Effects of Cytoplasmic and Luminal pH on Ca^{2+} Release Channels from Rabbit Skeletal Muscle

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ABSTRACT Ryanodine receptor (RyR)-Ca^{2+} release channels from rabbit skeletal muscle were incorporated into lipid bilayers. The effects of cytoplasmic and luminal pH were studied separately over the pH range 5–8, using half-unit intervals. RyR activity (at constant luminal pH of 7.5) was inhibited at acidic cytoplasmic pH, with a half-inhibitory pH (pH_I) ~6.5, irrespective of bilayer potential and of whether the RyRs were activated by cytoplasmic Ca^{2+} (50 μM), ATP (2 or 5 mM), or both. Inhibition occurred within ~1 s and could be fully reversed within ~1 s after brief inhibition or within ~30–60 s after longer exposure to acidic cytosolic pH. There was no evidence of any hysteresis in the cytoplasmic pH effect. Ryanodine-modified channels were less sensitive to pH inhibition, with pH_I at ~5.5, but the inhibition was similarly reversible. Steady-state open and closed dwell times of RyRs during cytoplasmic pH inhibition suggest a mechanism where the binding of one proton inhibits the channel and the binding of two to three additional protons promotes further inhibited states. RyR activity was unaffected by luminal pH in the pH range 7.5 to 6.0. At lower luminal pH (5–5.5) most RyRs were completely inhibited, and raising the pH again produced partial to full recovery in only ~50% of cases, with the extent of recovery not detectably different between pH 7.5 and pH 9. The results indicate that isolated skeletal muscle RyRs are not inhibited as strongly by low cytoplasmic and luminal pH, as suggested by previous single-channel studies.

INTRODUCTION

The ryanodine receptor (RyR)-Ca^{2+} release channel is the major pathway for Ca^{2+} efflux from the sarcoplasmic reticulum (SR) in skeletal muscle and so plays a crucial role in the control of force production. In vivo the RyRs are arranged in long arrays along the terminal cisternae of the SR, and their opening is in some way controlled by voltage-sensor/dihydropyridine receptor (DHPR) molecules in the apposing transverse tubular (T-) system (Melzer et al., 1995). RyR activity is modulated by many intracellular factors, such Ca^{2+}, Mg^{2+}, ATP, and pH (Meissner, 1994). In the absence of cytoplasmic Mg^{2+}, RyRs can be activated by μM Ca^{2+} and by mM ATP, both in situ (Endo, 1985; Lamb and Stephenson, 1991, 1994) and when isolated in SR vesicles or artificial lipid bilayers (Meissner, 1994).

The intracellular pH in skeletal muscle fibers is ~7.0–7.1 at rest, but can drop to ~6.5 after prolonged activity (Fitts, 1994). Consequently, it is important to understand what effect such a decrease in pH has upon the functioning of the RyRs, and whether this could play any role in the reduction of Ca^{2+} release that underlies the late stages of muscle fatigue (Allen et al., 1995). Although experiments with skinned muscle fibers indicate that voltage-sensor control of Ca^{2+} release is not markedly reduced, even at pH 6.1–6.2 (Lamb et al., 1992; Lamb and Stephenson, 1994), a number of studies with isolated RyRs in bilayers show very pronounced inhibition of Ca^{2+}-activated channel activity over the range pH 7.4 to 6.8 (Ma et al., 1988; Rousseau and Pinkos, 1990; Shomer et al., 1994a). However, there are major differences in findings between these single channel studies, as well as differences with another recent study (Ma and Zhao, 1994). Ma et al. (1988) used purified RyRs and found that lowering the pH on either the cytoplasmic or luminal side greatly inhibited channel open probability (P_o), with the pH giving half-maximum inhibition (pH_I) being ~7.2 in both cases. Using RyRs from native SR, Rousseau and Pinkos (1990) found that cytoplasmic pH inhibited channel activity with pH_I ~7.2, but that a decrease in luminal pH from 7.4 to 6.8 did not alter P_o and instead reduced channel conductance by ~27%. Another study with purified RyRs (Shomer et al., 1994a) observed a 5- to 10-fold inhibition of P_o by both cytoplasmic and luminal pH over the range 7.4 to 6.6, with no change in conductance when reducing luminal pH at cytoplasmic pH 7.4. Finally, Ma and Zhao (1994), using native RyRs, reported an entirely novel phenomenon in the pH inhibition. They simultaneously altered both the cytoplasmic and luminal pH and found that when the pH is lowered, activity actually increases between pH 7.4 and 7.0, and then declines to zero with pH_I ~6.5. Furthermore, when the pH is subsequently raised again on the same channel, activity only returns at pH ≥7.0, which they describe as "hysteresis" in the pH effect. However, when the pH was altered in this latter study and in that of Rousseau and Pinkos (1990), [Ca^{2+}] also changed substantially (~6 μM to 100 μM and ~2.5 μM to 40 μM, respectively), because the Ca^{2+} was buffered with EGTA, and the affinity of EGTA for Ca^{2+} is reduced 100-fold by a

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decrease of one pH unit (Harrison and Bers, 1987). Furthermore, Ma and Zhao (1994) did not examine the time dependence of recovery or what happened to RyR activity if the pH was subsequently reduced a second time. Consequently, their results do not distinguish whether the RyR activity shows true "hysteresis," slow recovery, or simply irreversible change. In all, it is currently unclear to what extent RyR activity is affected by changes in cytoplasmic and luminal pH, and whether any "hysteresis" is involved.

Here we examine the effects of independently altering the cytoplasmic and luminal pH on the activity of native RyRs from rabbit skeletal muscle. A multiple perfusion system was used to apply various solutions of known pH and [Ca^{2+}], using Ca^{2+} buffers with low pH sensitivity. We were thus able to rapidly and reproducibly apply such solutions in any sequence. Our results indicate that RyRs are much less sensitive to inhibition by both cytoplasmic and luminal pH than previously suggested. We find no "hysteresis" in the effect of cytoplasmic pH and find that treatment of the luminal face of the RyR with unphysiologically low pH ($\leq$5.5) results in channel inhibition, which is poorly reversible at either pH 7.5 or pH 9 and is exacerbated by longer exposures at low luminal pH. We discuss how the findings relate to the role of intracellular acidification in muscle fatigue.

MATERIALS AND METHODS

Preparation of SR microsomes

Native SR vesicles were isolated from New Zealand rabbit skeletal muscle with techniques based on those of Chu et al. (1988), as previously described by Kourie et al. (1996). Briefly, cubes of muscle were homogenized in a Waring blender in homogenizing buffer (20 mM imidazole, 300 mM sucrose, pH 7.1 with HCl), and centrifuged (11,000 $\times$ *g*, 15 min), and the pellet was resuspended, rehomogenized, and centrifuged as above. The supernatant was filtered through cotton gauze and pelleted by centrifugation (110,000 $\times$ *g* for 60 min) to yield a crude microsomal fraction, which was fractionated by loading onto a discontinuous sucrose gradient. Heavy SR vesicles were collected from the 35–45% (w/v) interface, snap frozen, and stored at -70°C.

Bilayer preparations

Bilayer preparations consist of one or more RyRs that are incorporated into a lipid bilayer membrane. Lipid bilayers were formed from phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (5:3:2) (Avanti Polar Lipids, Alabaster, AL) in *n*-decane, across an aperture of 150–250-μm diameter in a Delrin cup. The bilayer separated two solutions: *cis* (1.5 ml) and *trans* (1 ml). Vesicles were usually added to the *cis* solution, and incorporation with the bilayer occurred as described by Miller and Racker (1976). Fusion of vesicles with the bilayer was facilitated by an osmotic gradient across the bilayer (*cis* higher osmolality, see below) and the presence of 1 mM Ca^{2+} in the *cis* bath (incorporating solution). Because of the orientation of RyRs in the SR vesicles, RyRs added to the *cis* chamber were incorporated into the bilayer with the cytoplasmic face of the channel orientated to the *cis* solution. In some experiments it was desirable for the luminal side of the RyR to be facing the *cis* chamber (where the perfusion apparatus was located). Therefore, in these experiments, vesicles were added to the *trans* chamber.

Solutions

Cesium methanesulfonate (CsMS) was the principal salt used in the bathing solutions to obviate the problems associated with other SR ion channels interfering with RyR current signals. A small amount of Cl^- (20 mM as cesium salt) was included in the bath solutions to allow proper functioning of the $AgCl_2$-coated electrodes. Solutions used in local perfusion of the bilayer (see below) did not require Cl^-. In initial experiments the osmotic gradient across the bilayer needed for vesicle fusion was established using a [Cs^+] gradient (250 mM *cis* and 50 mM *trans*). In later experiments the osmotic gradient was produced by a [mannitol] difference across the membrane (500 mM mannitol *cis* and zero *trans*) in the presence of symmetrical 250 mM Cs^+. In previous investigations of RyRs there was no difference in the way RyRs were regulated by Mg^{2+} and Ca^{2+} in these two types of experiments (Laver et al., 1997a). The use of symmetrical [Cs^+] (250 mM) in both chambers allowed measurement of RyR activity at both positive and negative bilayer potentials. When cytoplasmic pH effects and some cases of luminal pH effects were measured, the mannitol was absent because the bilayer was perfused locally with mannitol-free solutions. A summary of the principal solutions is given in Table 1.

During measurements of channel activity, *cis* [Ca^{2+}] and pH were varied by bath perfusion (see below) according to the experimental needs. Unless otherwise stated, *trans* [Ca^{2+}] was low (either 10^{-9} M or $\sim 10^{-4}$ M) for measurements of cytoplasmic pH inhibition. In some of the luminal pH experiments where SR vesicles were added to the *trans* bath, the *trans* bath also contained 1 mM cytoplasmic Ca^{2+} and 500 mM mannitol. This was because exchanging the *trans* bath to replace the vesicle incorporating solution frequently resulted in rupture of the bilayer. This technical problem was avoided in some of the luminal pH experiments by adding SR

TABLE 1 Composition of frequently used solutions

Solution Identifier	Solution composition
Incorporating solution	CsMS (230), CsCl (20), $CaCl_2$ (1), mannitol (500), pH 7.5
Cytoplasmic pH effect	
ATP-activating (cytoplasmic)	CsMS (250), $\sim$1 nM free Ca^{2+} (BAPTA (1), no added Ca^{2+}), ATP (5) & various pH
Ca^{2+}-activating (cytoplasmic)	CsMS (250), 50 μM $CaCl_2$ & various pH
Ca^{2+}/ATP-activating (cytoplasmic)	CsMS (250), 50 μM free Ca^{2+}, ATP (2) & various pH
250-Cs^+ (luminal)	CsMS (230), CsCl (20), $CaCl_2$ (0–5), pH 7.5
50-Cs^+ (luminal)	CsMS (30), CsCl (20), $CaCl_2$ (0.1), pH 7.5
50-Ca^{2+} (luminal)	$CaCl_2$ (50), pH 7.5
Luminal pH effect	
Cytoplasmic	CsMS (230) CsCl (20), $CaCl_2$ (1), mannitol (500), pH 7.5 or Ca^{2+}/ATP-activating solution (see above)
Luminal	CsMS (250) $CaCl_2$ (0.1–1) & various pH

Concentrations in parentheses are mM. The concentrations of Ca^{2+} and pH buffers are given in Materials and Methods.

vesicles to the *cis* chamber and lowering the pH of the *trans* chamber by aliquot addition of HCl. In this case the luminal pH could not be accurately changed back and forth.

The pH buffers were 5 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) (pK_a 7.4) and 5 mM 2-[*N*-morpholino]ethanesulfonic acid (MES) (pK_a 6.1), and solutions were titrated to pHs of between 5.0 and 9.0 with CsOH. (The combination of TES and MES used here only provided weak buffering of pH outside the range 5–8.4. Despite this, the pH at the bilayer should have reached the specified value because solutions of the nominated pH were continuously flushed onto the bilayer.) Free [Ca^{2+}] was buffered to 10^{-9} to 10^{-7} M with 1–2 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid (BAPTA), and to 10^{-6} to 10^{-5} M with 1 mM dibromo-BAPTA. These solutions were titrated to the required free [Ca^{2+}] with $CaCl_2$. Solutions containing $\geq$50 μM free Ca^{2+} usually did not contain Ca^{2+} buffers. Free [Ca^{2+}] was estimated using published association constants (Marks and Maxfield, 1991) and the program *Bound and Determined* (Brooks and Storey, 1992), and a Ca^{2+}-selective electrode (Radiometer ION83) was used to verify free [Ca^{2+}] $\geq$ 0.1 μM.

Solution exchange

Three different methods were used for solution changes, depending on experimental requirements. First, exchange of the entire solution in the *cis* chamber was achieved by perfusing the bath with at least 6 volumes (10 ml), using back-to-back syringes in which perfusion and waste withdrawal were maintained at equal rates. It was established that this perfusion method produced more than 99% exchange by measuring the optical density of samples from the *cis* bath (which initially contained ruthenium red) both before and after perfusion. Second, a more rapid solution exchange at the bilayer ($\sim$1 s) was achieved by washing away the *cis* bath solution with the solution from a tube ($\sim$0.5-mm internal diameter) positioned with its outlet within 50 μm of the *cis* face of the bilayer. The tube was connected to a single reservoir, which was adjusted to produce a flow of $\sim$10 μl/s. The duration of the flow was kept to a minimum (i.e., $<$10s) to prevent disruption of the bilayer or significant changes in bath composition. Solution exchange rates were determined as described by Laver and Curtis (1996). Third, in the majority of experiments measuring effects of pH changes, the solution at the *cis* face of the bilayer was exchanged between any one of 16 available solutions. This was done by flowing solutions from reservoirs (2.5 ml) via 16 vinyl tubes (0.28 mm internal diameter), through a manifold and then through a single outlet tube (vinyl, $\sim$0.5 mm diameter, 50 μm from bilayer), directly onto the bilayer surface. At a flow rate of $\sim$1 μl/s, the solution at the bilayer surface could be completely replaced with the perfusing solution within 5–10 s (limited by the dead space of the manifold and outlet tube), and perfusion could be maintained for many minutes. The flow of solution onto the bilayer was confirmed visually by the refractive index discontinuity between the flow and bath solutions, which was clearly visible under the microscope when the bath solution (incorporating solution) contained mannitol and the flow solution did not. Two aspects of the method were assessed: 1) The rate of solution exchange at the bilayer surface was deduced from the conductance time course of SR Cl^- channels in these bilayers after the flow was switched between solutions with different [Cl^-]. 2) The pH of drops appearing at the manifold outflow was checked with pH indicator paper (Merck pH 6.5–10 and pH 4–7). When the solution flow was switched between pH 8 and 6 the manifold attained pH values within 0.2 pH units of either pH in 8–16 s (the times of appearance of the first and second drops, respectively).

Data acquisition and analysis

Bilayer potential was controlled and currents recorded with an Axopatch 200A amplifier (Axon Instruments). The *cis* chamber was electrically grounded to prevent electrical interference from the perfusion tubes, and the potential of the *trans* chamber was varied. All electrical potentials are expressed here as the cytoplasmic side of the RyR relative to the luminal side at virtual ground, irrespective of whether the SR vesicles were added to the *cis* or *trans* chamber. During experiments, current was recorded on videotape at a bandwidth of 5 kHz. For measurements of unitary current and open probability (P_o), the current signal was replayed through a 1-kHz, low-pass, 8-pole Bessel filter and sampled at 2 kHz. Unitary current was determined from inspection of the current records. Channel open probability was determined using an in-house analysis program, *Channel 2* (developed by P. W. Gage and M. Smith). To calculate P_o from single-channel records ($\sim$50% of bilayer preparations), a threshold discriminator was set just above the baseline to detect channel opening and closing events. For bilayer preparations that contained more than one active RyR, the time-averaged current was divided by the unitary current and the number of channels. This method of calculating P_o gives a result similar to that obtained when P_o of single channels is measured with a threshold discriminator (Laver et al., 1997a).

The inhibition of RyRs by protons was characterized by fitting a Hill equation to the relation between P_o and [H^+]. The Hill equation is expressed in terms of pH as follows:

$$P_o = \frac{P_{max}}{1 + 10^{H(pH_I - pH)}} \quad (1)$$

where H is the Hill coefficient, pH_I is the pH for half-inhibition, and P_{max} is the open probability at noninhibiting pH. Wherever possible, individual Hill fits were made to the P_o data for each preparation separately, to show the spectrum of pH_I values in the population of RyRs studied and to provide the best estimate of the steepness of the pH dependence (i.e., the Hill coefficient) in individual RyRs. Such an individual fit could only be done reliably when P_o data were available for the given bilayer across a sufficiently wide pH range to show both maximum and minimum activity. Table 2 shows the mean of the fit parameters obtained by performing individual fits in a number of preparations. In addition to performing

TABLE 2 Effect of activator and voltage on cytoplasmic pH inhibition

cis conditions	Luminal [Ca^{2+}]	pH_I	H	P_{max}	n
0 ATP, 50 μM Ca^{2+}, +40 mV	50–100 μM	6.60 $\pm$ 0.10	2.6 $\pm$ 0.2	0.07 $\pm$ 0.02	5
2 mM ATP, 50 μM Ca^{2+}, +40 mV	0–100 μM	6.43 $\pm$ 0.10	2.5 $\pm$ 0.3	0.19 $\pm$ 0.05	7
5 mM ATP, $\sim$1 nM Ca^{2+}, +40 mV	0–5 mM	6.76 $\pm$ 0.11	2.2 $\pm$ 0.3	0.36 $\pm$ 0.07	9
As previous +10 μM ryanodine	0–5 mM	5.49 $\pm$ 0.11	2.6 $\pm$ 0.6	0.82 $\pm$ 0.08	6
*5 mM ATP, $\sim$1 nM Ca^{2+}, −40 mV	0–5 mM	6.58 $\pm$ 0.23	2.8 $\pm$ 0.6	0.60 $\pm$ 0.08	5
*5 mM ATP, $\sim$1 nM Ca^{2+}, +40 mV	0–5 mM	6.69 $\pm$ 0.19	2.0 $\pm$ 0.4	0.48 $\pm$ 0.02	5

Mean $\pm$ SEM of the parameters were obtained by fitting the Hill equation (Eq. 1) to the P_o data for each preparation separately. Reliable fits were only possible when four or more P_o measurements were obtained over a sufficiently wide pH range. n indicates the number of preparations.
*Data obtained from a subset of preparations in which complete pH dependencies of P_o were obtained at both positive and negative voltages.

individual fits, we also pooled the P_o data from all preparations for a given set of conditions (including the cases where individual fits could not be reliably made), and then fitted the Hill equation to the mean data (e.g., Fig. 3). This procedure should provide a reasonably accurate estimate of the average pH_I in the total population of RyRs examined, but gives no indication of the spread of the individual pH_I values. It also underestimates the Hill coefficient in individual RyRs, because the mean P_o data are spread out across a comparatively large pH range, owing to the pooling of data from RyRs with different pH_I.

For measurements of channel open and closed dwell times, the current signal was replayed through a 5-kHz filter and sampled at 10 kHz. The dwell times were obtained from single-channel recordings with steady baseline and apparently stationary gating kinetics (i.e., no gating mode changes were apparent). Frequency histograms of dwell-time duration were compiled from channel records varying in duration from 10–60 s and containing on the order of 10^3 to 10^5 events. Event durations were extracted from idealized, two-level signals (namely, open and closed), modeled on the current records by the hidden Markov model algorithm (Chung et al., 1990). Details of our hidden Markov model analysis and justification of data filtering and sampling methods are given by Laver et al. (1997a). The histograms are presented as probability distributions, using variable bin widths with equal separation on a log scale as described by Sigworth and Sine (1987). Amplitude histograms of single-channel events (e.g., Fig. 5 *D*) were calculated using the hidden Markov model algorithm on data that were filtered at 1 kHz and sampled at 2 kHz.

Other statistics

The number of observations included in the analysis, *n*, refers to the number of bilayer preparations (bilayer preparations can contain more than one RyR; see above). Unless otherwise stated the data are presented as mean ± standard error of the mean (SEM). The quality of fit parameter used was the root mean square of the residuals. The Wilcoxon-Mann-Whitney test and Fisher exact test were used for nonparametric analysis of independent samples. One-way analysis of variance (ANOVA) with Bonferonni's multiple comparison post-test was used to test significance between the means of different pairs of data sets. In all tests, a value of statistical probability, *P,* less than 0.05 was considered to indicate a significant difference.

RESULTS

General observations

Rabbit skeletal RyRs studied here had ligand-dependent gating properties and conductance similar to those reported in our previous studies (Laver et al., 1995, 1997a,b; Laver and Lamb, 1998). RyRs had a conductance of ~520 pS in symmetrical 250 mM CsCl at [Ca^{2+}] less than 100 μM (Tinker et al., 1992; Shomer et al., 1994b). They were activated by μM cytoplasmic Ca^{2+} and inhibited by mM Ca^{2+} and Mg^{2+}. RyRs could also be activated by mM cytoplasmic ATP (Meissner et al., 1986). The addition of 10 μM ryanodine to the cytoplasmic bath markedly slowed RyR gating activity and reduced channel conductance, as reported previously (Rousseau et al., 1987). Ryanodine-modified RyRs had a relatively high open probability and were insensitive to regulation by cytoplasmic Ca^{2+}, Mg^{2+}, and ATP. With some RyRs, reversing the polarity of the membrane potential would rapidly activate them and then inactivate them after several seconds (voltage-dependent inactivation), as we have described recently (Laver and Lamb, 1998). RyRs with an intraburst open probability of less than 0.2 did not show such inactivation.

Single-channel recordings of skeletal RyRs obtained over the pH range 5–8 revealed that acid conditions on the cytoplasmic side had a strong inhibitory effect on RyR activity. This occurred regardless of whether RyRs were activated by Ca^{2+} or ATP or both in combination. Fig. 1 shows episodes from a typical experiment ($n = 9$; see Table 2) in which a RyR, activated by 5 mM ATP, was more than 50% inhibited when the pH was decreased to between 7 and 6.5. It is also apparent from Fig. l that inhibition is due to a decrease in channel open probability, which in turn is caused by both a decrease in the channel's mean open duration and an increase in its mean closed duration (see also Fig. 13).

Rate of RyR response to changes in cytoplasmic pH

With a rapid decrease in pH, RyR activity decreased to a steady level within the time (the settling time) expected for solution exchange at the bilayer surface (1–10 s, depending on the perfusion method; see Materials and Methods). With the faster perfusion method, solution exchange and, hence, pH changes occurred in ~1 s or less. This estimate was based on the duration of the capacitive current transient induced by the solution exchange (Laver and Curtis, 1996).

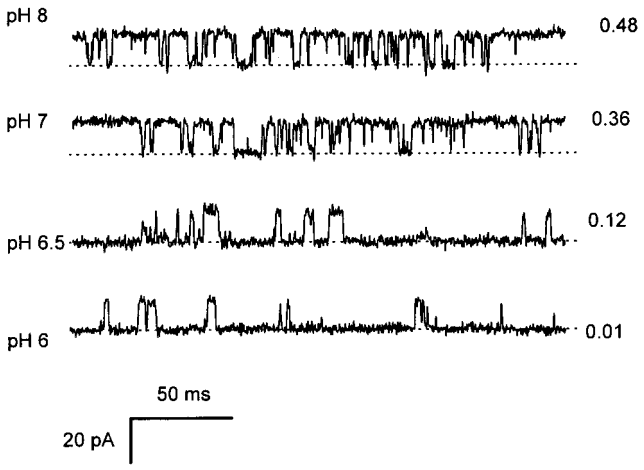

FIGURE 1 Effect of pH on a single skeletal RyR. The ATP-activating cytoplasmic solution (250 mM Cs^+, 5 mM ATP, 1 nM Ca^{2+}; see Table 1) was in the *cis* bath, and the *trans* bath contained the 250-Cs^+ luminal solution with 0.1 mM $CaCl_2$. The membrane potential was held at +40 mV, and channel openings are shown here by upward deflections of the current from the baseline (*dashed lines*). The pH in the cytoplasmic bath is shown at the left of each trace, and the P_o for 20–40 s of record is shown at the right. Lowering pH decreased the open event duration and increased the closed event duration of the RyR but had no effect on channel conductance. Hill fits to P_o values from these records indicated half-inhibition at pH 6.8 with a Hill coefficient of 1.8.

In the experiment shown in Fig. 2 *A*, a RyR was fused with the bilayer (*cis* bath contains the incorporating solution; see Materials and Methods and Table 1). The pH of the cytoplasmic bath was dropped to pH 5.3 by aliquot addition of HCl while keeping a solution (pH 7) flowing from a tube onto the bilayer. The solution stream contained sufficient ATP and Ca^{2+} to strongly activate the channel. The pH at the bilayer was decreased to pH 5.3 by removing the flow tube, which totally inhibited the channel within 2 s. The pH was successively raised and lowered between 5.3 and 7 by moving the solution stream (pH 7) onto or away from the bilayer. At pH 7 the channel activity was rapidly and completely restored. These experiments show that inhibition and recovery are both fast (probably faster than 1 s if solution exchange is taken into account). The faster recovery of activity (pH 5.3 to 7) compared with the onset of inhibition (pH 7 to 5.3) was largely or entirely due to the difference in the time taken for solution change at bilayer surface when applying a flowing solution as compared to removing the flow and allowing the bath solution to move back. This was apparent from other experiments in which the pH was changed at the bilayer by switching the solution flowing onto the bilayer (e.g., Fig. 2 *B*). In these cases, both the onset of inhibition at low pH and the recovery of activity at high pH occurred within 1 s.

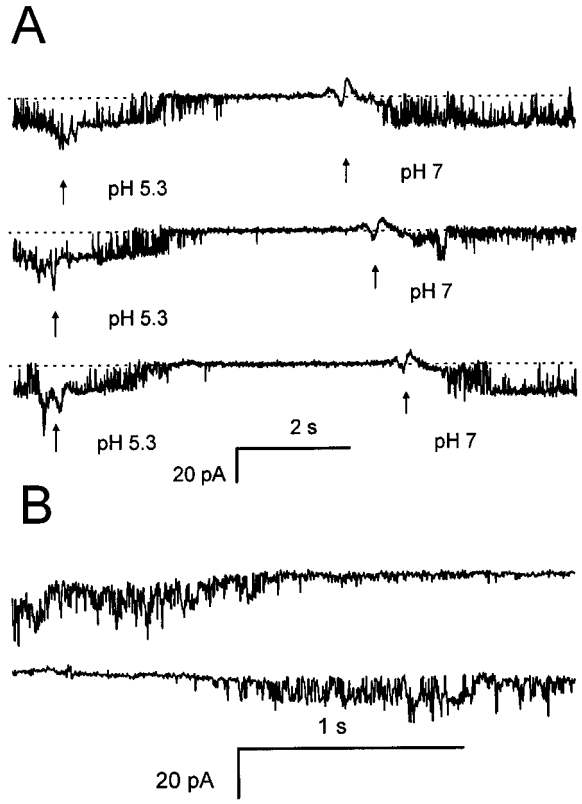

FIGURE 2 Onset and reversibility of pH inhibition with rapid solution changes at −40 mV. (*A*) Recordings of a single RyR. Continuous recordings, except for a 5-s gap between the second and third traces. The 250-Cs^+ luminal solution with 0.1 mM free Ca^{2+} (1 mM BAPTA and 1.1 mM $CaCl_2$) was in the *trans* bath. The cytoplasmic pH was rapidly changed (~1 s) by moving a solution stream from a tube onto and away from the bilayer. The cytoplasmic bath contained 230 mM CsMS and 20 mM CsCl plus 1 mM $CaCl_2$ (~700 μM free Ca^{2+}), 2 mM ATP, and 500 mM mannitol at pH 5.3; the free $[Ca^{2+}]$ only causes partial inhibition at this high ionic strength. The solution stream contained the Ca^{2+}/ATP-activating cytoplasmic solution at pH 7. The arrows indicate the times when the solution stream was either moved onto (pH 7) or removed from (pH 5.3) the bilayer. (*B*) Inhibition and recovery of two RyRs in response to ATP-activating cytoplasmic solutions flowing from either of two tubes (pH 7.5 and pH 6) placed within ~200 μm of the bilayer. Until the beginning of the upper trace, the RyRs were bathed in pH 7.5. Switching the solution flow from one tube to the other (i.e., pH 7.5 to pH 6) at the beginning of the upper trace inhibited the RyRs within 1 s. After ~10 s the pH was returned to 7.5 by again switching the flow at the beginning of the lower trace. In *A*, the faster recovery at pH 7 (~1 s) compared to the rate of inhibition at pH 5.3 (~2 s) is predominantly due to faster change at the bilayer surface when solution is allowed to flowed onto it, compared to when flow is stopped and the bath solution returns. In *B*, where both activating and inhibiting solutions are actively perfused, there are similar rates of onset and reversal of inhibition.

The time course for the recovery of RyR responses when the pH is raised from inhibiting levels (i.e., where $P_o < 0.01$) varied between channels from 10 to 150 s, depending on the duration of the low pH exposure. Provided RyRs had been exposed to inhibiting pH for less than 30 s, they fully recovered in 10 ± 3 s (SEM, $n = 11$) when the pH was raised to activating levels, which in most cases was as fast as could be expected for the solution exchange method utilized (see Materials and Methods). Returning the pH to 7.5 after longer exposures (30–220 s) to inhibiting pH produced a significantly slower recovery, with a mean of 30 ± 5 s ($n = 42$) ($p < 0.05$).

Effect of cytoplasmic pH on RyR open probability

To obtain a reliable measurement of RyR activity at each pH, we waited for 10–120 s to allow channel activity to settle to a steady level after each pH change before making measurements. The channel activity was considered steady if the open probability failed to show a trend over a 30-s period. The 30-s period was long enough for P_o to be little affected by stochastic fluctuations in channel activity and yet short enough to allow a reasonable likelihood that the experiment would be completed before the bilayer spontaneously ruptured. Steady-state open probability was determined from recordings of steady activity lasting 30–60 s at each pH. In two experiments, RyRs with open probabilities of ~0.5 exhibited voltage-dependent inactivation (see above and Laver and Lamb, 1998). In these instances P_o was determined over the time interval (~10 s) between voltage-step-induced activation of the RyR and its subsequent inactivation; the procedure was repeated several times, and records were concatenated for analysis. The RyRs that showed inactivation at high pH failed to show inactivation when the pH was lowered to levels where P_o was ~0.2 or less. This result is in keeping with our previous

finding (Laver and Lamb, 1998) that the RyR inactivation rate depends on P_o (and does not occur at $P_o < 0.2$) rather than on the particular activator (μM Ca^{2+}, ATP, caffeine, or ryanodine) or inhibitor present (mM Ca^{2+} or Mg^{2+}). The half-inhibitory pH for these two channels was 6.1 and 6.6, which was within the range of values obtained for RyRs that did not show inactivation.

Fig. 3 shows the relationship between cytoplasmic pH and mean P_o for the pooled data of all RyRs activated by cytoplasmic 50 μM free Ca^{2+} alone (*open circles*, 11 preparations) and for those activated by cytoplasmic 50 μM free Ca^{2+} plus 2 mM ATP (*filled circles*, 10 preparations) at +40 mV. In each case, the luminal solution was maintained at pH 7.5 and the pH on the cytoplasmic side was adjusted over the range 5–8. The activity of RyRs showed a sigmoidal dependence on cytoplasmic pH, with near-total inhibition occurring at pH 6. The presence of 2 mM ATP in the cytoplasmic bath caused higher channel activity but had no discernible effect on the sensitivity of RyRs to inhibition at low pH. The Hill fits (see Materials and Methods) indicate that half-inhibition of RyRs occurred at pH ~6.6, irrespective of the presence or absence of 2 mM ATP.

Hill curves were also fitted individually to each bilayer preparation (7 with 2 mM ATP and 5 without ATP), in which P_o was measured over a sufficient pH range to obtain a reliable fit. The means of the Hill parameters for the individual fits are summarized in Table 2. pH_I varied considerably between preparations, ranging from 6.1 to 6.8. The distribution of Hill coefficient estimates had a mean of 2.6 ± 0.2 (SEM). Note that the Hill coefficients for the individual bilayer preparations in Table 2 are typically higher than values derived from the pooled P_o data listed in the figure captions. This is because pooling P_o data from RyRs with very different pH_I reduces the apparent Hill coefficient (see Materials and Methods). Thus the values for the individual bilayer preparations are considered to be the more reliable estimates of the Hill coefficient.

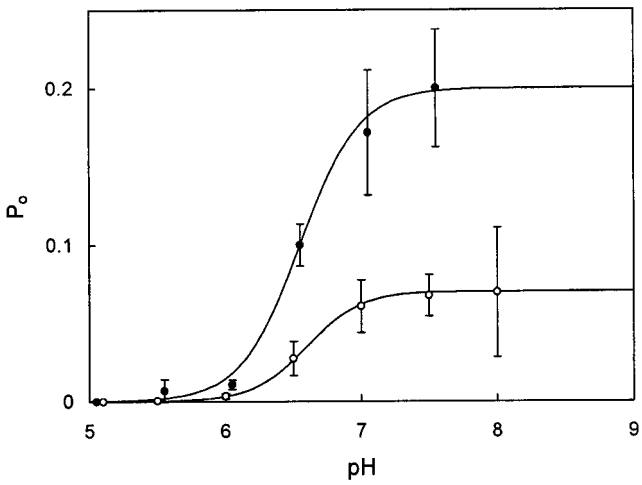

FIGURE 3 Pooled data showing pH inhibition of RyRs in the presence and absence of cytoplasmic ATP. ○, RyRs in the absence of cytoplasmic ATP (Ca^{2+}-activating cytoplasmic solution and 50-Cs^+ luminal solution with low (0.05–0.1 mM) Ca^{2+}). ●, RyRs in the presence of 2 mM cytoplasmic ATP (Ca^{2+}-ATP-activating cytoplasmic solution and 250-Cs^+ luminal solution with low (0–0.1 mM) Ca^{2+}). Bilayer potential was +40 mV in both conditions. The data points show the means and SEM (standard error on the mean) pooled from 11 preparations in the absence of ATP and 10 preparations in the presence of ATP. The solid curves show least-squares fits of the Hill equation (Eq. 1) to the data with the following parameters: open probability of noninhibited RyR (P_{max}), pH of half-inhibition (pH_I), and Hill coefficient (H). No ATP: $P_{max} = 0.07$, $pH_I = 6.60$, $H = 2.2$; 2 mM ATP: $P_{max} = 0.20$, $pH_I = 6.55$, $H = 2.0$.

We also investigated the pH dependence of RyRs activated by 5 mM ATP alone (i.e., with only 1 nM Ca^{2+}). In these experiments the pH was generally lowered and raised through the pH range in sequential half-unit steps, and the pH range was commonly covered several times for a given bilayer preparation. There was no significant difference in the pH_I (or Hill coefficient) obtained by fits to individual bilayer preparations when a luminal [Ca^{2+}] of either 1 nM or 5 mM was used (Table 3), and the fit parameters obtained under these two conditions were combined in the presentation in Table 2. Measurements were also made at 50 mM luminal [Ca^{2+}] (see conditions in Table 1), and the means for pH_I and the Hill coefficient obtained by individual fits in the three cases possible were not significantly different from those for either of the other two luminal [Ca^{2+}] (Table 3). Furthermore, the fit to the pooled P_o data for all preparations studied with 50 mM Ca^{2+} (which included a further five cases where individual Hill fits could not be made reliably) gave a pH_I very similar to that found at lower luminal [Ca^{2+}] (Table 3).

The mean values of pH_I and H obtained by fitting the P_o data in each preparation individually (Table 2) were not significantly different between the cases where the RyRs were activated by either Ca^{2+} or ATP or both (ANOVA; see Materials and Methods). Further statistical analysis of the data with the Wilcoxon-Mann-Whitney non-parametric test

TABLE 3 Effect of luminal [Ca^{2+}] on cytoplasmic pH inhibition

Luminal [Ca^{2+}]	pH_I	H	P_{max}	n
~1 nM	6.85 ± 0.15	2.3 ± 0.3	0.37 ± 0.07	4
5 mM	6.68 ± 0.18	2.0 ± 0.4	0.35 ± 0.10	5
50 mM	6.29 ± 0.14	1.8 ± 0.7	0.14 ± 0.11	3
50 mM	6.89*	2.5*	0.21*	8

The parameters in the top three rows are the means (± SEM) derived from fitting the Hill equation to P_o data for each preparation separately. There was no significant difference in the means at different luminal [Ca^{2+}] for either the pH_I or H values (ANOVA, $p > 0.05$; see Materials and Methods).

*The bottom row indicates the parameters obtained from a single fit to the pooled P_o data from all preparations recorded with 50 mM luminal Ca^{2+}, which included a further five preparations in which reliable individual fits could not be obtained. The *cis* solutions contained 5 mM ATP and ~1 nM Ca^{2+} (+40 mV) in all cases.

also failed to show any significant difference between the pH_I (or H) distributions for RyRs activated by either Ca^{2+} or ATP or both. We also attempted to determine whether the variability in pH_I was linked to differences in P_{max}, but found no correlation between values of P_{max} and pH_I (correlation coefficient $r < 0.3$, for individual P_{max} and pH_I values used in Tables 2 and 3, excluding the ryanodine modified channels).

The effect of voltage on the pH dependence was examined by measuring P_o at both +40 mV and −40 mV in the same preparation (with activation by 5 mM ATP). The pH_I and H values obtained by individual fits in five experiments (see last two rows in Table 2) were not significantly different at the two voltages (paired t-test, $p > 0.05$). Fig. 4 shows plots of the mean P_o at each pH for the two voltages, obtained by pooling P_o data from the above bilayer preparations plus additional preparations in which reliable individual fits could not be made at both +40 and −40 mV. The data from each bilayer preparation were normalized to their respective P_o values at pH 7.5 and +40 mV. RyRs tended to be slightly more active at −40 mV than at +40 mV, and similar results were also obtained in three bilayer preparations where the luminal $[Ca^{2+}]$ was less than 100 μM (e.g., Fig. 5 *A*). Hill fits to the pooled P_o data (*solid lines*) in Fig. 4 give pH_I 6.5–6.6 at both voltages. In summary, voltage had no apparent effect on pH_I.

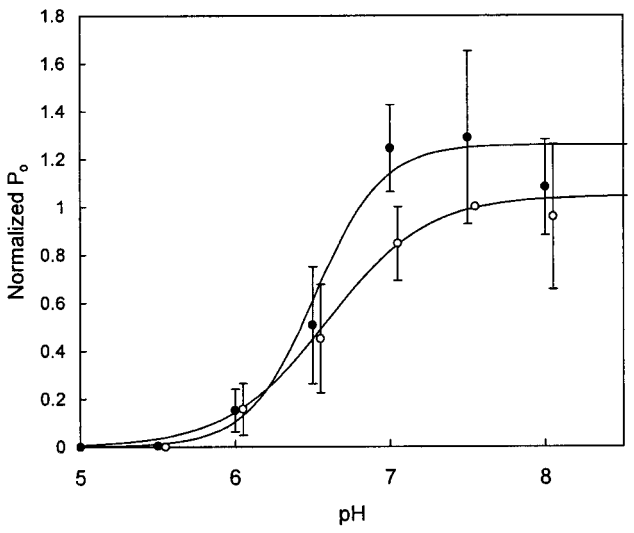

FIGURE 4 Pooled data showing inhibition by low pH at +40 mV (○) and at −40 mV (●). The baths contained the ATP-activating cytoplasmic solution and the 250-Cs^+ luminal solution with 5 mM $CaCl_2$. The data points show the mean and SEM obtained from six preparations at both +40 mV and −40 mV. The data from each preparation are normalized to their respective P_o values at pH 7.5 and +40 mV, which ranged from 0.15 to 0.5 (0.29 ± 0.15, mean ± SD). The solid curves are fits of Eq. 1 to the data with the following parameters: −40 mV: $P_{max} = 1.26$, $pH_I = 6.51$, $H = 2.0$; +40 mV: $P_{max} = 1.05$, $pH_I = 6.59$, $H = 1.4$.

Effect of cytoplasmic pH on ryanodine-modified RyRs

Fig. 5 *A* shows results from an experiment that measured the open probability of a single RyR in the presence of the ATP-activating cytoplasmic solution, both before and after the addition of 10 μM ryanodine to the cytoplasmic bath. The addition of ryanodine considerably increased the channel open probability and reduced the sensitivity of the RyR to inhibition by low pH. Fig. 5 *B* shows the effect of pH on the mean activity of ryanodine-modified RyRs pooled from seven preparations. These data have been fitted with a Hill plot (*solid line*) with $pH_I \sim 5.5$. Differences in the pH_I of different RyRs (see Fig. 6) cause the relative broadening of the pH dependence of the pooled data (i.e., they lower the Hill coefficient). Fits were also made to the P_o data from individual preparations, and the mean values are given in Table 2. Full recovery from low pH inhibition occurred in ryanodine-modified RyRs in 25 ± 9 s ($n = 4$) after the pH was raised above pH 7, which is similar to that found for unmodified channels (see earlier). In the presence of ryanodine, there was also obvious substate activity at low pH (in seven of eight experiments; e.g., see Fig. 5, *C* and *D*), as reported by Ma and Zhao (1994).

Fig. 6 shows the frequency distributions of all pH_I values obtained from the fits to individual bilayer preparations in the presence (*open bars*) and absence (*solid or hatched bars*) of ryanodine. There was a large and significant difference between the values of pH_I obtained in the presence and absence of ryanodine ($p < 0.01$, Wilcoxon-Mann-Whitney test). Channel-to-channel variation in pH_I (range 6.1–7.2; see Fig. 6) was much larger than would be expected from the statistical uncertainty in the fitting procedure for each case (typically ± 0.06). Hence the variation in pH_I reflects real variability in the RyR population under study. The uncertainty in the H value obtained in each Hill fit, however, was relatively large (typically ±1) and so must have contributed substantially to the observed variation in H between individual RyRs (not shown). This means that it is unclear how much H really does differ between different RyRs. The mean H value (e.g., Table 2) should nevertheless still be a reliable estimate.

Investigation of hysteresis in cytoplasmic pH inhibition

As an earlier study (Ma and Zhao, 1994) reported "hysteresis" in the effect of pH on RyRs, we sought to confirm this observation in the present study. Here the open probability of RyRs was measured as the pH in the cytoplasmic solution was progressively increased or decreased. RyRs required up to 150 s to recover from inhibition after prolonged exposure to low pH (see above), but we found no evidence for hysteresis in the response of RyRs to cytoplasmic pH changes. Fig. 7 *A* shows P_o from a preparation where

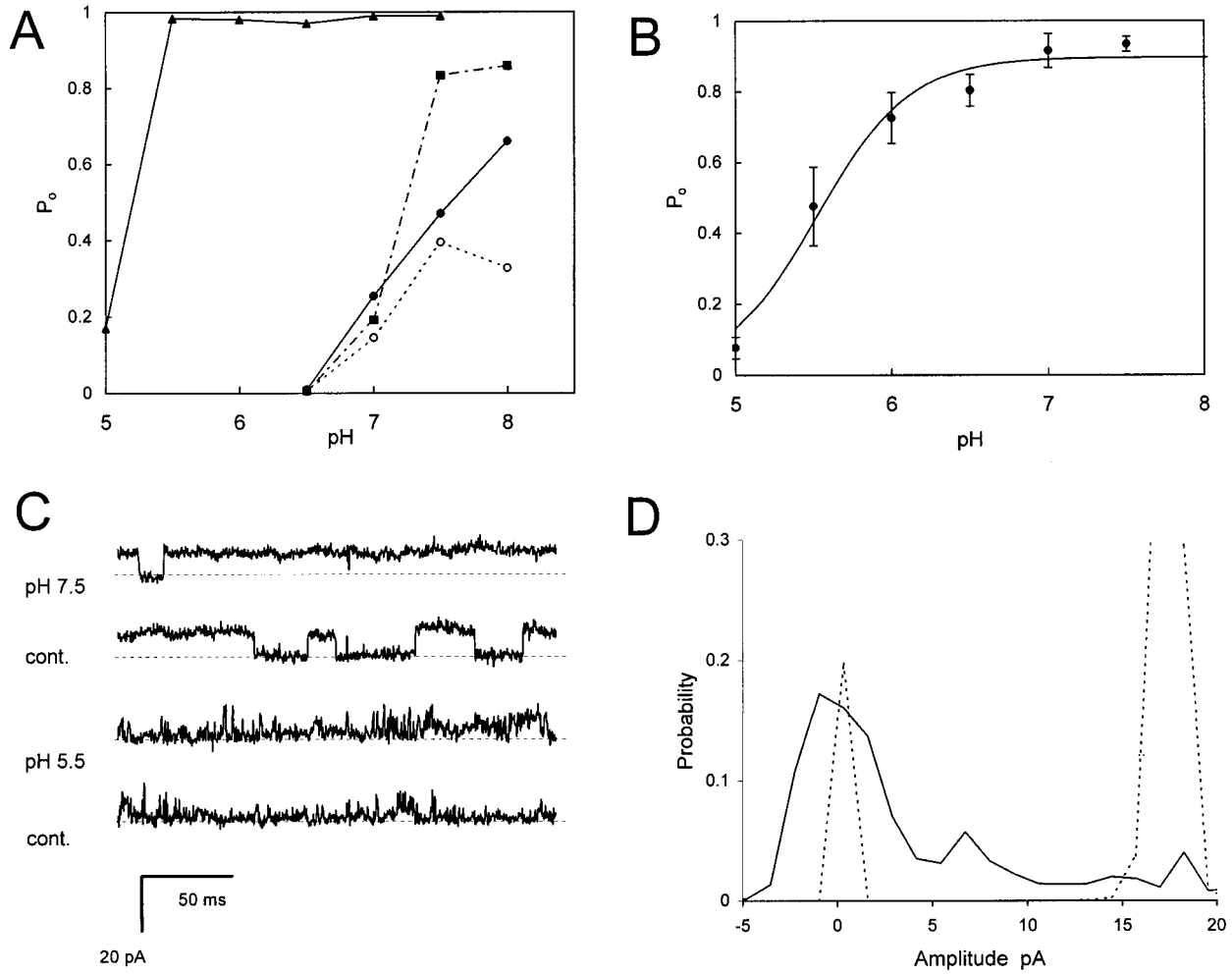

FIGURE 5 Effect of pH on ryanodine-modified RyRs. (*A*) Inhibition by low pH of a single RyR activated solely by 5 mM cytosolic ATP at +40 mV (○), −40 mV (●), and −60 mV (■). The baths contained the ATP-activating cytoplasmic solution and the 250-Cs^+ luminal solution with 100 μM $CaCl_2$. RyRs tended to be more active at the more negative membrane potentials. Addition of 10 μM ryanodine (▲) considerably reduced the sensitivity of the RyRs to inhibition by low pH at +40 mV. (*B*) Mean and SEM from seven experiments with ryanodine-modified RyRs at +40 mV. The solutions are the same as in *A*. The solid curve shows a fit of Eq. 1 to the data with the following parameters: $P_{max} = 0.90$, $pH_I = 5.52$, $H = 1.5$. (*C*) Recording of a ryanodine-modified channel showing the presence of conductance substates at low cytoplasmic pH. (*D*) Maximum likelihood probability distributions of event amplitude calculated from the data in *C*, using the hidden Markov model. ·····, pH 7.5; ——, pH 5.5.

cytoplasmic pH was progressively lowered (*open circles*) and raised (*filled circles* and *filled triangles*). Fig. 7 *B* shows the mean, normalized, pH dependence of P_o obtained from four such preparations. Hill fits (not shown) to the mean data give values of pH_I for rising and falling pH of 6.65 and 6.49, respectively. The results in Fig. 7 indicate that provided RyR open probability is given sufficient time to settle before commencing measurements (10–150 s; see above), it is not dependent on the cytoplasmic pH to which the RyRs had previously been exposed.

Effect of cytoplasmic [Ca^{2+}] on pH inhibition of RyRs

We investigated the possibility that protons inhibit RyRs by binding to the Ca^{2+} activation site on the protein, thus preventing cytoplasmic Ca^{2+} from activating the channel. If this were the case then the effects of H^+ and Ca^{2+} would be competitive and inhibition at low pH could be overcome by sufficiently increasing the [Ca^{2+}]. In this series of experiments, P_o of each RyR (at +40 mV) was measured at both pH 7.5 and pH 6.5, for [Ca^{2+}] ranging from ~0.2 μM to 50 μM (in the absence of ATP). Fig. 8 shows the ratio of P_o at pH 6.5 and pH 7.5 in individual RyRs over a wide range of cytoplasmic calcium concentrations. If protons inhibited channel activity by competing with Ca^{2+} for the activation site on the RyR, their inhibitory effect should be reduced at high [Ca^{2+}], with the P_o ratio approaching asymptotically a value of 1. No such behavior was evident. An analysis of variance (see Materials and Methods) showed no significant difference between the means for any calcium concentrations. In addition, a linear regression analysis of the data in

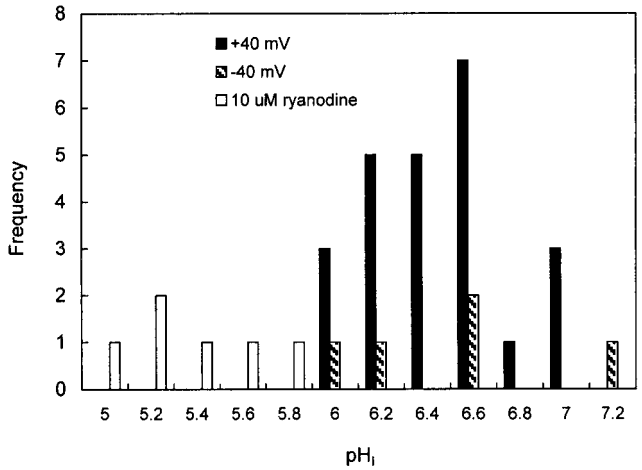

FIGURE 6 Frequency distributions of pH_I derived from cytoplasmic pH inhibition of individual RyRs. Values were obtained by fitting the data for each bilayer experiment separately as described in the text. ■, RyRs at +40 mV, which are activated by ATP and/or Ca^{2+}. ▨, RyRs at −40 mV that are activated by ATP alone. □, RyRs that have been modified by 10 μM ryanodine in the presence of ATP-activating cytoplasmic solution. The mean and SEM of these parameters obtained under the various experimental conditions are given in Tables 2 and 3, and they reveal no significant difference in sensitivity to inhibition by low pH of RyRs that are activated by either Ca^{2+} or ATP separately or together (in the absence of ryanodine).

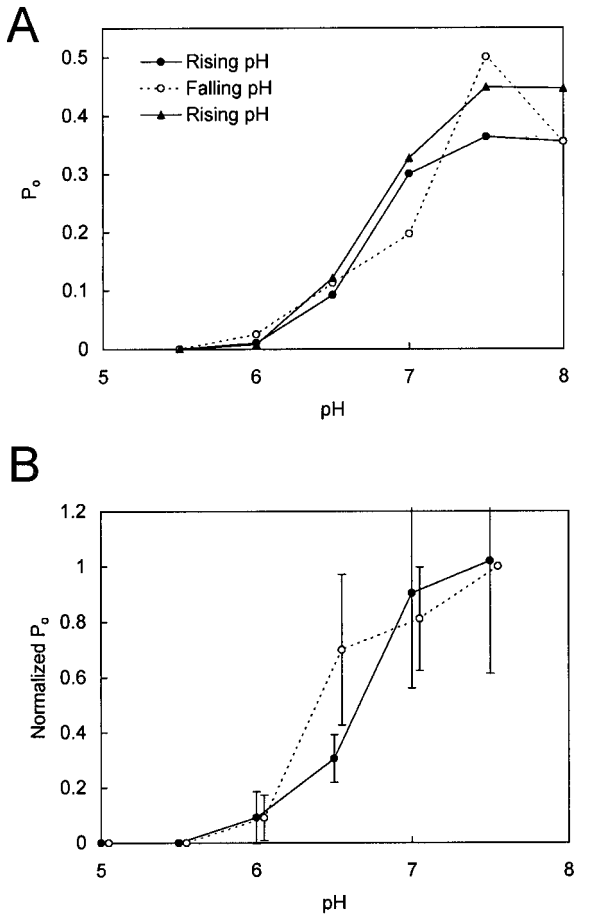

FIGURE 7 (*A*) RyR open probability in one preparation (mean of four RyRs present in the bilayer) to cytoplasmic pH when it was progressively increased (●, ▲) or decreased (○). The baths contained the Ca^{2+}-ATP-activating cytoplasmic solution (2 mM ATP) and 50-Cs^+ luminal solution with 0.1 mM Ca^{2+}. (*B*) The mean normalized P_o (and SEM) obtained from four separate preparations with increasing (●) and decreasing (○) cytoplasmic pH. Values of P_o were normalized in each preparation to their respective values at pH 7.5 before the first pH decrease. The normalizing values ranged from 0.05 to 0.44 (0.21 ± 0.15, mean ± SD). Fitting the Hill equation to the data (not shown) gave values of pH_I for rising and falling pH of 6.65 and 6.49, respectively.

Fig. 8 showed no indication of a relationship between the P_o ratio and pCa ($r^2 = 0.02$). Furthermore, in other experiments with ATP present, there was no significant difference in the mean of the P_o ratio at 1 nM Ca^{2+} (i.e., pCa 9: 0.28 ± 0.15, $n = 9$) and at 50 μM Ca^{2+} (0.42 ± 0.06, $n = 6$); these P_o ratios were also similar to those seen when the channel was activated by Ca^{2+} alone (see Fig. 8). In other words, the relative inhibition at low pH was similar irrespective of whether the channel was activated by Ca^{2+} alone, ATP alone, or Ca^{2+} and ATP together. In summary, the data are not consistent with cytoplasmic H^+ inhibiting the RyR by competing with Ca^{2+} for the activation site.

Effect of luminal pH on RyR open probability

A series of experiments were carried out in which the pH of the luminal solution was varied over the range 7.5 to 5 (in half-unit steps or in one large step) while the cytoplasmic solution remained at pH 7.5. To achieve this, the SR vesicles were added to the *trans* chamber (except where mentioned below), so that the luminal side of the RyRs, when fused with the bilayer, would face the solution flow apparatus, allowing application of solutions of known composition. Decreasing the luminal pH did not alter channel conductance (520 ± 30 pS at both pH 7.5 and pH 6, at 100 μM luminal Ca^{2+}).

In one series of experiments ($n = 8$) the RyRs were activated by 1 mM Ca^{2+} and studied at +40 mV. When the luminal pH was progressively lowered from 7.5 to pH 6 there was no significant change in the level of activity (mean $P_o = 0.15 \pm 0.04$ at pH 7.5, 0.13 ± 0.04 at pH 6.0, $n = 8$). However, when the luminal pH was further reduced to 5.5 or 5, channel activity usually stopped completely within 5–50 s (typically ~15 s; e.g., Fig. 9, *top trace*). Specifically, in the five preparations where the pH was lowered from 6.0 to 5.5, activity abruptly ceased completely in three cases, dropped to 20% of control in another (and subsequently ceased at pH 5), and was unaffected in the remaining case. In the three preparations where the pH was decreased from 6 straight to 5, activity ceased completely in two cases but was quite unaffected in the other case. In a further 17 preparations in which the luminal pH was decreased from 7.5 straight to 5 (see section below), activity

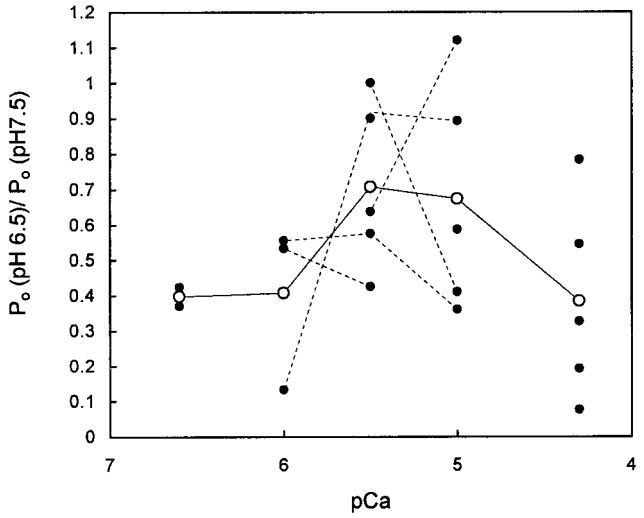

FIGURE 8 Effect of cytoplasmic $[Ca^{2+}]$ on the sensitivity of RyRs to pH inhibition. The ordinate gives the ratio of P_o at pH 6.5 compared to pH 7.5. RyRs activated solely by Ca^{2+} (no ATP) at +40 mV. $[Ca^{2+}]$ is indicated as pCa ($= -\log_{10}[Ca^{2+}]$). ●, The P_o ratio for individual preparations; – – –, connecting values obtained from the same preparation at different $[Ca^{2+}]$. The mean of the data at each $[Ca^{2+}]$ is also given (○). There was no consistent trend in individual preparations and no evidence that the P_o ratio was larger at high $[Ca^{2+}]$, as would be expected if Ca^{2+} and protons compete for the Ca^{2+} activation site on the channel protein. The large variability in the P_o ratio predominantly stems from the large range in P_o values at pH 6.5, where the response varies steeply with pH.

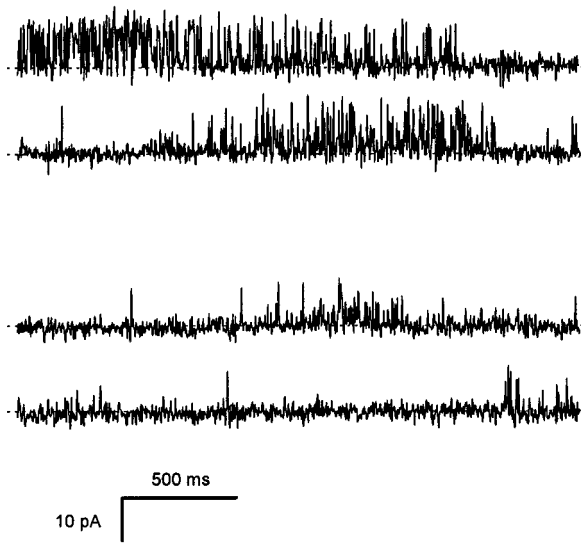

FIGURE 9 Effect of luminal pH on activity in a single RyR (40 mV). The cytoplasmic and luminal bath solutions are listed under "Luminal pH effect" in Table 1. The flickery appearance of the channel activity is characteristic of partial RyR inhibition caused by 1 mM cytoplasmic Ca^{2+}. (*Trace 1*) This shows the onset of inhibition when the luminal pH was decreased from 7.5 to 5 by switching the solution flow by ~1 s before the start of the record. The activity at the left was unchanged from that at pH 7.5 (P_o = 0.17). The channel activity decreased shortly after exposure of the receptor to pH 5 and ceased (P_o = 0) at the right. (*Trace 2*) Partial recovery of activity 40 s after the pH was restored to 7.5 (P_o = 0.05, measured over >10 s). After this, the channel was again inhibited by lowering the pH to 5 (not shown), and it recovered more poorly over a 60-s exposure to pH 9 (P_o = 0.02) (*Trace 3*). When the luminal pH was then decreased to 7.5, channel activity remained unchanged (P_o = 0.02) (*Trace 4*).

ceased abruptly and completely in every case within 50 s. Together, these data illustrate that 1) luminal pH in the range 5.5 to 5 causes sudden and complete inhibition of RyR activity and 2) full inhibition occurs over a narrow pH range in some if not all cases, with the exact threshold level varying considerably between different RyRs.

In further experiments (n = 4), we verified that the inhibitory effect of luminal pH was apparently independent of the activating conditions used on the cytoplasmic side of the RyRs. In these cases the SR vesicles were added to the *cis* chamber and the cytoplasmic face of the RyRs perfused with the Ca^{2+}/ATP activating solution (at pH 7.5), while the luminal pH was lowered from pH 7.5 to 5 in half-unit steps by the addition of calibrated amounts of HCl. In all bilayer preparations examined, RyR activity was unchanged at pH 6.0 (mean $P_o = 0.051 \pm 0.021$ at pH 7.5, 0.055 ± 0.022 at pH 6.0, n = 4, paired t-test, $p > 0.05$), but ceased completely by pH 5. Fig. 10 compares the mean relative effect of luminal (*filled circles*) and cytoplasmic (*empty circles*) pH. The data were normalized to their respective P_o values at pH 7.5 in each bilayer preparation.

The recovery of RyRs from inhibition at low luminal pH was also investigated. In these experiments, the luminal pH was decreased from 7.5 to 5 for sufficient time to shut the channels (17 preparations, luminal perfusion method) and then raised to pH 7.5 or 9. In general, recovery of RyR activity was poor and highly variable at both pH 7.5 and 9, with activity remaining extremely low (<5% of that before inhibition) in many cases, or suddenly recovering partially or fully after a period of 10 s to 10 min. Activity only recovered to 20–35% (of the preinhibition level) within 1–2 min in three of five preparations at pH 9, and to 10–150% in four of 12 preparations at pH 7.5. In the cases where activity did not return at pH 7.5 (within 1–2 min), subsequently raising the pH to 9 resulted in partial recovery in two of five cases (after 10 s and 6 min). Statistical analysis indicated that neither the percentage of RyRs showing recovery (Fisher exact test) nor the extent of recovery (Wilcoxon-Mann-Whitney test) differed significantly between pH 7.5 and pH 9.

In three preparations it was also possible to measure P_o in a RyR over several inhibition-recovery cycles. In all three cases the extent of recovery upon raising the pH was much smaller after the second or third exposure to pH 5 (P_o recovered to 0–11% of original control level) than after the first exposure (recovery to 30–60%) (e.g., Fig. 9, which of the three cases studied, shows the greatest relative recovery observed after a second exposure to pH 5). This suggests that longer exposure to luminal pH 5 reduces the extent of

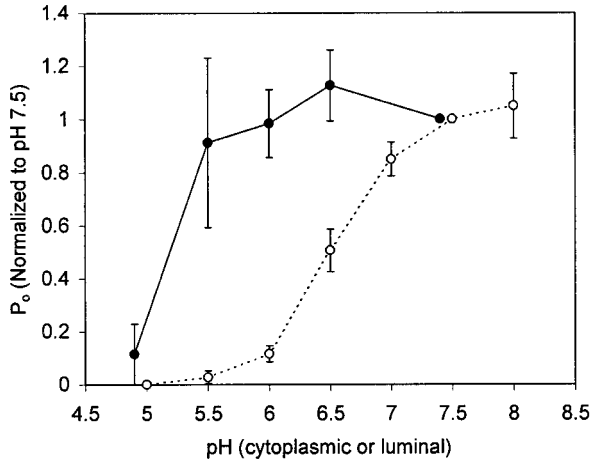

FIGURE 10 Comparison of the effects of luminal (●) and cytoplasmic (○) pH on mean P_o at +40 mV. Inhibition by luminal pH was measured under the conditions listed in Table 1 with activation by either 1 mM cytoplasmic Ca^{2+} ($n = 8$) or Ca^{2+}/ATP activation ($n = 4$). The data for the cytoplasmic pH effect are the means of all bilayer preparations without ryanodine ($n = 38$). When the luminal pH was varied, the cytoplasmic pH was constant at 7.5, and vice versa. The P_o values at different pH in each preparation were normalized to their respective values at pH 7.5. The mean absolute values of P_o at pH 7.5 are 0.12 ± 0.03 for the luminal inhibition data and 0.21 ± 0.026 for the cytoplasmic inhibition data.

recovery occurring at higher pH. Finally, in the two cases where it was examined, the level of activity reached after recovery at pH 9 (P_o: 11% and 80% of preinhibition value) remained unchanged when the pH was subsequently lowered to 7.5 again (e.g., *bottom two traces* in Fig. 9). This confirms that the activity seen at pH 9 reflects partial recovery of normal channel activity rather than some specific stimulatory effect of highly alkaline pH.

Analysis of dwell times at different cytoplasmic pH

To further characterize the mechanism of RyR inhibition at low cytoplasmic pH, we compiled histograms of open and closed dwell times for five individual RyRs, using the ATP-activating cytoplasmic solutions over a range of pH and membrane potentials. Fig. 11 shows dwell-time histograms from a single RyR at +40 mV at each of four cytoplasmic pH conditions. Dwell-time distributions were fitted with functions with two, three, and, in one case, four exponential components. Fig. 12 shows the effect of cytoplasmic pH on the open and closed exponential time constants in one RyR, at both −40 mV (Fig. 12, *A* and *B*) and +40 mV (Fig. 12, *C* and *D*). At −40 mV there were only two closed time constants (Fig. 12 *B*), which became progressively larger at lower pH but remained of approximately equal weighting. In contrast, at +40 mV (Fig. 12 *D*), there were four closed time constants. The relative weighting of two of these varied considerably over the pH range, though

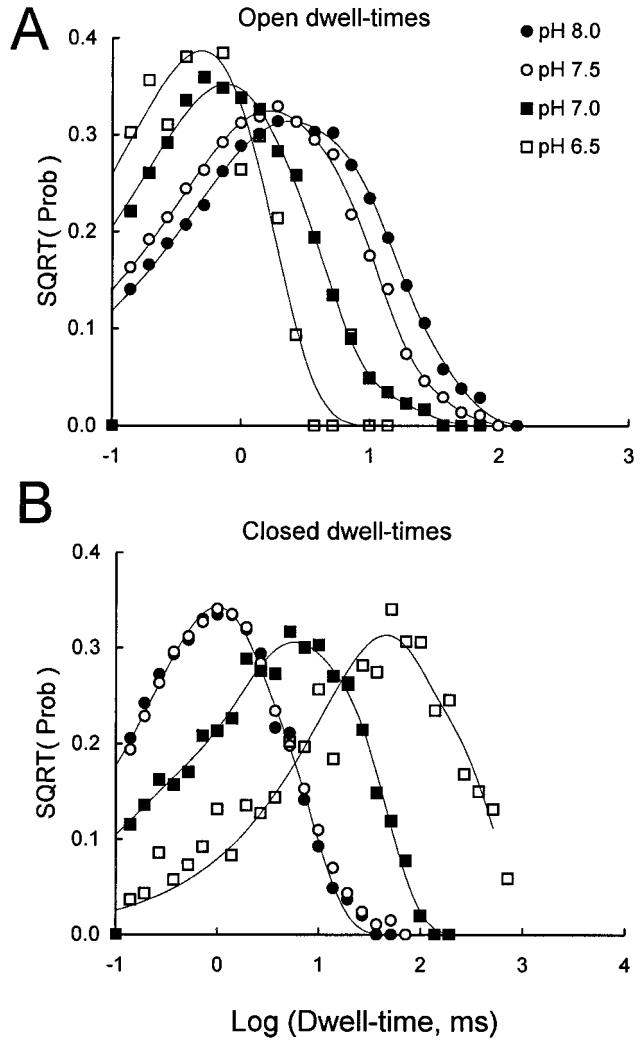

FIGURE 11 Probability histograms of open (*A*) and closed (*B*) dwell times compiled from recordings of a single RyR at +40 mV. The baths contained the ATP-activating cytoplasmic solution and the 250-Cs^+ luminal solution. The RyR activity went from noninhibited at pH > 7.5 to 99% inhibited at pH 6.5. The solid curves show fits of multiexponential functions to the data. The curve through the pH 7.5 data in *B* has been omitted for clarity. This is the same RyR as in Fig. 5 *A*.

there was still a general trend for the time constant values to increase by 20- to 50-fold as the pH was reduced from 8 to 6.5. The open dwell-time distributions could be described by three exponential time constants at both +40 mV and −40 mV (Fig. 12, *A* and *C*). The size of the time constants stayed relatively constant or decreased slowly with decreasing pH, but there was a progressive increase in the proportion of events associated with the shortest open time constant (e.g., at pH 6.5 only a single time constant was apparent). In general, all five RyRs examined showed trends broadly similar to those in Fig. 12, with the individual open time constants decreasing by about one- to fivefold and the closed time constants increasing by ~30–100-fold over the

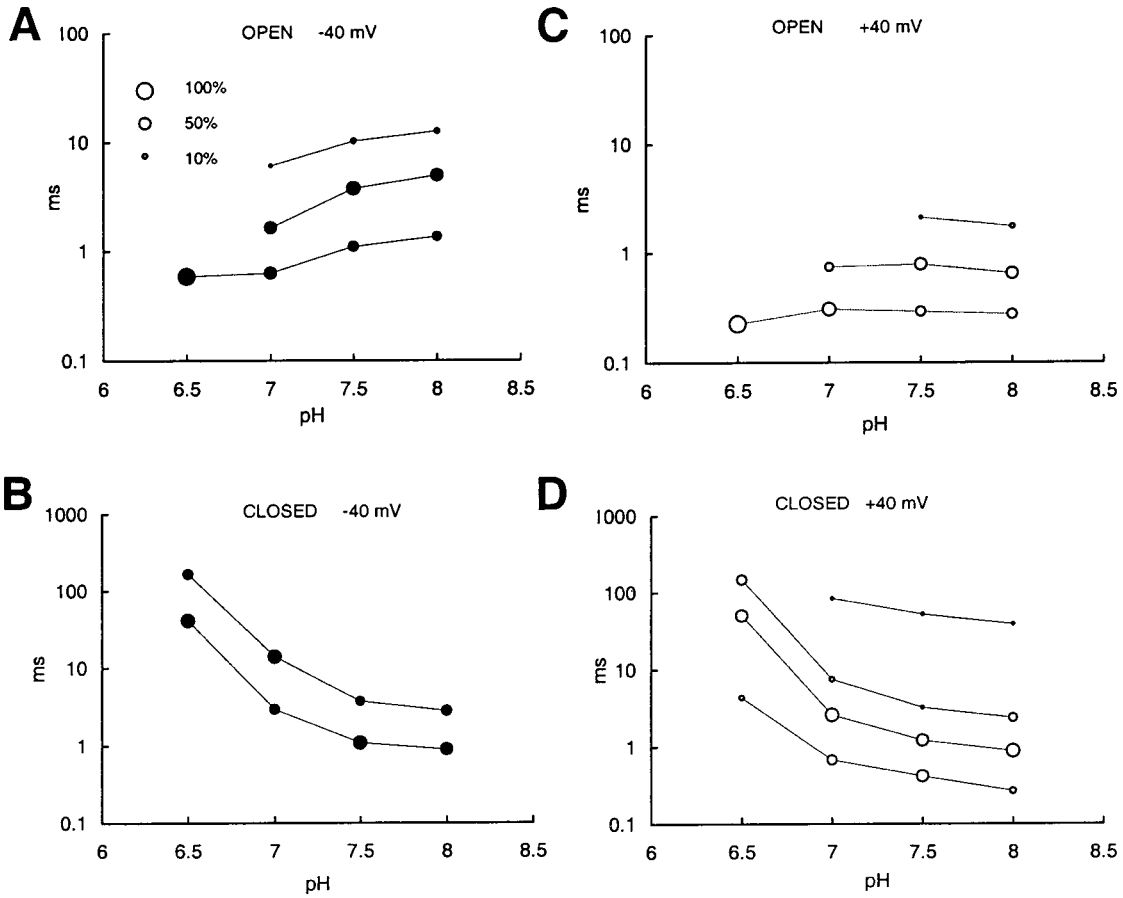

FIGURE 12 The effect of cytoplasmic pH on the parameters of exponential fits to open (*A, C*) and closed (*B, D*) dwell-time distributions from one RyR, at both −40 mV (*A, B*) and +40 mV (*C, D*). At each pH, the relative proportion of events associated with each exponential within the distributions are indicated by the size of the symbol. (Note that the sum of all of the proportions at a given pH is 100%.)

pH range 8 to 6.5, even though the number of time constants and their absolute values differed considerably.

The overall pH dependence of the dwell-time distributions can be analyzed more simply by their mean dwell time rather than by their individual exponential components. The mean dwell-time approach makes no assumptions about the number of exponential components actually present in the distributions, thus avoiding erroneous fitting of dwell-time distributions that would occur if the number of components is underestimated. Such an analysis also allows comparison between cases where there are different numbers of time constants present (e.g., Fig. 12, *B* and *D*). The mean open and closed dwell times obtained from the histograms in Fig. 11 plus data from two other membrane potentials are shown in Fig. 13. In Fig. 13 the effect of membrane potential and pH on RyR mean dwell times is quite apparent. The mean open dwell time is significantly shorter at positive membrane potentials, whereas the mean closed dwell time was relatively unaffected by membrane voltage. This is also apparent from the individual time constants in Fig. 12. Thus the voltage dependence of P_o seen in Fig. 4 is evidently mediated mainly by changes in the open dwell time.

The pH dependence of mean dwell times showed trends similar to that seen for individual time constants (see above). Cytoplasmic pH had a much stronger effect on the RyR closed dwell time than on open dwell time. The order of the pH dependencies was investigated by fitting the mean open (τ_{open}) and closed (τ_{closed}) dwell times with polynomial equations:

$$\tau_{open}^{-1} = \tau_{o\,max}^{-1} + k_o[H^+]^{n_o} \quad (2)$$

$$\tau_{closed} = \tau_{c\,min} + k_c[H^+]^{n_c} \quad (3)$$

where τ_{omax} and τ_{cmin} are the mean open and closed times of the RyR in the absence of pH inhibition, n_o and n_c are the orders of each equation, and k_o and k_c are constants that reflect the RyR sensitivity to pH inhibition. The parameters n_o and n_c are indicative of the minimum number of bound protons needed to close the channel (n_o) and the number of additional protons that can bind to the closed channel (n_c). The parameters τ_{omax} and τ_{cmin} describe RyR properties in the absence of proton binding, whereas k_c, k_o, n_c, and n_o describe characteristics of proton inhibition. The data from one experiment are fitted with Eqs. 2 (Fig. 13 *A*) and 3 (Fig.

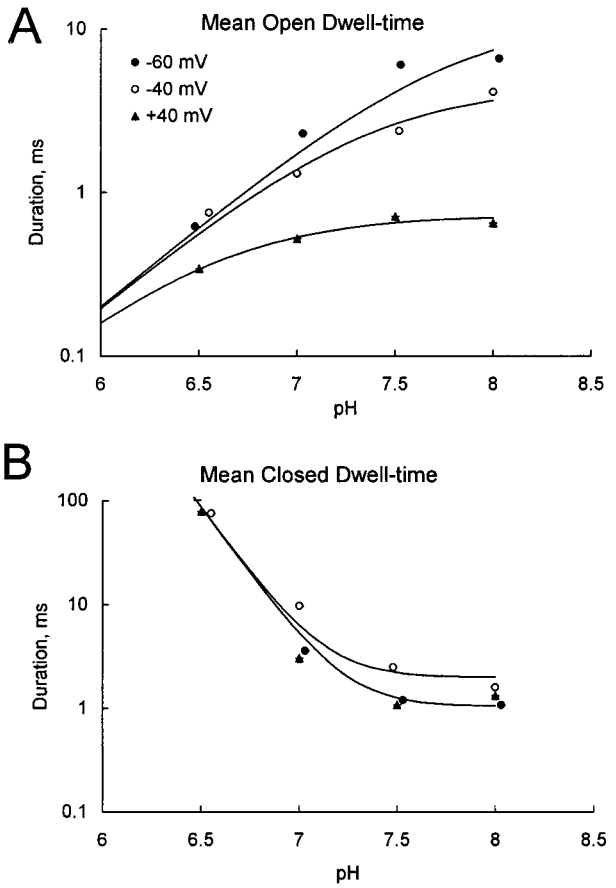

FIGURE 13 Mean open (*A*) and closed (*B*) dwell times at three membrane potentials and over a range of pH. The data were obtained from the same experiment as used for Figs. 11 and 12. The mean open duration of RyRs is shorter at positive membrane potentials, whereas the mean closed times were relatively unaffected by voltage. The solid curves show fits of Eqs. 2 (in *A*) and 3 (in *B*) to the data. The parameter values common to the three membrane voltages describe the pH inhibition of the RyR; these are $k_o = 5 \times 10^6$, $n_o = 1.0$, $k_c = 8 \times 10^{18}$, and $n_c = 2.6$ (units for k_o and k_c depend on the values for n_o and n_c; see Eqs. 2 and 3). The parameters that depended on membrane voltage describe the opening and closing of the noninhibited RyR; these are $\tau_{omax} = 0.7$ ms (+40 mV), 4.5 ms (−40 mV), 11.8 ms (−60 mV), and $\tau_{cmin} = 1.0$ ms (+40 mV), 2.0 ms (−40 mV), 1.1 ms (−60 mV).

13 *B*). The same values of k_c, k_o, n_c, and n_o were able to adequately fit the data at all voltages, whereas τ_{omax} and τ_{cmin} were voltage-dependent. Thus it appears that the pH inhibition- and voltage-dependent regulation mechanisms work independently of each other. Fits to the five experiments gave $n_c = 2.3 \pm 0.3$ and $n_o = 1.2 \pm 0.2$. When Eqs. 2 and 3 were instead fitted (where possible) to the pH dependence of each time constant obtained in the same experiments (e.g., for data in Fig. 12), the mean values obtained for n_c (1.8 ± 0.2, $n = 20$) and n_o (0.88 ± 0.11, $n = 16$) were smaller, though each was still of a similar order (i.e., ~2 and ~1, respectively). (This difference is the result of the pH dependence of the mean dwell time, reflecting change in both the size and the relative weighting factor of the individual time constants; e.g., in Fig. 12 *A*, see the shift toward briefer openings and the shortest time constant as the pH decreases.) Thus, irrespective of whether one considers the mean dwell times or the individual components, such an analysis suggests an inhibition mechanism whereby 1) the binding of a single proton inhibits the channel and 2) an additional two protons (or perhaps three protons, based on mean dwell-time analysis) are able to bind to the inhibited RyR, with the RyR only being able to open after all of these protons have dissociated. One possible physical arrangement of this mechanism is illustrated in Fig. 14.

DISCUSSION

This study has characterized the effect of both cytoplasmic and luminal pH on the activity of isolated RyRs from skeletal muscle, using solutions with accurately known pH and $[Ca^{2+}]$. Decreasing the pH below 7.0 on the cytoplasmic side of the RyR reduced channel open probability (P_o), with no change in conductance. Channel activity was more than 90% inhibited at cytoplasmic pH 6, with a pH causing half-maximum inhibition (pH_I) of ~6.5, irrespective of whether the channel was activated by either Ca^{2+} or ATP or by both together (Figs. 3 and 4 and Table 2). Bilayer potential also had no noticeable effect on the pH_I. The onset of inhibition was very rapid (~1–2 s) and may have been limited primarily by the speed of solution exchange at the bilayer surface (Fig. 2). If the exposure to inhibitory levels of cytoplasmic pH was only for several seconds, RyR activity could be restored very rapidly (≤1 s) by returning to pH 7 (Fig. 2). However, if the exposure was prolonged (30

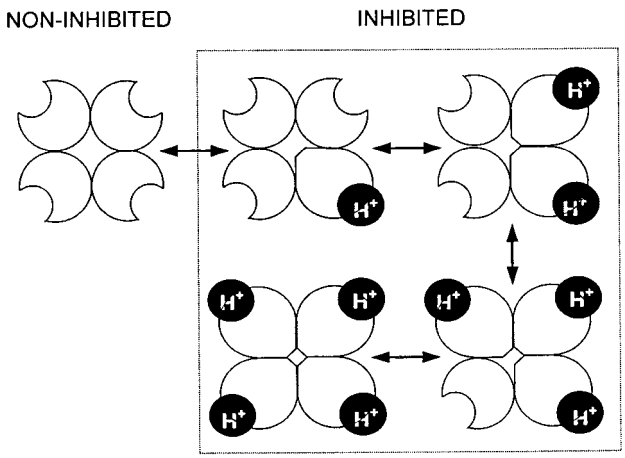

FIGURE 14 A diagram of a simple model for proton inhibition that is consistent with both the data and the homotetrameric structure of RyRs. The noninhibited state shown here embraces a whole range of RyR open and closed states, which can be discerned from normal channel function other than pH inhibition (e.g., modal gating, voltage- and ligand-dependent regulation). In this scheme, all subunits are able to bind protons, and the binding of a single proton to an inhibitory site on any subunit can inhibit the RyR.

s to >5 min), it took 30 s or more for the activity to return to the control level. Importantly, inhibition by cytoplasmic pH showed no noticeable "hysteresis," with the RyR activity at a given pH being approximately the same, regardless of whether the pH had been acidic or alkaline for several minutes (Fig. 7). This was also the case when the RyR had been modified with ryanodine, which favors channel activation even in the absence of Ca^{2+} and ATP. To cause inhibition in ryanodine-modified channels, cytoplasmic $[H^+]$ had to be raised ~10-fold higher than in unmodified channels (pH_I ~5.5; Fig. 5), but the inhibition was readily reversible and showed no evidence of hysteresis.

There was a high degree of variability in the gating behavior of different RyRs at physiological pH and in their sensitivity to inhibition by low pH. Variations in RyR function have also been reported in other studies of channel gating by Mg^{2+} and Ca^{2+} (Copello et al., 1997) and voltage-dependent inactivation (e.g., Ma, 1994). Functional variations may stem from RyRs that are differently coupled to accessory proteins known to affect RyR function, such as DHPR (Melzer et al., 1995), calsequestrin (Kawasaki and Kasai, 1994), FK506-binding protein (Timerman et al., 1993; Ahern et al., 1994), and calmodulin (Tripathy et al., 1995) or from differences in RyR oxidation or phosphorylation states (Witcher et al., 1991; Herrmann-Frank and Varsanyi, 1993; Hain et al., 1994; Marengo et al., 1998). The significance of this heterogeneity is not clear (a detailed discussion of this is given by Copello et al. (1997)). It may reflect an important functional diversity of RyRs in muscle (Copello et al., 1997) or could have been introduced during the process of isolating SR membranes (Marengo et al., 1998).

The way in which acidification of the cytoplasmic solution causes inhibition is not clear. Potentially, channel activity might be inhibited by the protonation of any of many sites on the RyR. Alternatively, chemical changes in the lipid bilayer at extreme pH (5 and 9) might alter RyR function by altering protein-lipid interactions. The slow recovery of RyRs from inhibition at low pH and irreversible effects of luminal pH could conceivably arise by such a mechanism. The effect of lipid bilayer composition on RyR function is not known. However, given that inhibition of Ca^{2+} release from SR vesicles (Meissner, 1984; Michalak et al., 1988) exhibits the same pH sensitivity as RyRs in artificial bilayers, it is apparent that the pH effects reported here are not due simply to some peculiarity of the lipid bilayer composition.

The findings that the cytoplasmic pH inhibition 1) did not depend on bilayer potential, 2) was not accompanied by a change in conductance, and 3) was quite distinct from the inhibition occurring with luminal acidification (see below) suggest that the inhibitory site is not inside the channel pore, as had previously been suggested (Ma et al., 1988). The inhibition apparently was not due to protons competing with Ca^{2+} for the cytoplasmic activation site, because the level of inhibition was not reduced when the concentration of activating Ca^{2+} was raised more than 100-fold (Fig. 8). Because acidic cytoplasmic pH also inhibited channel activation by ATP, with the pH_I indistinguishable from that for Ca^{2+} activation, it seems most likely that the protons can bind and inhibit RyRs independently of their method of activation (ryanodine activation excepted). The open and closed dwell times of channels at different levels of steady-state inhibition by cytoplasmic pH (Figs. 11–13) indicated an inhibition mechanism in which the binding of one proton closed the channel and the binding of two or three additional protons promoted further inhibited states, with the binding rates independent of membrane potential. Given that the RyR is composed of four identical subunits (Meissner, 1994) that normally function together to produce all-or-none channel opening, this inhibitory effect of pH suggests that proton binding to the cytoplasmic face of any one of the subunits is sufficient to prevent channel activation by ATP or Ca^{2+} (e.g., see Fig. 14). More complex inhibition models may also be consistent with the data and the homotetrameric structure of RyRs. For example, the data could also be explained by a model in which some, but not all, of the inhibited states reflect modes of relatively low open probability rather than zero open probability. Ryanodine-modified channels required a 10-fold higher concentration of protons to inhibit channel opening, and there was an increase in substate activity (e.g., Fig. 5 *C*) like that reported by Ma and Zhao (1994), possibly indicating that in ryanodine-modified channels full channel closure only occurred at a much higher $[H^+]$ when protons were simultaneously bound to most or all of the subunits. The cause of the relatively slow recovery of RyRs from inhibition with relatively long exposures to low pH is not clear.

The effect of pH on the SR luminal side of the RyR was very different from the effect on the cytoplasmic side. First, channel activity was virtually unaffected by any pH change in the physiological range (7.5 to 6.5); there was inhibition only at pH <6 (Figs. 9 and 10). Second, when exposure of RyRs to a luminal pH of 5.5 to 5 for several seconds abolished activity, raising the pH again could not readily restore it. There was little or no recovery in about half of the RyRs examined, with no significant difference in the extent of recovery at pH 7.5 or pH 9. Furthermore, there was poorer recovery when RyRs were exposed to the luminal pH 5 solution for a second or third (brief) period, possibly indicating a progressively greater level of irreversible change in the RyR properties with longer exposure periods at very acidic pH.

Comparison with other findings

It is pertinent that Ma and Zhao (1994) found that when native skeletal RyRs were first subjected to acid conditions, that is, before there was any possible irreversible change in properties, the pH_I (for a simultaneous change in both the

cytoplasmic and luminal pH) was ~6.5. This is in good accord with the findings here (i.e., pH_I 6.5 for cytoplasmic pH changes and no effect of luminal pH until <6). Interestingly, in purified skeletal RyRs the cytoplasmic pH_I is much higher than this (~7.2) (Ma et al., 1988; Shomer et al., 1994a). Furthermore, the luminal pH_I is also much higher in purified RyRs (pH_I ~7.2) than in native channels ($pH_I < 6$; this study and Rousseau and Pinkos, 1990). Thus it is possible that purification of the skeletal RyRs renders them more sensitive to inhibition by both cytoplasmic and luminal protons. A further reason to suspect that the pH inhibition may be altered in purified RyRs is that the pH_I for Ca^{2+} release and ryanodine binding in skeletal SR vesicles is much lower (~6.5) (Meissner, 1984; Michalak et al., 1988), close to that found here in isolated native RyRs.

Ma and Zhao (1994) also reported that after the RyRs were inhibited by simultaneous lowering of both the cytoplasmic and luminal pH to ~5.5, the pH_I, upon the subsequent rise in pH, was found to be considerably higher (~7.4) than the original value and, in fact, was similar to that found in the studies with purified channels. They described this phenomenon as "hysteresis." Interestingly, when these authors examined ryanodine-modified channels, they had to drop the pH (both cytoplasmic and luminal) even lower (to ~pH 5) to get full inhibition (as found here too; see Fig. 5), and then they found that the activity did not recover even at pH 8.5–9 in a high proportion of cases. It seems possible to explain these results in terms of our findings that 1) recovery from low cytoplasmic pH was complete though often quite slow after prolonged exposure, and 2) a lower luminal pH did not affect the response until it reached a critical level in the range of pH 5.5 to 5, where it induced very slowly reversible or possibly irreversible changes that increased with the duration of the exposure. Thus, when Ma and Zhao exposed unmodified RyRs to progressively lower pH levels down to 5.5, they may have induced a slowly but fully reversible inhibition due to changes on the cytoplasmic side of the RyR, whereas when they exposed the ryanodine-modified channels to even lower pH, they may have also elicited irreversible changes occurring at the luminal side of the RyR. It is possible that low luminal pH could cause irreversible change in the RyR properties by altering the interaction between the RyR and some normally associated luminal protein, such as calsequestrin. Nevertheless, it is likely that at least some of the effect of low luminal pH is due to change in the skeletal RyR itself (or the bilayer-RyR interaction; see earlier), because a similar, apparently irreversible, abolition of activity is also observed when purified cardiac RyRs are exposed to low luminal (and cytoplasmic) pH (Xu et al., 1996).

It is not clear why Rousseau and Pinkos (1990) obtained a higher estimate of cytoplasmic pH_I in native skeletal RyRs (~7.2) than obtained here and by Ma and Zhao (1994) and in SR vesicles studies (Meissner, 1984; Michalak et al., 1988). One could propose that it resulted from their use of a very high [Ca^{2+}] (50 mM) on the luminal side of the RyR. This may have modified the characteristics of RyR activation either directly or via an associated protein such as calsequestrin (Hidalgo and Donoso, 1995; Sitsapesan and Williams, 1997) or by accessing the cytoplasmic Ca^{2+} activation site (Tripathy and Meissner, 1996). However, we found no noticeable difference in the pH_I, regardless of whether we had 1 nM, 5 mM, or 50 mM Ca^{2+} on the luminal side (Table 3). Instead, it is possible that the data of Rousseau and Pinkos (1990) reflect only a limited subset of RyRs, because their brief report does not specify the number of RyRs actually examined, and it was found here that some RyRs are inhibited at relatively high cytoplasmic pH levels (Fig. 6).

In this study we did not observe the "biphasic" response to pH changes reported by Ma and Zhao (1994), where channel activity showed a moderate depression at pH 7.5, then increased ~25% at pH 7.0, before declining progressively to zero at more acidic pH. This behavior was possibly due to changes in the [Ca^{2+}] that accompanied the pH changes. Ma and Zhao (1994) used 1 mM EGTA to try to buffer the [Ca^{2+}] and added 2 mM ATP to a cytoplasmic solution (at pH 7.5) with excess Ca^{2+} (1.1 mM total, 100 μM free), thereby lowering the free [Ca^{2+}] to ~10 μM. As the affinity of EGTA for Ca^{2+} is reduced 100-fold per unit decrease in pH (Harrison and Bers, 1987) and the affinity of ATP for Ca^{2+} is also reduced, the free [Ca^{2+}] would have increased toward more optimal activating levels at pH 7.0 (e.g., ~20–30 μM), thereby possibly explaining the increase in channel activity. When the pH was reduced further (below pH 7), the activity would decline again, because of the inhibitory effect of protons on the RyR. The change in free [Ca^{2+}] on the luminal side, where EGTA was also used and the pH was altered in tandem with the cytoplasmic side, may also have contributed to the observed channel behavior.

In a final comparison, we did not find any change in channel conductance with reductions in either cytoplasmic or luminal pH, in agreement with the findings of Xu et al. (1996) in purified cardiac RyRs. Rousseau and Pinkos (1990) reported that the channel conductance in native skeletal RyRs was reduced 27% by decreasing the luminal pH from 7.4 to 6.8. However, this pH change was achieved by exchanging the luminal solution from a 50 mM Ca^{2+}/250 mM HEPES solution at pH 7.4 to a 50 mM Ca^{2+}/250 mM piperazine-*N,N′*-bis(2-ethanesulfonic) acid (PIPES) solution at pH 6.8. Hence the reduced conductance was quite possibly due to the additional (unspecified) cations (250 mM mEq) that must also have been present to counter the average of 1.5 negative charges per PIPES molecule at pH 6.8.

Relationship to muscle fatigue

With repeated activity the pH inside a muscle fiber can decline from ~7.1 to as low as ~6.5 (Fitts, 1994). The

almost complete inhibition of channel activity at pH 6.5 observed in previous single-channel studies (Ma et al., 1988; Rousseau and Pinkos, 1990) is often cited as evidence that a decrease in pH could cause the reduction in Ca^{2+} release that underlies the late stage of muscle fatigue. The data in this study indicate that RyR activity is far less sensitive to inhibition by either cytoplasmic or luminal acidification than suggested by the previous studies, and that the pH would never reach a low enough level physiologically to cause irreversible changes in the activation characteristics of the RyRs. Nevertheless, if RyR activation in a muscle fiber were inhibited by pH in the same way as found here with Ca^{2+} and ATP activation of isolated channels, one would still expect to see an appreciable reduction in Ca^{2+} release at pH 6.5. In contrast to such an expectation, depolarization-induced Ca^{2+} release is hardly reduced if at all in skinned fibers from rat and toad at pH 6.2 (Lamb et al., 1992; Lamb and Stephenson, 1994) or in intact fibers from mouse at pH 6.5 (Bruton et al., 1998) or frog at pH 6.3 (Baker, Brandes and Weiner, 1995). It is possible that the pH in the highly restricted space of the triad junction is not well controlled in such experiments, even with the heavy pH buffering used in the case of the skinned fibers (28 mM PIPES and 60 mM HEPES), because the acidification of the SR lumen occurring during rapid Ca^{2+} efflux (Kamp et al., 1998) probably involves an appreciable influx of protons from the triadic junction. Consequently, if RyR activity in vivo were only depressed to the extent found in this study with Ca^{2+}/ATP activation of the isolated RyRs (e.g., to ~20% of normal, at pH 6.2), it is possible that the initial Ca^{2+} release, though small, might be enough to cause an increase in pH in the triad junction, thereby reducing the pH inhibition and allowing full release. (We note, however, that if RyR activation were inhibited by pH as strongly as suggested in previous single-channel studies, such an explanation would not be tenable, as the initial Ca^{2+} release rate would be too low to induce relief of pH-inhibition.) Alternatively, the discrepancy between the findings with isolated RyRs and functioning fibers might simply reflect that Ca^{2+}/ATP activation of the RyR is not the normal physiological activation mechanism and that voltage sensors in the T-system can potently activate the RyRs, even at low pH, because they either overcome or simply bypass the inhibitory effect of protons on the RyRs, as they evidently do with Mg^{2+} inhibition (Lamb and Stephenson, 1991, 1994). It should also be noted that the pH inhibition data were obtained here (as in other single-channel studies) in the absence of Mg^{2+}, because Mg^{2+} potently inhibits activation of isolated RyRs. Consequently, we cannot be sure that the pH inhibition observed here would be the same as in an intact fiber in the presence of physiological [Mg^{2+}]. Furthermore, it could be argued that the properties of RyRs in bilayers are different from those in situ. Finally, another possible explanation for the discrepancy is that some other factor present in skinned and intact fibers and not in the isolated RyRs shifts the pH dependence of the inhibition somewhat lower than observed here (e.g., pH_I at 6.0 rather than 6.5). This could be due to the physical coupling between the voltage sensors and the RyRs or might be due to the accompanying acidification of the SR lumen upon Ca^{2+} release, as such acidification has been shown to augment Ca^{2+} release markedly over the pH range 7 to 6 (Donoso et al., 1996).

In conclusion, the results of this study indicate 1) that isolated RyRs from mammalian skeletal muscle are not inhibited as strongly by low cytoplasmic and luminal pH as previously suggested, and 2) that the intracellular pH would never decrease sufficiently in a functioning fiber to cause any "hysteresis" or irreversible change in the RyR properties. It is not yet known for certain why low pH apparently has even less effect on E-C coupling than on Ca^{2+}/ATP activation of isolated RyRs.

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