mM anthracene-9-carboxylic acid was added to the solution bathing the same bundle as in e to show that it was able to generate a typical myotonic response. Note the after-activity when a muscle is in chloride-free solution [EMG trace above force response (b); similar after-activity was recorded with responses c, d, and f].

presence of 1 mM furosemide (Fig. 1d), the relaxation occurred in two phases: a normal fast one and a subsequent slow one that was usually preceded by a plateau or even a small increase in force. The plateau and the slow relaxation phase were associated with electrical after-activity (compare the EMGs in the upper traces of Fig. 1a and b) and resembled the slowed muscle relaxation in clinical myotonia. In the presence of 1 mM clofibrate, there was a small and variable prolongation of relaxation (Fig. 1e). Additional administration of 0.05 mM anthracene-9-carboxylic acid showed that the muscle bundle was capable of myotonia-

like responses (Fig. 1f). In another muscle bundle the application of a high concentration of clofibrate (25 mM) resulted in electrical and mechanical responses that were very similar to those in chloride-free solution (Fig. 1b). All results were reproducible provided the muscles were stimulated once every 5 minutes. With more frequent stimulation, the warm-up phenomenon lead to a disappearance of both the mechanical and electrical myotonia within 8–15 contractions. The contraction pattern usually returned to normal 20–30 minutes after washout of the drugs, showing that the effects were reversible.



**FIGURE 2.** Intracellularly recorded responses to 120 msec constant current pulses in the absence of drugs (a,b), in the presence of 0.05 mM anthracene-9-carboxylic acid (c–e), and in the presence of 1 mM clofibrate (g,h). Trace f shows spontaneous repetitive activity in fibers exposed to 0.05 mM anthracene-9-carboxylic acid.

**Table 1.** Resting potentials (RP), electrical threshold potentials (TP), action potential overshoots (OS), rheobasic currents (I), and latencies (L) of human intercostal muscle fibers at 37°C.

Solution	Fibers (patients)	RP (mV)	TP (mV)	OS (mV)	I (nA)	L (msec)
Bretag's	34 (5)	-82.3 ± 3.4	-58.4 ± 3.4	16.8 ± 4.3	40.7 ± 6.7	11.2 ± 3.0
0.05 mM 9-AC	14 (3)	-81.1 ± 2.5	-57.4 ± 2.9	12.3 ± 4.7	15.3 ± 1.4	100.1 ± 16.5
1 mM furosemide	12 (3)	-83.6 ± 2.4	-59.7 ± 3.3	12.7 ± 2.8	17.3 ± 2.3	97.0 ± 18.3
1 mM clofibrate	10 (2)	-82.7 ± 3.1	-59.4 ± 3.7	13.8 ± 3.7	25.7 ± 4.0	73.4 ± 15.6

Note: In the presence of the drugs, I and L were significantly different from control ( $P < 0.01$ ).

**Excitability Parameters.** These were taken from recordings as shown in Figure 2. Fibers exposed to normal solution mostly responded with a single action potential, even when the long-lasting depolarization provided by the DC pulse was well above the threshold potential (Fig 2a and b). In fibers exposed to anthracene-9-carboxylic acid, the rheobase was much smaller, and the time interval between the onset of the current pulse and the beginning of an action potential (i.e., the latency) was prolonged (Fig. 2c and Table 1). When the depolarizing current was increased, the latency was shortened and additional action potentials were generated (Fig. 2d). At two to three times rheobase the spiking often continued after the termination of the current pulse (Fig. 2e). Sometimes trains of repetitive action potentials appeared spontaneously, perhaps elicited by mechanical noise in the surroundings (Fig. 2f). With 1 mM furosemide, the results were very similar (not illustrated). In fibers exposed to clofibrate, the latency was also increased, and multiple spiking occurred during the flow of stimulating current (Fig. 2g and h), but self-sustained activity after termination of the current pulse was observed only once in 10 experiments. The excitability parameters obtained from these experiments are summarized in Table 1. The threshold and the overshoot of the action potential were not significantly changed in the presence of the drugs.

**Voltage-clamp Experiments.** Table 2 summarizes the passive membrane parameters of the muscle fibers determined before and after application of the drugs. To suppress the electrical activity, all measurements were made in the presence of 0.3 mg/l tetrodotoxin. None of the drugs affected the membrane resting potential, but the specific membrane resistance was significantly increased by each of the drugs; most by anthracene-9-carboxylic acid, least by clofibrate (see Table 2). Measurements in the chloride-free solution showed that this increase was due to a lowered chloride conductance (Table 3). The resting potassium conductance was not significantly changed by either drug.

Steady-state current-voltage relationships are illustrated in Figure 3. The dashed line represents the control current-voltage relationship in Bretag's solution. In the presence of the drugs (see legend for explanation of symbols) the membrane currents were smaller throughout the potential range investigated. The curves for the different drugs were almost indistinguishable from each other and were very similar to the curve obtained in the chloride-free solution (solid line), indicating that the main effect of the drugs is a decrease of the chloride conductance. This conclusion was corroborated when we looked for an effect of anthracene-9-carboxylic acid on the membrane resistance of fibers bathed in the chloride-free solu-

**Table 2.** Resting potentials (RP), calculated fiber diameters ( $d$ ), length constants ( $\lambda$ ) and specific membrane resistances ( $R_m$ ) of human intercostal fibers at 37°C.

Solution	Fibers (patients)	RP (mV)	$d$ ( $\mu\text{m}$ )	$\lambda$ ( $\mu\text{m}$ )	$R_m$ ( $\Omega \cdot \text{cm}^2$ )
Bretag's	64 (8)	-82.3 ± 3.2	48.9 ± 3.8	2367 ± 205	5721 ± 1021
0.05 mM 9-AC	28 (5)	-80.6 ± 2.7	52.5 ± 4.1	4075 ± 630	16417 ± 3534
1 mM furosemide	27 (6)	-84.6 ± 3.6	53.1 ± 5.7	4059 ± 749	15165 ± 3302
1 mM clofibrate	15 (3)	-83.0 ± 3.0	54 ± 5.0	3211 ± 529	9747 ± 1846

Note: In the presence of the drugs,  $\lambda$  and  $R_m$  were significantly different from control ( $P < 0.01$ ).

**Table 3.** Specific membrane conductances ( $g_m$ ) and component conductances ( $g_{Cl}$  and  $g_K$ ) of human intercostal muscle fibers at 37°C.

Solution	Fibers (patients)	$g_m$ ( $\mu\text{S}/\text{cm}^2$ )	$g_{Cl}$ ( $\mu\text{S}/\text{cm}^2$ )	$g_K$ ( $\mu\text{S}/\text{cm}^2$ )	$g_{Cl}$ (%)	$g_K$ (%)
Bretag's	101 (8)	$174.8 \pm 31.2$	$132.7 \pm 23.2$	$42.1 \pm 8.5$	76	24
0.05 mM 9-AC	33 (5)	$60.9 \pm 13.2$	$18.4 \pm 3.9$	$42.5 \pm 7.4$	30	70
1 mM Furosemide	32 (6)	$65.9 \pm 14.3$	$24.2 \pm 4.6$	$41.7 \pm 8.3$	37	63
1 mM Clofibrate	21 (3)	$102.6 \pm 18.9$	$53.2 \pm 6.8$	$49.4 \pm 6.2$	52	48

Note: The last two columns give the component conductances in percent of the total membrane conductance under the assumption that  $g_{Na}$  is negligible. In the presence of the drugs,  $g_m$  and  $g_{Cl}$  were significantly different from control ( $P < 0.01$ ).

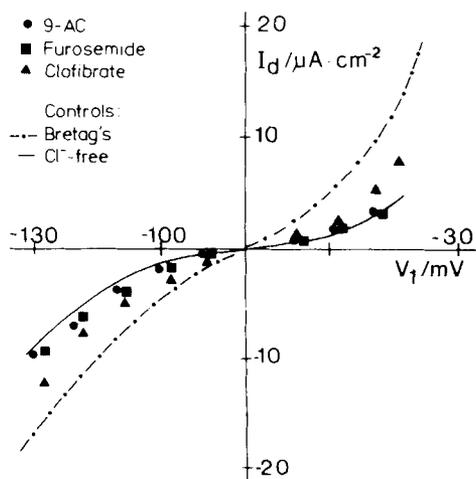
tion. There was no effect of the drug under these conditions.

The membrane current flowing in the potential range negative to the resting potential was slightly larger when the bundles were bathed in the standard solution containing the drugs than in the chloride-free solution. Some chloride current may thus have been flowing in the presence of the drugs. Alternatively, the potassium current may have been increased. To distinguish between these possibilities we performed additional experiments in which the effects of the drugs on the potassium currents were studied. The current through the inwardly rectifying channels was investigated in a chloride-free solution with the potassium concentration increased to 60 mM (with 0.3 mg/liter tetrodotoxin.<sup>15</sup> None of the drugs had any significant effect on the current-voltage relationship. The delayed outward current was

studied in the standard solution, with 0.9 mg/l tetrodotoxin and 4 mg/l dantrolene sodium added, to prevent muscle twitching during depolarizing voltage-clamp pulses. No significant change was detected in the presence of either drug. Thus, our results did not confirm the earlier finding of an increased potassium conductance in the presence of aromatic carboxylic acids.<sup>8</sup>

## DISCUSSION

The experiments showed that the myotonia produced by anthracene-9-carboxylic acid, furosemide, and clofibrate in human skeletal muscle is associated with a lowering of the steady-state chloride conductance of the muscle fiber membrane. A lower than normal chloride conductance has also been found in the Thomsen<sup>18</sup> and Becker<sup>22</sup> types of human myotonia congenita, in paramyotonia congenita with myotonia in warm environment,<sup>17</sup> and in the hereditary myotonia of the goat.<sup>10</sup> Also, the changes of the excitability parameters in the presence of the drugs were in the same direction as those observed in intercostal muscles from myotonic goats.<sup>1</sup> Such similarities strongly support the notion that the pathomechanism of chemically induced myotonia and of hereditary myotonia is the same. However, there are also differences between the results obtained with the drugs and at least one type of hereditary myotonia. The potassium conductance of normal muscles was not affected by the drugs, whereas current-voltage relations of fibers from a patient with recessive generalized myotonia revealed—in addition to a decreased chloride current—an increased potassium current through the inward-going rectifier. This resulted in an N shape of the curve, with a region of negative slope between  $-70$  and  $-55$  mV.<sup>22</sup> For dominant myotonia congenita, the current-voltage relationship of the muscle fiber membrane has not yet been determined. In this disease too, not only the chloride



**FIGURE 3.** Current-voltage relationships recorded in the presence of 0.05 M anthracene-9-carboxylic acid (●), 1 mM furosemide (■), and 1 mM clofibrate (▲) in comparison to the curve measured in chloride-free solution in absence of the drugs (solid line). The dashed line shows the normal current-voltage relation in Bretag's standard solution.

conductance was found to be reduced, but also the potassium conductance seemed abnormal. Surprisingly, the latter was found decreased in Thomsen's disease and increased in the hereditary myotonia of the goat.<sup>18</sup> In particular, the membrane of muscle fibers from myotonic goat is distinguished by a two-fold higher than normal density of the outwardly rectifying potassium channels<sup>10</sup> which is certainly not the case in normal fibers made myotonic by the drugs investigated by us. Another difference concerns the mechanical threshold, which is lowered by anthracene-9-carboxylic acid in fibers from normal goats but is increased in fibers from myotonic goats.<sup>9</sup>

There is a certain structural similarity between anthracene-9-carboxylic acid and furosemide, and this might explain their similar effect on the chloride conductance.<sup>7</sup> Clofibrate was the least effective in producing myotonia, and, at 1 mM, it re-

duced the chloride conductance to just 40% of the control value, which, according to Barchi,<sup>3</sup> is not enough to account for the myotonia-producing electrical instability of the membrane. Indeed, after-activity was almost never observed during intracellular stimulation of fibers in the presence of clofibrate. Nevertheless, on direct tetanic stimulation electrical and mechanical myotonia could be elicited. By analogy to the effect of clofibrate on the cholesterol metabolism, one would expect clofibrate-induced myotonia to resemble diazacholesterol-induced myotonia. For the latter, an effect on the sodium conductance has been held essential.<sup>4</sup> Considering the normal threshold and overshoot of the action potentials measured in its presence, our results do not suggest that clofibrate affects the sodium current. It has already been pointed out that the biochemical basis of clofibrate-induced myotonia is different from that of diazacholesterol-induced myotonia.<sup>19</sup>

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