did not acount for the asymmetrical relationship between conjugate branch pairs. Our observation of a self-similarity ratio for conjugate tube lengths also complements descriptions of the fractal architecture of the lung which implies a scaling relationship from one generation of tubes to the next[2,8,9].

Fibonacci scaling has been observed in multiple other biological systems and anatomic relationships[10–12]. For example, the historical observation that the ratio of the total height in humans to the vertical height of the navel approximates the golden mean has been verified in a systematic study[13]. From a morphogenetic viewpoint, the generation of complex, irregular structures based in part on principles of fractal self-similarity[9] and Fibonacci proportionality may serve to minimize constructional error[3]. How the information which regulates this type of stable growth pattern is encoded and processed are major unanswered questions. From an evolutionary point of view such scaling mechanisms are also of interest because they lead to complex anatomic structures without apparent recourse to natural selection. The relevance of natural selection to organismic complexity is a current area of investigation and debate[14]. It remains to be seen whether Fibonacci geometry of the lung will generalize to enhance the understanding of other irregular branching networks, as seen in the nervous system, the vascular tree, the biliary ducts, and the His-Purkinje network.

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Frequency-dependent effect of verapamil on rat soleus muscle

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Summary. In the presence of verapamil (0.1 mM) rat soleus muscle fibers failed to generate action potentials with overshoots. In fibers with their V_m set to a local level of −90 mV, verapamil produces a gradual reduction in the amplitude of the repetitive action potentials; this effect is more pronounced at high rates of stimulation (100 Hz). Our results suggest a local anesthetic action of this drug that could contribute with its calcium channel blocking effect to the diminished mechanical tension observed in the presence of the drug.

Key words. Rat skeletal muscle; verapamil; frequency-dependent effect; mechanical activity; electrical activity.

There are several reports that suggest the necessity for the participation of external calcium ions in activation of mammalian skeletal muscle[2–6]. Recently, we reported a decrease in the twitch and tetanus tension of rat soleus muscle exposed to verapamil[4]; the effect of this calcium channel blocker on the mechanical activity is more pronounced when the muscle is repetitively stimulated. Several hypotheses have been suggested to explain this phenomenon (frequency-dependent or use-dependent effect)[7–10]. In addition, verapamil does not have any significant effect on caffeine contracture tension[4].

It has been suggested that many of these drugs have an anesthetic effect besides their calcium channel blocker effect[11–13]. Verapamil (0.1 mM) does not hinder the generation of single action potentials (APs) recorded in hyperpolarized rat soleus muscle fibers[4] but there are no data available on the iterative generation of APs in the presence of this drug. So it seemed to be of interest to find out whether verapamil would modify the electrical activity elicited at low and high frequencies of stimulation.

Material and methods. Experiments were performed in rat (Wistar strain) soleus muscle in vitro at 32 °C. APs were generated and recorded using a double microelectrode technique. The fiber was impaled with two microelectrodes (6–15 MΩ, 3 M KCl); a recording and a stimulating electrode (inserted at about 100 µm from one another). In some cases, the fiber was hyperpolarized to −90 mV with anodal current through the stimulating electrode, 5–10 s before a depolarizing pulse of 2–3 ms duration via a WPI electrometer. The action potential and its

Action potential characteristics in control and verapamil (0.1 mM) solutions

	Control (n: 39) without hyperpolarization	with hyperpolarization	Verapamil (n: 41) with hyperpolarization
Overshoot (mV)	10.9 ± 5.4	16.5 ± 5.1	11.6 ± 5.0
Duration (ms)	0.9 ± 0.2	0.8 ± 0.2	1.2 ± 0.3
+ dV/dt (V/s)	203 ± 64	309 ± 40	267 ± 26
− dV/dt (V/s)	140 ± 37	157 ± 37	95 ± 19

Values are mean ± SD. The action potentials were measured in fibers with their normal resting membrane potential (without hyperpolarization) and hyperpolarized to − 90 mV (see methods) previous to depolarizing pulse. The duration of the AP was measured at − 40 mV. + dV/dt: maximum rate of rise; − dV/dt: maximum rate of fall.

first derivative were displayed on the oscilloscope. In those experiments in which the fiber was repetitively stimulated (3–5–100 Hz), dantrolene sodium (5 mg/l) was added to the solution to reduce movement artifacts to a minimum. No effect of Dantrolene on the APs of mammalian skeletal muscle was found[14]. The isometric twitch and tetanus (100 Hz, 500 ms) were generated through massive stimulation (0.3 msec pulse duration) and recorded through a Grass force transducer on a Grass poligraph. D-Tubocurarine (10^{-5} g/ml) was added to normal Ringer solution. The normal Ringer solution contained (mM) NaCl 135, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, Na_2HPO_4 1, $NaHCO_3$ 20, glucose 11. The pH was 7.2–7.4 (bubbled with 95% O_2 and 5% CO_2). Verapamil (0.1 mM, Knoll Pharmaceutical Co.) was added to normal solution. The values given are means ±SD.

Results. Figure 1A shows mechanical responses (twitch and tetanus) obtained in normal Ringer solution; there is no modification in the twitch tension of slow muscle stimulated at low frequency or following tetanic stimulation. The tetanus/twitch ratio in normal solutions is 5.7 ± 1.1 (n: 21). Figure 1B shows the same type of response when verapamil (0.1 mM) is present (30–50 min of equilibration). As can be seen there is a reduction in the twitch and tetanus tension and a progressive decline in the successive twitches when the muscle is stimulated at 3 and 5 Hz. The amplitude of the 20th twitch in comparison with the first twitch was 0.66 ± 0.1 and 0.38 ± 0.1 for 3 and 5 Hz respectively (4 experiments). The control values for these frequencies were very close to 1: 0.96 ± 0.1 and 0.99 ± 0.2 respectively (10 experiments). The effect of the drug on the tetanus response is more manifest than on the twitch tension; the tetanus/twitch ratio measured at the time of eliciting the tetanic response was 2.6 ± 0.4 (n: 7). When the stimulation was interrupted for several minutes the amplitude of the twitch began to increase although it remained below the control values.

Reversal of the effects of verapamil on the mechanical activity by washing out the drug was partial and slow; a significant effect observed after 3 h is a smaller decrement of the successive twitches at 3 and 5 Hz (fig. 1C); the tetanus response remains reduced relative to the control values.

In normal Ringer solution the AP generation is reduced in fibers with low V_m values. In order to minimize the effect of variations in the V_m the potential of the fibers was set to a local level of −90 mV (see methods). The maximum rate of rise as well as the overshoot of the AP increased in the hyperpolarized fibers in comparison with the values obtained in fibers with normal V_m (fig. 2, table). Verapamil (0.1 mM) had no significant effect on the V_m: control: −65.2 ± 3.3 mV (39 fibers), verapamil: − 64.9 ± 4.3 mV (n: 41 fibers). The APs were recorded in fibers which were locally hyperpolarized (fig. 2C) but in the absence of this procedure the depolarizing pulse usually evoked a slow response without overshoot (fig. 2D).

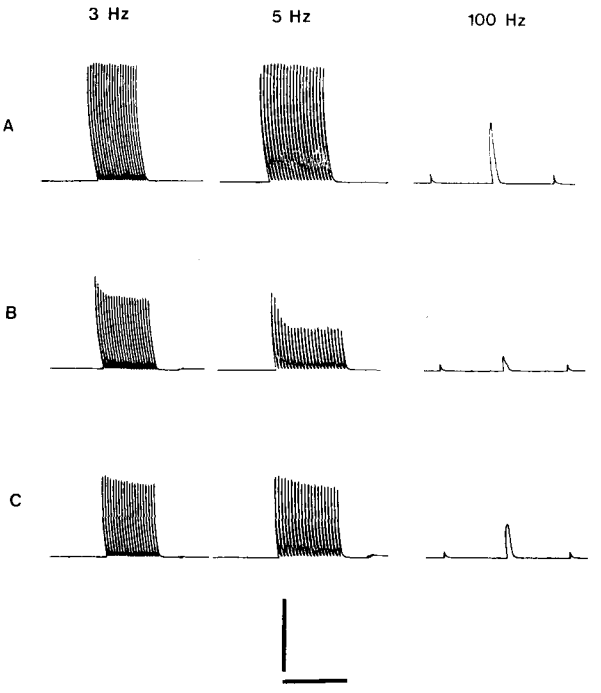

Figure 1. Repetitive mechanical activity in soleus muscle in normal solution (A), in the presence of 0.1 mM verapamil (B) and after 3 h of washout of the drug (C). Horizontal calibration: 4 s (left and central columns), 8 s (right column). Vertical calibration: 5 g (left and central columns), 50 g (right column).

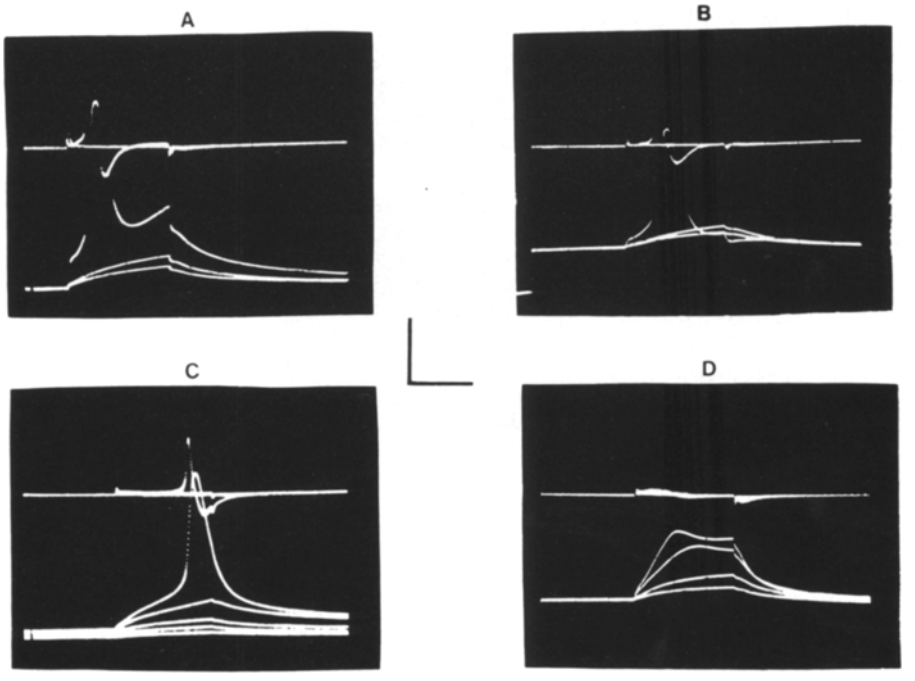

Figure 2. Action potentials and their first derivative (upper trace) generated in normal Ringer solution (A, B) and in the presence of 0.1 mM verapamil (C, D). In panels A and C the fibers were hyperpolarized to − 90 mV previously to the depolarizing pulse. Horizontal calibration: 2 ms. Vertical calibration: 40 mV, 400 V/s.

Figure 3 depicts APs evoked at different frequencies of stimulation in the presence of verapamil (0.1 mM, 50 min of equilibration). There is a small and gradual reduction in the amplitude of the APs at 3 and 5 Hz but during the whole time of the stimulation most of the fibers gave APs with overshoots (fig. 3, A and B).

The ratio of the 20th overshoot/1st overshoot elicited at 3 and 5 Hz in the presence of verapamil was 0.65 ± 0.2 and 0.69 ± 0.2 respectively (18 fibers).

Figure 3 C and D shows trains of APs generated at 50 and 100 Hz (100 ms duration). At 100 Hz the amplitude of the evoked APs declines so rapidly, that after 50 ms after the beginning of the stimulation the APs did not have overshoots and remained under the zero line of potential. The ratio of the amplitude of the 10th AP/1st AP was 0.48 ± 0.1 (18 fibers). There is no modification in the amplitude of the APs in normal saline at low or high frequencies of stimulation; figure 3 F is a typical response of a fiber at 100 Hz after 1 h of equilibration in normal Ringer solution.

We also tested the effect of withdrawal of verapamil on the APs. Figure 3 E shows a train of APs at 100 Hz after 60 min of washout of the drug; a significant recuperation in the amplitude of the APs can be seen. The faster recuperation in the amplitude of the APs in comparison with the mechanical activity after the washout of the drug could be explained by the different methodologies employed; the APs were generated in the surface of the muscle whereas the mechanical activity output represents the sum of the whole muscle fibers where diffusion of the drug from deep fibers may be retarded.

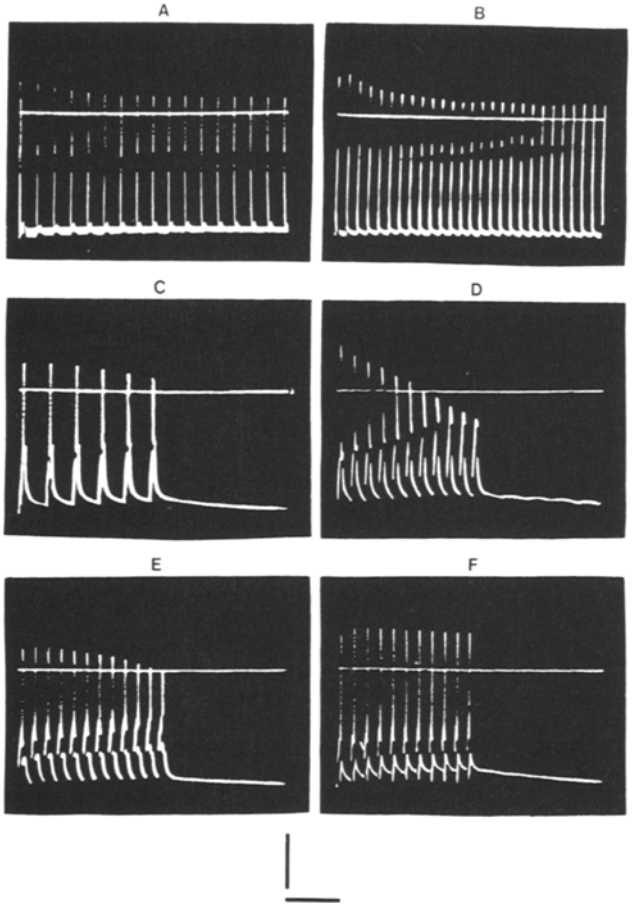

Figure 3. Action potentials generated in hyperpolarized fibers at different frequencies: 3 Hz (A), 5 Hz (B), 50 Hz (C), 100 Hz (D) in the presence of verapamil (0.1 mM). Panel E shows a train of APs (100 Hz) elicited 60 min after the washout of the drug. Panel F shows a train of APs (100 Hz) in normal solution. Horizontal calibration: 40 ms (C, D, E, F), 1 s (A, B). Vertical calibration: 40 mV.

Discussion. Two interpretations may be suggested to explain our results. The first one is to consider the local anesthetic effect of verapamil which has been reported in other preparations[11–13]. We have found that 0.1 mM verapamil does not affect the generation of single APs in hyperpolarized fibers although the responses are smaller and slower than in the control solution. When the muscle is repetitively stimulated in the presence of verapamil there is a reduction in the amplitude of the successive APs, this effect being more pronounced at high frequencies: Frequency-dependent effect[7–10]. In this regard, it was demonstrated in nerve fibers that local anesthetic agents act intracellularly and exhibit both frequency and voltage features[15]. The action of verapamil became more appreciable in fibers which were not previously hyperpolarized with anodal current; in these fibers the depolarizing pulse triggered a response without overshoots.

These responses with a very slow rate of rise are not totally ineffective since twitches are reduced but not abolished in the presence of the drug. However, we do not have data about the generation of APs in the presence of verapamil in deep fibers. The second mechanism of action could be the well known calcium channel blocker effect of verapamil. Eisenberg et al.[16] have proposed several sites of action for D 600, a drug related to verapamil; a calcium channel in the T-tubule and in the SR and charged molecules in the T-Tubule membranes essential for the EC coupling process. Recently, an inward current carried by calcium ions and blocked by several types of drugs was demonstrated in mammalian skeletal muscle[17,18]. This calcium current is fast enough to represent an entry mechanism for calcium as a consequence of an AP[17], and it might play a role in the mechanical activation of mammalian skeletal muscle. It may be possible that both mechanism, the local anesthetic effect and the inhibition of a calcium current are responsible for the mechanical changes observed by us. It should be mentioned that this is merely a first investigation of the problem as far as the technique employed in our experiments is concerned. Voltage clamp analysis in isolated muscle fibers will be needed to clarify this point.

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