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Modulation of the Ca^{2+} release channel of sarcoplasmic reticulum by amiloride analogs

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Abstract

Dichlorobenzamil, phenamil and other amiloride analogs (1–100 μM) elicit transient tension in rabbit skinned muscle fibers. Tension requires preloading of Ca^{2+} into the sarcoplasmic reticulum, is facilitated by low-[Mg^{2+}] solutions, abolished by ruthenium red or by functional disruption of the sarcoplasmic reticulum, and is followed by inhibition of the caffeine-evoked tension. Bilayer recording of Cs^+ currents through the sarcoplasmic reticulum Ca^{2+} release channel reveals that phenamil (10–100 μM) increases the open channel probability, whereas dichlorobenzamil affects the channel activity in a complex concentration- and time-dependent manner: stimulation occurs throughout exposure to 10 μM, but is followed by channel blockade when 100 μM dichlorobenzamil is used. It is concluded that stimulation of the sarcoplasmic reticulum Ca^{2+} release channel accounts for the dichlorobenzamil- or phenamil-induced tension in skinned fibers, whereas depletion of sarcoplasmic reticulum Ca^{2+} stores and channel block (with dichlorobenzamil) explains the inhibition of the caffeine-evoked tension by amiloride analogs.

Keywords: Ryanodine receptor; Skinned fiber; Dichlorobenzamil; Phenamil; Caffeine

1. Introduction

Amiloride and various structurally related analogs have been extensively used as probes for a wide variety of transport systems (Kleyman and Cragoe, 1988). Notably, terminal guanidino-nitrogen derivatives block epithelial Na^+ channels, while analogs substituted at the five-amino group of the pyrazine ring inhibit Na^+–H^+ exchange. Both structural classes, as well as molecules disubstituted at these two positions, are also effective inhibitors of Na^+–Ca^{2+} exchange (Kaczorowski et al., 1985, 1989). Amiloride analogs have been used to elucidate the physiological role of Na^+–H^+ or Na^+–Ca^{2+} exchange in controlling ionic homeostasis (Lazdunski et al., 1985; Grinstein et al., 1986; Levitsky et al., 1998; Matalon and O'Brodovich, 1999) mitogenesis (L'Allemain et al., 1984) and stimulus–secretion coupling (Sweatt et al., 1985) in intact cells and tissues. However, interpretation of the results of these studies is often complicated by the demonstrated ability of amiloride derivatives to block both L-type and T-type voltage-dependent Ca^{2+} channels (Suarez-Kurtz and Kaczorowski, 1988; Tang et al., 1988; Garcia et al., 1990). Since Ca^{2+} channels are distributed in a wide variety of cell types, and Ca^{2+} plays such a fundamental role in many different cellular processes, the effects reported with amiloride analogs might be related to Ca^{2+} channel blockade rather than to the Na^+-transport system being investigated. For example, the negative inotropic effect of 3,4-dichlorobenzamil (dichlorobenzamil) — one of the amiloride derivatives examined in the present study — could be a consequence of Ca^{2+} channel block (Suarez-Kurtz and Kaczorowski, 1988; Suarez-Kurtz et al., 1988; Garcia et al., 1990) either alone or in addition to inhibition of Na^+–Ca^{2+} exchange, as originally postulated (Siegl et al., 1984).

In the present study, single channel recordings from lipid bilayer membranes and isometric tension measurements from skinned muscle fibers were used to investigate the effects of dichlorobenzamil and other amiloride analogs on the Ca^{2+} release channel of skeletal muscle sarco-

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plasmic reticulum. This channel binds the plant alkaloid ryanodine with high affinity and specificity and hence is also known as the ryanodine receptor (for reviews see Franzini-Armstrong and Protasi, 1997; Sutko et al., 1998). The ryanodine receptor is an essential component of excitation–contraction coupling in skeletal muscle fibers, whereby depolarization across the transverse tubule membrane leads to the rapid release of sarcoplasmic reticulum-stored Ca^{2+} into the myoplasm. The Ca^{2+} release channel is a large conductance pore, with a linear conductance of ~100 pS for Ca^{2+} and 400–700 pS for monovalent cations, such as K^+ or Cs^+ (Lai et al., 1988; Smith et al., 1988), and its activity is modulated by endogenous effectors, notably Ca^{2+}, Mg^{2+} and ATP, and by several exogenous substances (reviewed by Zucchi and Ronca-Testoni, 1997). The effects of the latter are often complex and/or not completely clarified, and several exogenous modulators of the ryanodine receptor channel are capable of both stimulating and inhibiting Ca^{2+} release, depending on drug concentrations, incubation time and other experimental variables. The present study identifies a novel group of modulators of the ryanodine receptor, namely amiloride analogs, and thoroughly investigates their mechanisms of action. Preliminary results have appeared in abstract form (Estrela et al., 1998).

2. Materials and methods

2.1. Materials

Dichlorobenzamil and benzamyl were kindly provided by Dr. G.J. Kaczorowski from Merck Sharp and Dohme Research Laboratories, USA, phenamil methanesulfonate was purchased from Research Biochemicals International (Natick, MA, USA), while the other amiloride derivatives used, namely 5-*N*-ethyl-*N*-isopropyl amiloride and 5-*N*-methyl-*N*-isobutyl amiloride, were gifts from Prof. G. Malnic, Universidade de São Paulo, Brazil. Caffeine and ruthenium red were purchased from Sigma (St. Louis, MO, USA), while 1-palmytoil-2-oleoylphosphatidylcholine (POPC) and 1-palmytoil-2-oleoylphosphatidyl-ethanolamine (POPE) were from Avanti Polar Lipids (Alabaster, AL, USA). All other reagents were of high-purity grade.

2.2. Skinned fiber experiments

2.2.1. Skinned fibers

Small bundles of fibers were dissected from rabbit psoas muscle, chemically skinned by soaking in EGTA-containing ''skinning'' solution (see below) and stored at −20°C in the presence of glycerol until use (Eastwood et al., 1979). This procedure eliminates the sarcolemma as a diffusion barrier to large solutes, making it possible to modify the composition of the medium bathing the myofibrils and the sarcoplasmic reticulum membranes. On the day of the experiment, a segment of a single fiber was isolated and mounted in an experimental chamber containing 0.5 ml of skinning solution and provided with continuous stirring and temperature control. The temperature of the bathing medium was maintained at 20 ± 1°C.

For recording isometric tension, one end of the fiber was attached to a clamp connected to a force-displacement transducer (FT-03C, Grass Instruments, Quincy, MA, USA) and the other end was fixed to a hook connected to a micromanipulator, which was used to stretch the fiber to about 120% of its slack length. The transducer signals were amplified and recorded on a Gould polygraph (2400S, Gould, Cleveland, OH, USA). Integration of the area under tension curves was performed with a Grass integrator (7P122).

2.2.2. Solutions

The composition of all solutions was calculated using the computer program developed by Fabiato (1988) and the constants given by Fabiato and Fabiato (1979). The association constants for CaEGTA and MgATP were those reported by Orentlicher et al. (1977). 3-(*N*-morpholino) propanesulfonic acid (MOPS) was used as pH buffer (pH 7.0). The control bathing medium (''washing solution'', designated W) had the following composition (in mM): 185 K propionate, 2.5 K_2Na_2ATP, 2.5 Mg^{2+} acetate and 10 MOPS; 50 μM K_2 EGTA was added to this solution to reduce the concentration of contaminating Ca^{2+}. The calculated concentrations of free ATP ([ATP]) and [Mg^{2+}] in this solution were 323 and 585 μM, respectively. In one series of experiments (Fig. 4), the concentrations of K_2Na_2ATP and Mg^{2+} acetate in solution W were modified in order to provide 1000 μM [ATP] and 50–200 μM [Mg^{2+}]; these are designated as low-[Mg^{2+}] solutions. The skinning solution and the ''relaxing'' solution (designated R) had the same composition (in mM): 170 K propionate, 2.5 K_2Na_2ATP, 2.5 Mg^{2+} acetate, 5 K_2EGTA and 10 MOPS. Solutions used to activate the myofibrils directly (''activating solution'' of pCa^{2+} 4.5) or to load Ca^{2+} into the sarcoplasmic reticulum (''loading solutions'' of pCa^{2+} 6.5 or 7.0) were prepared by partially replacing K_2EGTA in the relaxing solution with CaK_2EGTA, to obtain different ratios of K_2EGTA/CaK_2EGTA, while keeping the total [EGTA] constant at 5 mM.

2.2.3. Experimental protocols

At the beginning and at various times during each experiment, T_{max}, the maximum contractile response of the fiber when exposed to *p*Ca 4.5, was recorded. Whenever T_{max} declined by 20% or more of its initial value, the experiment was terminated.

2.2.3.1. Ca^{2+} uptake by the sarcoplasmic reticulum. The procedures used to load Ca^{2+} into the sarcoplasmic reticulum have been previously described in detail (Suarez-Kurtz et al., 1995; Vianna-Jorge et al., 1998). Briefly, the fiber

was initially exposed to 20 mM caffeine in solution R, to deplete sarcoplasmic reticulum Ca^{2+} stores. After two washes with W, the fiber was soaked in a loading solution of *p*Ca 6.5 or 7.0, for 1 min; Ca^{2+} loading was terminated by returning the fiber to solution W. Cumulated data from our laboratory ($N > 300$ rabbit skinned fibers) indicate that 1-min soaking in *p*Ca 6.5 allows maximum Ca^{2+} loading of the sarcoplasmic reticulum, as evaluated by the area under the caffeine-induced tension curve. When the *p*Ca of the loading solution is 7.0, the area under the caffeine-induced tension curve averages 56% of the value obtained in the same fibers ($N = 21$) following loading at *p*Ca 6.5. The expressions ''maximally loaded'' and ''submaximally loaded'' are used below to refer to fibers loaded for 1 min at *p*Ca 6.5 or 7.0, respectively.

2.2.3.2. Release of sarcoplasmic reticulum-stored Ca^{2+}. After Ca^{2+} was loaded into the sarcoplasmic reticulum, as described above, release of the stored Ca^{2+} was induced by caffeine (20 mM in solution W), low-[Mg^{2+}] solutions and/or one of the amiloride analogs studied.

2.2.3.3. Functional disruption of the sarcoplasmic reticulum. Incubation with the nonionic detergent Brij-58 (2% w/v in solution R for 10 min) was used to disrupt the sarcoplasmic reticulum membranes, while preserving the ability of the skinned fibers to contract when challenged with Ca^{2+} (Orentlicher et al., 1974). The damage to the sarcoplasmic reticulum membranes was confirmed by elimination of the caffeine-evoked tension after treatment with Brij-58.

2.2.3.4. Blockade of the sarcoplasmic reticulum Ca^{2+} release channel. Irreversible blockade of the sarcoplasmic reticulum Ca^{2+} channels was obtained by soaking the skinned fiber in solution R containing 5 mM ruthenium red for 10 min (Suarez-Kurtz et al., 1995).

Data from pooled experiments with skinned fibers are presented as means ± S.E.M. Student's *t*-test was used for statistical analysis of the data. The level of significance was set at $P < 0.05$.

2.3. Electrophysiological experiments

Heavy sarcoplasmic reticulum membrane fractions from rabbit leg and back muscles were prepared as described previously (Suarez-Kurtz, 1994; Catinot et al., 1997). The membrane fractions were stored at −70°C until used in the bilayer experiments. Planar lipid bilayers were prepared from a solution of POPE and POPC, in a 7:3 molar ratio in decane at 50 mg/ml, painted across a 250-μm hole in a partition separating two aqueous compartments. Membranes were added to the *cis* (cytosolic) solution, which was composed of 250 mM Cs methanesulfonate and 10 mM MOPS, titrated with CsOH to pH 7.4. The *trans* (luminal) solution was identical, except during fusion of the vesicles with the bilayers, when the Cs methanesulfonate concentration was 50 mM. The use of Cs^+ rather than Ca^{2+} as a current carrier avoids the build up of large Ca^{2+} gradients near the mouth of the channel, which affects channel gating (Xu and Meissner, 1998) and, in addition, virtually blocks the conductance of other sarcoplasmic reticulum cation channels (Cukierman et al., 1985). Methanesulfonate prevents current flow through the sarcoplasmic reticulum Cl^- channel, thus allowing isolation of the sarcoplasmic reticulum Ca^{2+} release channel currents. All experiments were carried out at room temperature (22 ± 2°C), and EGTA (1 mM) was used to buffer the Ca^{2+} concentration of the *cis* compartment at *p*Ca 5.0. Cs^+ currents flowing across the sarcoplasmic reticulum Ca^{2+} release channels were measured with a Bilayer Clamp BC-525C amplifier (Warner Instruments, Hamden, CT, USA), membrane potentials being referenced to the *trans* compartment. The holding potential was −35 mV. The current recordings were filtered at 1 kHz and digitized at a sampling frequency of 5 kHz. Data acquisition and analysis were accomplished with a software package (pCLAMP 6, Axon Instruments, Burlington, CA, USA) running on a 486 PC-compatible computer. Open probability and lifetimes of open and closed events were determined from several continuous recording segments, each of 1–10 s duration.

3. Results

3.1. Skinned fiber experiments

3.1.1. Amiloride analogs elicit tension in maximally loaded skinned fibers

Fig. 1 depicts the protocol used to investigate the effects of the amiloride analogs on the functional properties of the sarcoplasmic reticulum Ca^{2+} release channel of rabbit skinned fibers and shows representative results obtained with dichlorobenzamil in Ca^{2+}-loaded fibers. Addition of dichlorobenzamil (> 10 μM) to solution W, at the end of the Ca^{2+}-loading period, induced tension in maximally loaded (Fig. 1D–F), but not in submaximally loaded fibers (Fig. 1H). The time course of the dichlorobenzamil-induced tension was similar to that of the caffeine-evoked tension (Fig. 1B), displaying an initial fast rate of rise, leading to peak amplitude within 5 s, followed by a return to baseline tension in 15–35 s. A short (< 10 s) delay before the onset of tension development and/or tension oscillations was sometimes observed in maximally loaded fibers exposed to dichlorobenzamil (Fig. 1D–F).

The peak amplitude of the dichlorobenzamil-induced tension reached 80–100% of the maximum caffeine-evoked tension, and showed little difference among effective concentrations. Thus, increasing the concentration of dichlorobenzamil above that minimally required for inducing tension did not augment, and may have actually slightly

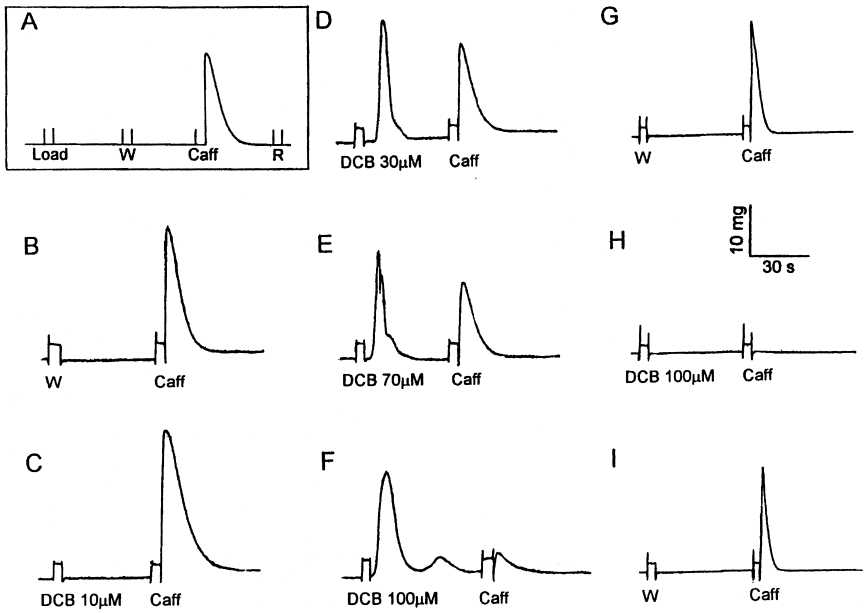

Fig. 1. Dichlorobenzamil induces tension per se and modulates the caffeine-induced tension in Ca^{2+}-loaded skinned muscle fibers. Tension recordings from two (B–F, G–I) fibers, studied with the protocol depicted in panel A, which consisted of exposing the fiber for successive 1-min periods to a loading solution of *p*Ca 6.5 or 7.0 (Load), washing solution (W) and then to 20 mM caffeine in solution W (Caff). This was followed by a brief exposure to solution R, and after two washes with W, a new loading cycle was initiated. The *p*Ca of the loading solution was 6.5 for one fiber (B–F) and 7.0 for the other (G–I). Dichlorobenzamil was added to solution W at the end of the loading period and remained in the bath throughout the challenge with caffeine. Further description in the text.

reduced the peak tension (Fig. 1D–F). However, fibers varied in their sensitivity to the tension-inducing effect of dichlorobenzamil or the other amiloride analogs studied, as shown in Fig. 2. The data are plotted as the percentage of maximally loaded fibers that developed detectable tension ($> 5\%$ T_{max}) when exposed to 10, 30 or 100 μM concentrations of the amiloride derivatives. Amiloride and 5-*N*-methyl-*N*-isobutyl amiloride failed to elicit tension, while the effectiveness of the other analogs increased in the sequence phenamil < 5-*N*-ethyl-*N*-isopropyl amiloride < benzamil = dichlorobenzamil. The time course and amplitude of the tension elicited by these analogs were in all respects similar to that elicited by dichlorobenzamil, as illustrated in Fig. 2C for phenamil. The latter compound and dichlorobenzamil were selected for use in the experiments described below.

3.1.2. Effects of ruthenium red and of Brij-58 on the tension induced by dichlorobenzamil or phenamil

The observation that dichlorobenzamil or phenamil elicited tension in maximally loaded, but not in submaximally loaded, skinned fibers (Figs. 1 and 2) points to the sarcoplasmic reticulum as the source of Ca^{2+} which activates the contractile proteins. Accordingly, the tension elicited by dichlorobenzamil or phenamil was abolished by pretreatment of the fibers (Methods) with either ruthenium red, which irreversibly blocks the sarcoplasmic reticulum Ca^{2+} release channel, or with the nonionic detergent Brij-58, which functionally disrupts the sarcoplasmic reticulum membrane (data not shown).

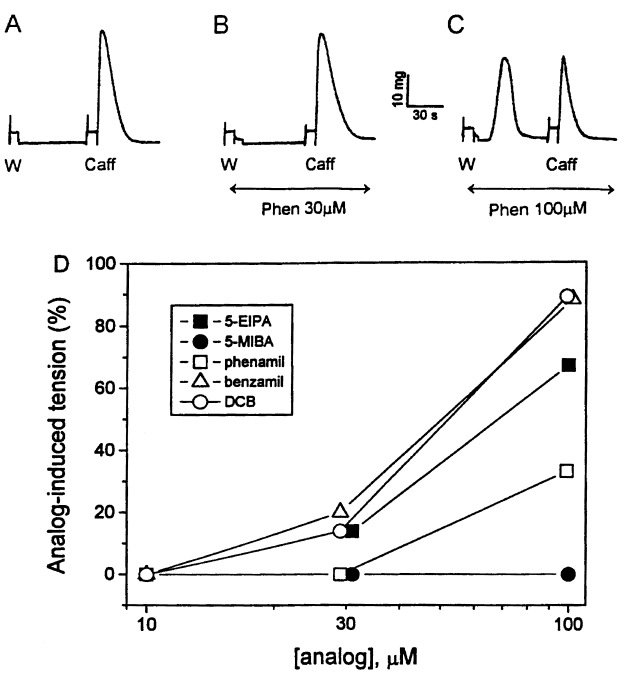

Fig. 2. (A–C) Phenamil induces tension per se and modulates the caffeine-induced tension in Ca^{2+}-loaded skinned muscle fibers. Tension recordings from a single fiber, studied with the protocol depicted in Fig. 1A. Phenamil was added to the bathing media during the periods indicated by the double-arrow lines. Plot: Quantal concentration–effect curve for the tension induced by amiloride analogs in Ca^{2+}-loaded skinned fibers. Frequency is expressed as the percentage of the number of experiments in which a given concentration of each analog elicited detectable tension ($> 5\%$ of T_{max}) in maximally loaded fibers. 5-EIPA, 5-*N*-ethyl-*N*-isopropyl amiloride; 5-MIBA, 5-*N*-methyl-*N*-isobutyl amiloride.

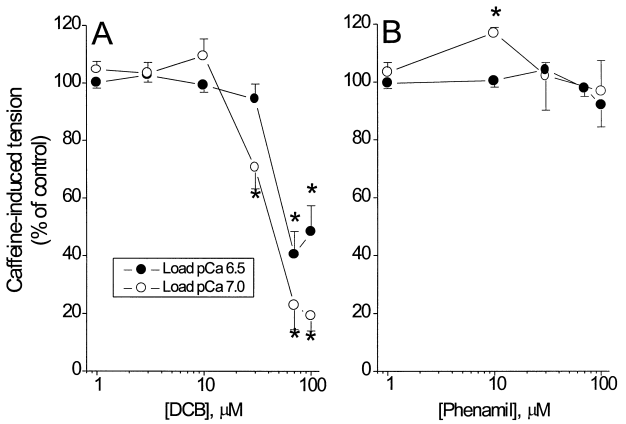

Fig. 3. Effects of dichlorobenzamil (A) or phenamil (B) on the caffeine-induced tension in skinned fibers. Pooled data from several experiments performed under the protocol depicted in Fig. 1A were used to construct the concentration–response curves for maximally (closed circles) or submaximally loaded fibers (open circles). The area under the caffeine-evoked tension curves expressed as a percentage of the control response to caffeine (in the absence of dichlorobenzamil or phenamil), is plotted against the concentration of each amiloride analog. $^{*}P < 0.05$ between the dichlorobenzamil- or phenamil-modified response and the respective control caffeine-evoked tension.

3.1.3. Pharmacological interaction between dichlorobenzamil or phenamil and caffeine

The experimental protocol depicted in Fig 1 was used to investigate the effects of dichlorobenzamil or phenamil on the caffeine-induced tension, and pooled data from several experiments are plotted in Fig. 3. Pre-exposure to dichlorobenzamil reduced, in a concentration-dependent and reversible manner, the caffeine-induced tension. Statistically significant inhibition was observed with > 30 μM dichlorobenzamil in submaximally loaded fibers, but higher drug concentrations (70 and 100 μM) were required for the same effect in maximally loaded fibers (Fig. 3A). In contrast to dichlorobenzamil, phenamil (1–100 μM) had no significant effect on the magnitude of the caffeine-induced tension in six maximally loaded fibers (Fig 3B); however, in the two fibers, which developed tension when challenged with 100 μM phenamil, the contractile response elicited subsequently by caffeine was reduced by 16% (Fig. 2C) and by 41%, relative to their respective control values. Phenamil (10 μM) increased by 17%, on average, the magnitude of the caffeine-induced tension in submaximally loaded skinned fibers ($P < 0.05$); this effect was not seen with other phenamil concentrations (1, 30 and 100 μM; Fig. 3B). A small (9%), but not statistically significant increase in the caffeine-induced tension in submaximally loaded fibers was also observed in the presence of 10 μM dichlorobenzamil (Fig. 3A).

3.1.4. Effects of low-[Mg^{2+}] solution on the dichlorobenzamil- or phenamil-induced tension

Ca^{2+}-loaded skinned fibers developed tension when [Mg^{2+}] in solution W was sufficiently reduced (50–200 μM) to prevent Mg^{2+} from opposing the stimulatory effect of ATP on the sarcoplasmic reticulum Ca^{2+} release channels (Reuben et al., 1975). Exposure of maximally loaded fibers to low-[Mg^{2+}] solutions provides, therefore, an experimental paradigm for facilitating the release of sarcoplasmic reticulum-stored Ca^{2+}, and was used to explore the mechanism of dichlorobenzamil- or phenamil-induced tension. In the representative experiments shown in Fig. 4, the two fibers remained quiescent during 1-min exposure to 100 μM [Mg^{2+}], but contracted vigorously when dichlorobenzamil (3 or 30 μM) or phenamil (10 μM) was added to the medium. These two fibers, however, failed to contract when challenged with the same concentrations of dichlorobenzamil or phenamil while bathed in normal solution W (not shown). Tension oscillations were sometimes observed during exposure of maxi-

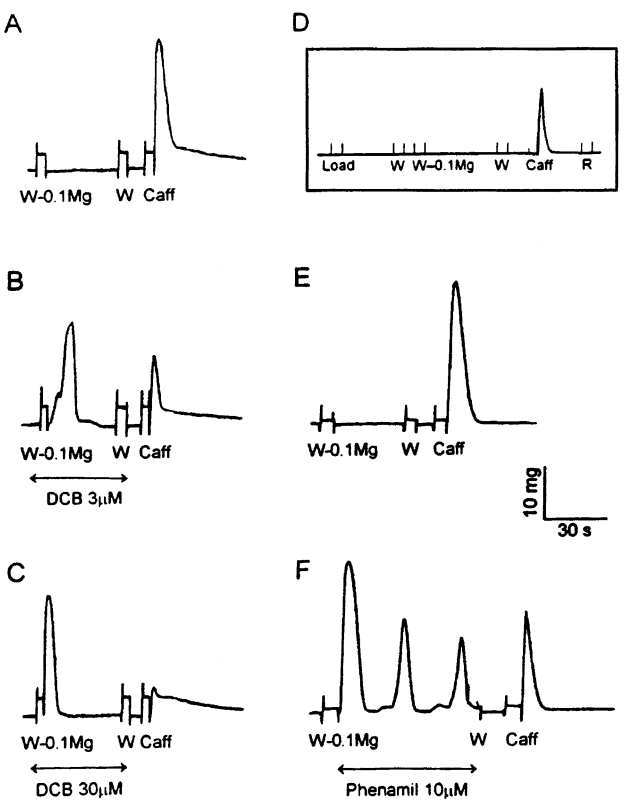

Fig. 4. Low [Mg^{2+}] facilitates the dichlorobenzamil- or phenamil-induced tension in maximally loaded skinned fibers. Tension recordings from two fibers (A–C, E–F) studied with the protocol depicted in panel D, which consisted of the following steps: 1-min soaking in loading solution of *p*Ca 6.5 (Load); a brief rinse with washing solution W; 1-min soaking in solution W containing 0.1 mM [Mg^{2+}]; a brief rinse with washing solution W; exposure to 20 mM caffeine in solution W (Caff). This was followed by a rinse with solution R, and after two washes with W, a new loading cycle was initiated. The recordings shown in B–F began immediately before the fiber was transferred to the low-[Mg^{2+}] solution and ended after the 30- to 45-s exposure to caffeine. Dichlorobenzamil or phenamil was added to the bathing media during the periods indicated by the double-arrow lines. Further description in the text.

mally loaded fibers to dichlorobenzamil or phenamil in low-[Mg^{2+}], solutions (Fig. 4F). Pooled data from six experiments using the same protocol as in Fig. 4 revealed 3- to 10-fold reductions in the minimal concentrations of dichlorobenzamil or phenamil required for inducing tension in maximally loaded fibers bathed in low-[Mg^{2+}], as compared to normal solution W. An additional observation in this series of experiments was that fibers which contracted when challenged with dichlorobenzamil or phenamil, while bathed in low-[Mg^{2+}] solution, developed relatively less tension when subsequently exposed to caffeine (Fig. 4).

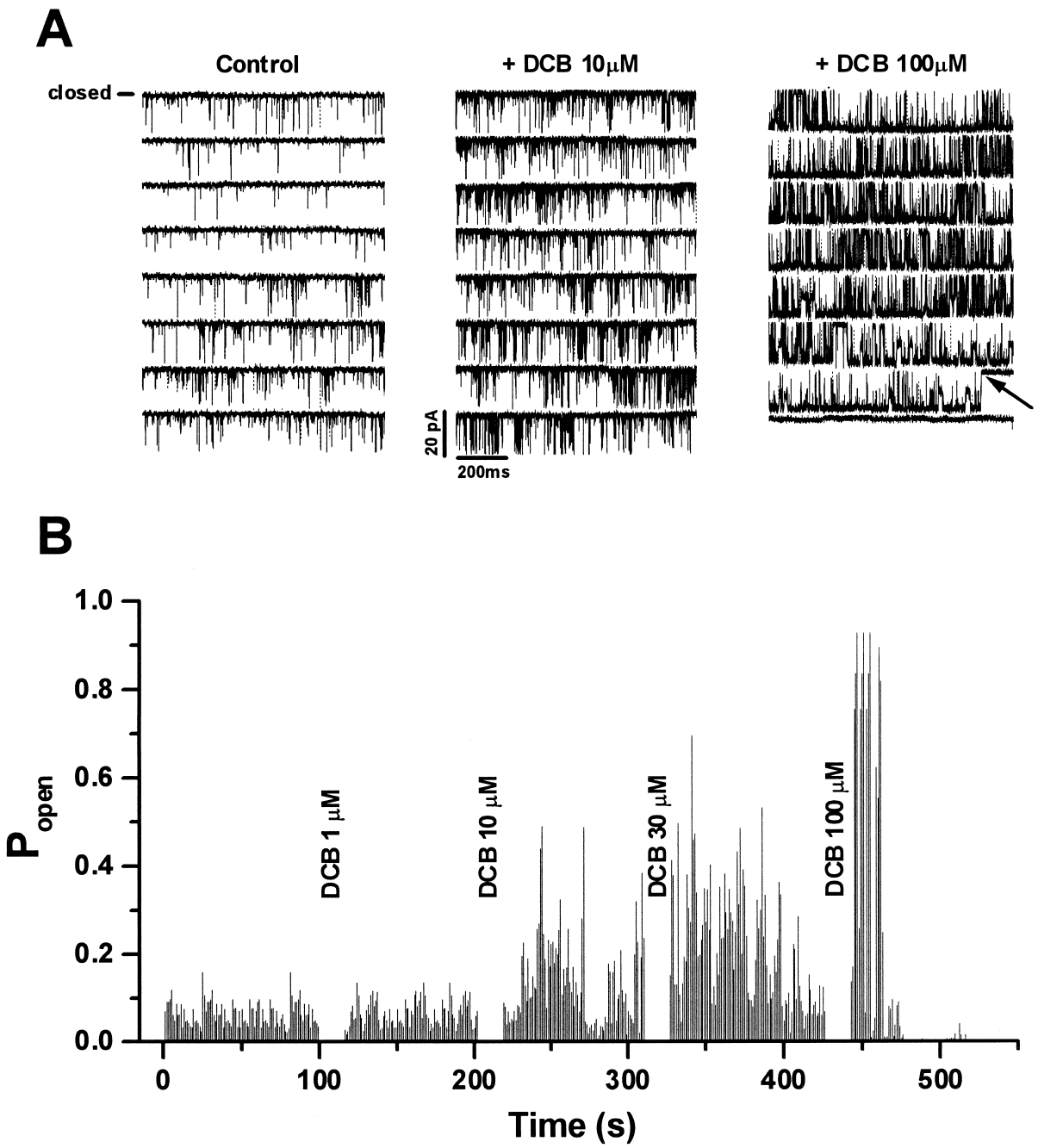

Fig. 5. Modulation of the sarcoplasmic reticulum Ca^{2+} release channel by dichlorobenzamil. (A) Representative single-channel recordings in control (left) and after 30-s exposure to 10 (center) and 100 μM dichlorobenzamil (right) in the *cis* compartment. The arrow on the right points to the abrupt transition from channel stimulation to blockade during exposure to 100 μM dichlorobenzamil. Channel openings are presented as downward deflections. Records were low-pass filtered at 1 kHz and digitized at 5 kHz. Holding potential: −35 mV. Current flows from *trans* (intraluminal) to *cis* (cytosolic) side. (B) Continuous recording (8 min) from another experiment were divided into 1-s intervals to construct a diary plot of P_o under control conditions and during exposure to increasing concentrations of dichlorobenzamil (1–100 μM).

3.2. Electrophysiological experiments

Dichlorobenzamil affected the Cs^+ currents flowing through the sarcoplasmic reticulum Ca^{2+} release channel in a complex, time- and concentration-dependent manner. In Fig. 5A, a single channel was recorded in the absence (control) and in the presence of 10 and 100 μM dichlorobenzamil. Short, often not fully resolved, channel opening was observed in the control (Fig. 5A, left). Addition of 10 or 100 μM dichlorobenzamil to the *cis* compartment stimulated channel activity (Fig. 5A, middle and right panels), but had no effect of the single channel current amplitude (control: 18.8 ± 2.1 pA). Channel stimulation persisted throughout the 3-min exposure to 10 μM dichlorobenzamil, but was transient in the presence of 100 μM dichlorobenzamil. Thus, after an initial period of intense stimulation, with long open events interrupted by brief closings, channel activity was virtually abolished after 1 min of exposure to 100 μM dichlorobenzamil. This biphasic effect, i.e., initial stimulation followed by channel block, was observed consistently with 100 μM dichlorobenzamil ($N = 5$; see Fig. 5B), and in two out of five experiments with 30 μM dichlorobenzamil. Fig. 5B shows a diary plot of the open channel probability (P_o) of the Ca^{2+} release channel in control and in the presence of increasing dichlorobenzamil concentrations (1–100 μM).

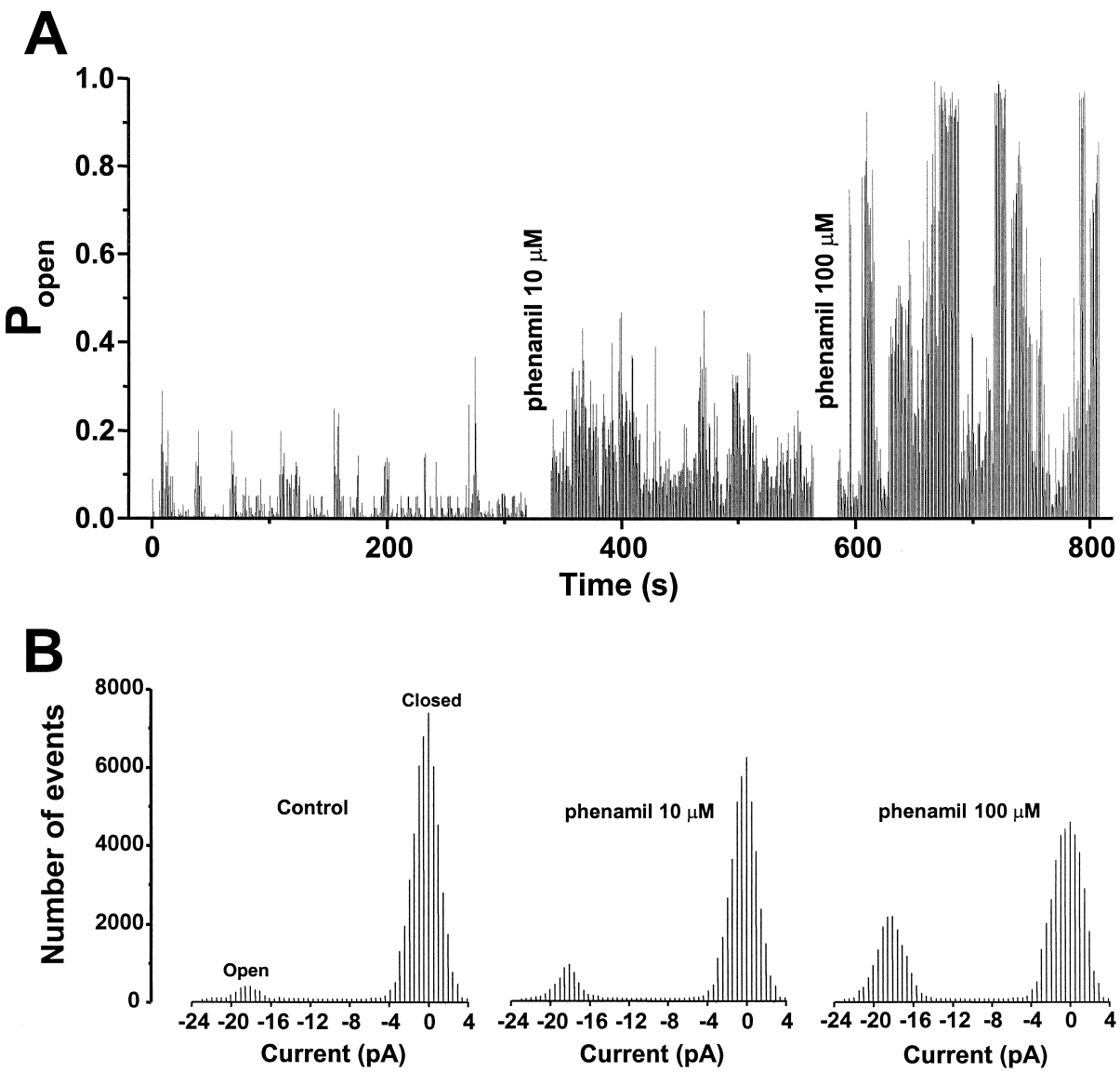

Fig. 6. Modulation of the sarcoplasmic reticulum Ca^{2+} release channel by phenamil. Experimental conditions identical to those for Fig. 5. (A) Continuous recordings (14 min) of the activity of skeletal ryanodine receptors were divided into 1-s intervals to construct a diary plot of P_o under control conditions and during exposure to 10 and 100 μM phenamil. (B) Data from the same experiment were used to construct current histograms for 150-s recording periods under each condition.

The data confirm the sustained stimulatory effect of 10 and 30 μM dichlorobenzamil and the biphasic effect elicited by 100 μM dichlorobenzamil.

In contrast to dichlorobenzamil, phenamil (1–100 μM) caused a monotonic, concentration-dependent activation of the sarcoplasmic reticulum Ca^{2+} release channel, as indicated by increases in P_o (Fig. 6); the single channel current amplitude (control: 18.3 ± 2.4 pA) was not affected by phenamil. Data from several experiments, similar to those shown in Figs. 5 and 6, were used to construct the plots depicted in Fig. 7, which reveal that both dichlorobenzamil- and phenamil-induced increases in P_o were accompanied by an increased number of events, reduction in the mean closed dwell-time and, to a lesser extent, increase in the mean open time.

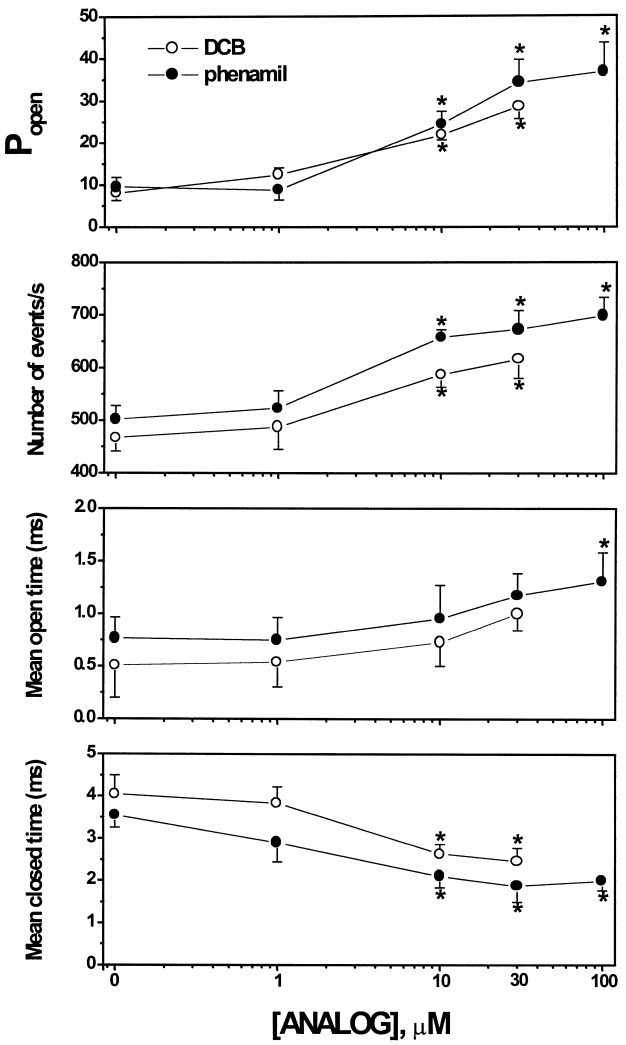

Fig. 7. Concentration–response curves for the effects of dichlorobenzamil and phenamil on gating parameters of the sarcoplasmic reticulum Ca^{2+} release channel. Data from three to five experiments with each drug concentration were used to construct the curves. Measurements were made between 30 and 180 s of drug exposure. The effects of 100 μM dichlorobenzamil were not plotted, since channel stimulation followed by blockade occurred during this time interval. In the two experiments in which 30 μM dichlorobenzamil had a biphasic effect on channel activity, only data collected during channel stimulation were used for the plots. $^{*}P < 0.05$.

4. Discussion

The present study provides converging evidence from functional experiments with skinned fibers and from electrophysiological recordings in lipid bilayer membranes for modulatory effects of amiloride analogs on the sarcoplasmic reticulum Ca^{2+} release channel. Both sets of experiments were carried out in preparations derived from rabbit skeletal muscle, which increases confidence when extrapolating results from one experimental paradigm to the other. The electrophysiological studies showed that phenamil caused a monotonic, concentration-dependent (1–100 μM) stimulation of the Ca^{2+} release channel, whereas in the case of dichlorobenzamil, stimulation was followed, at concentrations > 30 μM, by channel inhibition. With both compounds, channel stimulation was seen as increased P_o, reduction of the mean closed dwell-time and, to a lesser extent, increased mean open time. Gating of the sarcoplasmic reticulum Ca^{2+} release channel is thought to be modulated by cation binding sites, including high-affinity, stimulatory Ca^{2+} binding site(s) and a low-affinity, inhibitory Ca^{2+} binding site (reviewed by Zucchi and Ronca-Testoni, 1997). Accordingly, Mg^{2+} ions are thought to exert their inhibitory effects on sarcoplasmic reticulum channel activity and release of stored Ca^{2+} by competitively displacing Ca^{2+} from putative stimulatory site(s) or by interaction with the low-affinity site. Amiloride analogs are hydrophobic cations which easily partition into plasma membranes to generate a high local concentration at the external face of ion transporters or ionic channels, where the acyl guanidine group of amiloride is protonated at physiological pH (Garcia et al., 1990). It has been postulated that protonated amiloride analogs exert their effects on ion transporters and channels by competing with Ca^{2+} or other metal ions for specific binding sites (Slaughter et al., 1988). Whether similar mechanisms underlie the modulatory effects of dichlorobenzamil and phenamil on the sarcoplasmic reticulum Ca^{2+} release channel is speculative at present. Nevertheless, the electrophysiological data presented here provide a consistent explanation for the functional effects of dichlorobenzamil and phenamil on skinned muscle fibers. Thus, activation of the sarcoplasmic reticulum Ca^{2+} release channel, leading to an increased myoplasmic Ca^{2+} concentration, can account for the tension induced by dichlorobenzamil, phenamil and, by extension, the other amiloride analogs examined in the present study, namely benzamil and 5-*N*-ethyl-*N*-isopropyl amiloride. Several experimental observations are consistent with this interpretation. First, the dichlorobenzamil- or phenamil-induced tension depends on the Ca^{2+} loading of the sarcoplasmic reticulum, as sub-

maximally loaded fibers failed to develop tension. Second, the tension elicited by dichlorobenzamil or phenamil was abolished by ruthenium red, which irreversibly blocks the sarcoplasmic reticulum Ca^{2+} release channel (Salviati and Volpe, 1986), and by pretreatment of the skinned fibers with the nonionic detergent Brij-58, which functionally disrupts the sarcoplasmic reticulum membranes (Orentlicher et al., 1974). Third, lower concentrations of dichlorobenzamil or phenamil were required for eliciting tension in maximally loaded skinned fibers bathed in low-[Mg^{2+}] solutions than in fibers bathed in solution W. Mg^{2+} is an endogenous inhibitor of the sarcoplasmic reticulum Ca^{2+} release channel and opposes activation by several channel agonists, such as Ca^{2+}, ATP or caffeine (see above). Fourth, fibers which developed tension in the presence of dichlorobenzamil or, to a lesser extent, phenamil displayed reduced contractile responses to a subsequent challenge with caffeine. This inhibition of the caffeine-induced tension can be ascribed, at least in part, to a decrease in the sarcoplasmic reticulum Ca^{2+} content following activation of the Ca^{2+} release channels by the amiloride analogs. However, especially in the case of dichlorobenzamil, blockade of the Ca^{2+} release channel, observed in the electrophysiological experiments, could also have contributed to inhibition of the caffeine-induced tension in skinned fibers. Phenamil and, to a lesser extent, dichlorobenzamil, both at 10 μM, enhanced the caffeine-induced tension of submaximally loaded skinned fibers. This effect can be ascribed to synergism between caffeine and either dichlorobenzamil or phenamil in their abilities to stimulate the sarcoplasmic reticulum Ca^{2+} release channel.

In conclusion, the present study reveals that the Ca^{2+} release channel/ryanodine receptor of skeletal muscle sarcoplasmic reticulum represents yet another pharmacological target for dichlorobenzamil and related amiloride analogs. The concentrations of dichlorobenzamil required for modulation of the sarcoplasmic reticulum Ca^{2+} release channel are similar to those necessary for inhibition of Na^+–Ca^{2+} exchange in plasma membranes ($K_D = 17$ μM, Siegl et al., 1984). Based on the evidence that dichlorobenzamil affects other transport processes and ionic channels, Suarez-Kurtz and Kaczorowsky (1988) have previously cautioned against the use of this amiloride analog as an experimental probe for investigating the role of Na^+–Ca^{2+} exchange in intact cells. The present observations provide novel, additional evidence in support of this notion, which could be extended to phenamil and to other amiloride analogs that affect multiple ion channels transport systems.

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