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Physiology

Evidence for a Ca^{2+}-gated ryanodine-sensitive Ca^{2+} release channel in visceral smooth muscle

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ABSTRACT **Although a role for the ryanodine receptor (RyR) in Ca^{2+} signaling in smooth muscle has been inferred, direct information on the biochemical and functional properties of the receptor has been largely lacking. Studies were thus carried out to purify and characterize the RyR in stomach smooth muscle cells from the toad *Bufo marinus*. Intracellular Ca^{2+} measurements with the Ca^{2+}-sensitive fluorescent indicator fura-2 under voltage clamp indicated the presence of a caffeine- and ryanodine-sensitive internal store for Ca^{2+} in these cells. The (CHAPS)-solubilized, [^{3}H]ryanodine-labeled RyR of toad smooth muscle was partially purified from microsomal membranes by rate density centrifugation as a 30-S protein complex. SDS/PAGE indicated the comigration of a high molecular weight polypeptide with the peak attributed to 30-S RyR, which had a mobility similar to the cardiac RyR and on immunoblots cross-reacted with a monoclonal antibody to the canine cardiac RyR. Following planar lipid bilayer reconstitution of 30-S stomach muscle RyR fractions, single-channel currents (830 pS with 250 mM K^+ as the permeant ion) were observed that were activated by Ca^{2+} and modified by ryanodine. In vesicle-$^{45}Ca^{2+}$ efflux measurements, the toad channel was activated to a greater extent at 100–1000 μM than 1–10 μM Ca^{2+}. These results suggest that toad stomach muscle contains a ryanodine-sensitive Ca^{2+} release channel with properties similar but not identical to those of the mammalian skeletal and cardiac Ca^{2+}-release channels.**

In smooth muscle, as in other muscle, contractile activity appears to be principally controlled by the level of free cytoplasmic Ca^{2+} (1). Increases in cytosolic Ca^{2+} concentration result from entry of Ca^{2+} ions across the cell's surface membrane and/or release of Ca^{2+} from an intracellular membrane compartment, the sarcoplasmic reticulum (SR) (2). Two mechanisms have been suggested to be involved in physiological release of Ca^{2+} from the SR. One responds to a change in surface membrane potential (3, 4) and involves the activation of a ryanodine-receptor (RyR)–Ca^{2+}-release channel complex (5, 6). The voltage-dependent mechanism has been extensively studied in striated muscle (5, 6). The second mechanism involves the agonist-induced formation of inositol 1,4,5-trisphosphate (IP_3), which causes release of Ca^{2+} into the cytoplasm by activating an IP_3 receptor–Ca^{2+}-release channel complex located in the SR membrane (7, 8). There is ample evidence that the IP_3-mediated mechanism plays a major role in regulating levels of free cytoplasmic Ca^{2+} in smooth muscle cells (2, 9). The role of the RyR–Ca^{2+}-release channel complex is less certain in smooth muscle, however. Ca^{2+}-induced Ca^{2+} release, which is presumed to be the predominate mechanism of RyR-mediated Ca^{2+} release in heart (4, 10, 11), has been demonstrated in skinned smooth muscle (12), but evidence for this mechanism in intact smooth muscle has been largely lacking. While experiments in intact tissue have demonstrated an effect of ryanodine *per se* on both cytoplasmic Ca^{2+} levels and contractile activity (13, 14), clear evidence of Ca^{2+}-induced Ca^{2+} release that is ryanodine sensitive, similar to that in cardiac muscle (15), has been elusive. Perhaps the best evidence to date for a role of Ca^{2+}-induced Ca^{2+} release in smooth muscle has been obtained by studying the effects of pharmacological agents on ionic currents and Ca^{2+} levels in single smooth muscle cells. While a ryanodine-sensitive component of the Ca^{2+} signal resulting from depolarization was reported, secondary effects of ryanodine precluded characterization of the putative Ca^{2+}-induced Ca^{2+}-release mechanism even in this system (16).

Progress in understanding the physiological role of the RyR-mediated Ca^{2+}-release mechanism in smooth muscle has been hampered by the lack of detailed information about the chemical and functional properties of these channels in smooth muscle. Recently, the single-channel properties of a 30-S RyR from mammalian aortic tissue have been described (17), and the mRNA of the RyR has also been identified (18, 19). Upon reconstitution of an enriched, detergent-solubilized receptor preparation into planar lipid bilayers, a Ca^{2+}-gated, ryanodine- and caffeine-sensitive, cation-conducting channel was observed. Because of the low abundance of the RyR in the aorta, further biochemical and functional characterization of the RyR from this tissue was not possible.

In this report, we describe the first partial purification and preliminary characterization of the properties of a RyR from smooth muscle. We have chosen to carry out these studies with the stomach smooth muscle of the toad *Bufo marinus* because we found that the RyR is more abundant in this muscle and that relatively large amounts of a pure preparation of smooth muscle can be obtained from this source (20). This allowed us to obtain sufficient amounts, in contrast to an earlier report (17), to define the ryanodine-binding properties of the receptor and to obtain an estimate of its molecular weight and antigenic properties relative to ryanodine receptors from cardiac and skeletal muscle. Furthermore, we were able to demonstrate that these receptors function to release Ca^{2+} from internal stores in these smooth muscle cells by studying both intact cells and isolated membrane vesicles, the results thus providing direct evidence for both the properties of the RyRs and their role in Ca^{2+} signaling in smooth muscle.

EXPERIMENTAL PROCEDURES

Intracellular Ca^{2+} Measurements. Single smooth muscle cells were isolated by enzymatic disaggregation from thin

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Abbreviations: SR, sarcoplasmic reticulum; RyR, ryanodine receptor; IP_3, inositol 1,4,5-trisphosphate; CHAPS, 3-[3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate.

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slices of the stomach muscularis obtained from the toad *Bufo marinus* (20). Changes in intracellular Ca^{2+} and transmembrane ionic currents were simultaneously recorded in cells that were loaded with the Ca^{2+}-sensitive fluorescent dye, fura-2. Intracellular Ca^{2+} was measured with a high-speed dual-wavelength microfluorimeter described previously (1, 21, 22), and whole-cell membrane currents were recorded with an Axopatch-1D amplifier. The patch–pipette solution contained 130 mM CsCl, 4 mM $MgCl_2$, 3 mM Na_2ATP, 1 mM Na_3GTP, 0.05 mM fura-2 pentapotassium salt, and 20 mM Hepes (pH 7.2 with CsOH). At least 5 min at a holding membrane potential of −80 mV were allowed for equilibration of pipette contents and intracellular milieu. Three or four depolarizations during this period did not change the effectiveness of ryanodine as an inhibitor of caffeine-induced increase in intracellular Ca^{2+}. The extracellular medium contained 120 mM NaCl, 3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM tetraethylammonium chloride, and 5 mM Hepes (pH 7.6 with NaOH). Caffeine was applied to the cell by pressure ejection from a micropipette filled with 20 mM caffeine in extracellular medium.

Isolation of SR Vesicles and 30-S RyR Complex. Stomach muscle of *Bufo marinus* was rapidly dissected, frozen in liquid N_2, and stored at −75°C before use. Microsomal fractions enriched in [^{3}H]ryanodine binding activity were isolated at 4°C from thawed muscle as a pellet from centrifugation at 2,600–40,000 × *g* in the presence of protease inhibitors (23). The 40,000 × *g* pellets were resuspended in 0.6 M KCl medium (23); the suspension was layered at the top of a discontinuous sucrose gradient consisting of 10 ml each of 25% and 35% sucrose in 0.6 M KCl and centrifuged for 2 hr at 130,000 × *g* in a Beckman SW 28 rotor. Membranes sedimenting at the sample/25% sucrose and 25%/35% sucrose interfaces were diluted with 2 volumes of 0.3 M KCl, sedimented, resuspended in 0.3 M sucrose/5 mM K Pipes, pH 6.8, and stored in 0.3-ml aliquots at −135°C. Both microsomal fractions were used in this study because both exhibited a similar [^{3}H]ryanodine and Ca^{2+}-release activity. The combined yield of the two microsomal fractions was 0.82 ± 0.38 mg of protein per g of muscle (*n* = 5). The 3-[3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPS)-solubilized 30-S toad smooth muscle RyR–Ca^{2+}-release channel complex was isolated by rate density gradient centrifugation in the presence of protease inhibitors (23).

$^{45}Ca^{2+}$ Efflux Measurements. Toad microsomal fractions (10 mg of protein per ml) were passively loaded with 1 mM $^{45}Ca^{2+}$ in a medium containing 0.1 M KCl, 20 mM Pipes potassium salt (pH 6.8), and 1 mM $^{45}Ca^{2+}$ as described (23). $^{45}Ca^{2+}$ efflux was initiated by diluting vesicles 1:100 into isomolar efflux medium and was stopped by placing 0.4-ml aliquots at various times on a 0.45-μm filter (type HA, Millipore). Filters were washed with a quench solution containing 0.1 M KCl, 20 mM Pipes potassium salt (pH 6.8), 5 mM Mg^{2+}, 20 μM ruthenium red, and 0.2 mM EGTA. Radioactivity remaining with the vesicles on the filters was determined by liquid scintillation counting.

[^{3}H]Ryanodine Binding. [^{3}H]Ryanodine binding was determined as described (24). Membranes were incubated for 3 hr at 37°C in medium containing 1 M NaCl, 20 mM Pipes sodium salt (pH 7.0), 100 μM Ca^{2+}, 5 mM AMP, 1 mM diisopropyl fluorophosphate, 5 μM leupeptin, and 0.5–20 nM [^{3}H]ryanodine. Nonspecific binding was estimated by using a 1000-fold excess of unlabeled ryanodine.

Single-Channel Recordings. Single-channel measurements were performed by incorporating 30-S toad RyR fractions, purified in the absence of [^{3}H]ryanodine, into Mueller–Rudin type planar lipid bilayers containing phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine in the ratio 5:3:2 (50 mg of total phospholipid per ml in *n*-decane) (25). A symmetric solution of buffered KCl [(0.25 M KCl/20 mM Pipes potassium salt (pH 7.0)] was used to record single-channel currents. Bilayer currents were recorded and analyzed as described (25).

Materials. [^{3}H]Ryanodine (54.7 Ci/mmol; 1 Ci = 37 GBq) and $^{45}Ca^{2+}$ were obtained from DuPont/New England Nuclear; unlabeled ryanodine was from Agri Systems (Wind Gap, PA). Caffeine was from Sigma. Phospholipids were purchased from Avanti Polar Lipids; CHAPS, from Boehringer Mannheim; ruthenium red, from Fluka; SDS gel molecular weight markers, from Sigma; and peroxidase-conjugated secondary antibodies, from Calbiochem. All other chemicals were of analytical grade.

Statistics. Errors reported with all averages are the standard deviation of the mean.

RESULTS

The presence of a caffeine- and ryanodine-sensitive mechanism for the release of Ca^{2+} from internal stores in the smooth

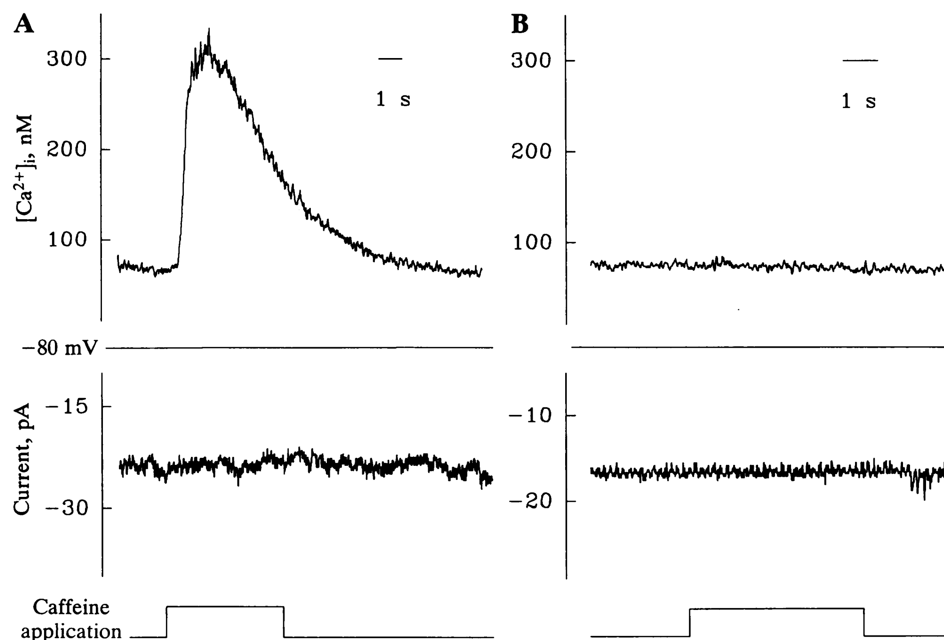

FIG. 1. Effect of caffeine and ryanodine on intracellular Ca^{2+} transients in single voltage-clamped toad stomach cells. (*A*) Effect of 20 mM caffeine applied by pressure ejection from a Buffer pipette to a cell voltage-clamped at −80 mV. (*B*) Effect of caffeine applied as in *A* to a cell exposed to 100 μM ryanodine in the patch pipette.

muscle cells of the stomach of *Bufo marinus* was assessed by determining the effects of these agents on cytoplasmic Ca^{2+} levels in single cells under voltage clamp. Fig. 1*A* shows that cytoplasmic Ca^{2+} increased when cells were exposed to caffeine. In these studies, the membrane potential was held at −80 mV to fully deactivate surface membrane voltage-sensitive Ca^{2+} channels (26). There was no detectable change in the holding current during the caffeine-induced increase in cytoplasmic Ca^{2+}, indicating that the increase was due to release from internal stores (Fig. 1*A*). On the other hand, in other such cells, when 100 μM ryanodine was present in the pipette solution (Fig. 1*B*), caffeine did not affect intracellular Ca^{2+} concentration. On average, caffeine caused a peak increase in free Ca^{2+} of 377 ± 75 nM from a resting level of 58 ± 26 nM in 14 cells, where it had no effect on the holding current. By contrast, in the presence of 100 μM ryanodine, caffeine failed to elicit significant transient Ca^{2+}, Ca^{2+} concentration changing only by 7.3 ± 21 nM from a resting level of 65 ± 19 nM in 14 cells, where it also had no effect on the holding current. These results suggest that the smooth muscle cells of the stomach of *Bufo marinus* contain a ryanodine- and caffeine-sensitive internal store for Ca^{2+}.

Fig. 2 illustrates the $^{45}Ca^{2+}$ efflux behavior of passively loaded toad microsomal membrane fractions in the presence of various concentrations of extravesicular Ca^{2+}. As previously observed for skeletal and cardiac SR vesicles enriched in Ca^{2+}-release activity (27), $^{45}Ca^{2+}$ efflux was slow in a medium containing <10 nM free Ca^{2+} and the two Ca^{2+} release inhibitors Mg^{2+} and ruthenium red. Omission of the two Ca^{2+}-release inhibitors from the nanomolar Ca^{2+} efflux medium resulted in an ≈2-fold increase in the $^{45}Ca^{2+}$ efflux rate from the microsomes. Further marked increases in $^{45}Ca^{2+}$ efflux rates were observed when vesicles were placed into media containing 1, 10, 100, or 1000 μM free Ca^{2+}. At 1 mM Ca^{2+}, vesicles released about half their $^{45}Ca^{2+}$ contents in 20 s. About one-third of the radioactivity remained with the vesicles for longer times, indicating the presence of a subpopulation of vesicles lacking a Ca^{2+}-gated Ca^{2+}-release pathway (27). The half times of $^{45}Ca^{2+}$ efflux from the Ca^{2+} permeable vesicle population are listed in Table 1.

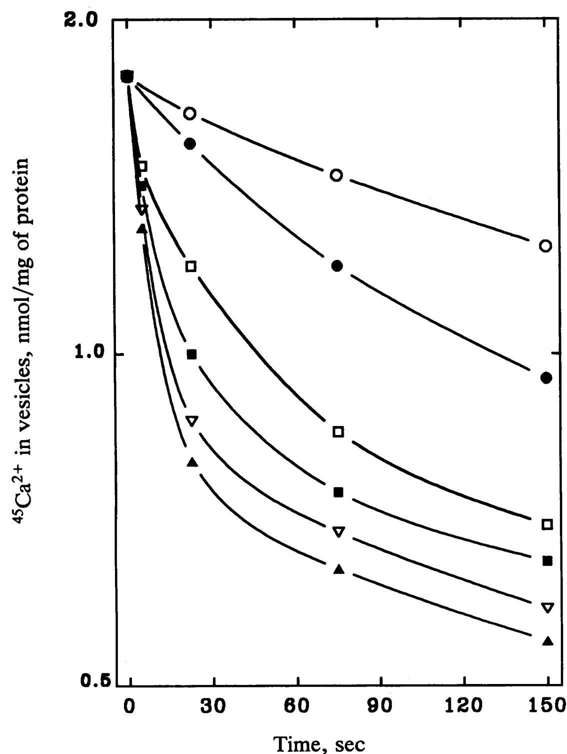

FIG. 2. Effect of extravesicular Ca^{2+} on $^{45}Ca^{2+}$ efflux rates from toad stomach muscle microsomes. Toad microsomes were passively loaded with 1 mM $^{45}Ca^{2+}$ and then diluted 100-fold into unlabeled 0.1 M KCl/20 mM Pipes potassium salt, pH 6.8, efflux medium (*i*) containing 10 mM Mg^{2+}, 20 μM ruthenium red, and 2 mM EGTA (○); or (*ii*) containing only 2 mM BAPTA (●); or (*iii*) containing 1 mM EGTA and various Ca^{2+} concentrations to yield 1 μM (□), 10 μM (■), 100 μM (▽), or 1 mM (▲) free Ca^{2+} (BAPTA = bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetracetate). $^{45}Ca^{2+}$ radioactivity remaining with the vesicles at the indicated times was determined by filtration as described in text.

The effects of several additional compounds reported to activate the mammalian skeletal and cardiac ryanodine-sensitive Ca^{2+}-release channels were tested (5, 6). Addition of 5 mM ATP to a medium with <10 nM Ca^{2+} increased the $^{45}Ca^{2+}$ efflux rate about 2-fold, whereas addition of 5 mM adenosine 5′-[α,β-methylene]triphosphate (a nonhydrolyzable ATP analog) to a 10 μM Ca^{2+}-containing medium was without a significant effect on the $^{45}Ca^{2+}$ efflux rate (Table 1). The addition of 10 mM caffeine to nanomolar and micromolar Ca^{2+}-release media resulted in a small (≈1.2-fold) stimulation of $^{45}Ca^{2+}$ efflux. The presence of 10 μM IP_3 in nanomolar and micromolar Ca^{2+} efflux media did not significantly alter the $^{45}Ca^{2+}$ efflux rate from toad microsomal membrane fractions. Table 1 further shows that the Ca^{2+}-activated efflux pathway in toad microsomes could be partially inhibited by 5 mM Mg^{2+}.

[^{3}H]Ryanodine-binding experiments indicated that the drug bound with nanomolar affinity to toad microsomal membrane fractions. Scatchard analysis of binding data indicated the presence of a specific high-affinity site with a B_{max} value of 0.4 pmol/mg of protein and K_d of 2 nM (not shown). On average, toad microsomes bound 0.50 ± 0.19 pmol of [^{3}H]ryanodine per mg of protein (*n* = 5), as compared with <0.1 pmol/mg of protein for aorta microsomal membranes (17) and 5–20 pmol/mg of protein for rabbit skeletal and canine cardiac SR vesicles (6).

The CHAPS-solubilized, [^{3}H]ryanodine-labeled toad RyR was partially purified by a double sucrose gradient procedure (23). Analysis of gradients revealed a bound peak of [^{3}H]ryanodine with an apparent sedimentation coefficient of 30 S (Fig. 3*A*). SDS gel analysis showed that [^{3}H]ryanodine peak

Table 1. $^{45}Ca^{2+}$ efflux from toad stomach microsomes

Free Ca^{2+}, M	Additions to efflux medium (mM)	$^{45}Ca^{2+}$ efflux half-times, sec (*n*)
$<10^{-8}$	Ruthenium red (0.02)	
	+ Mg^{2+} (10)	145 ± 60 (10)
	—	88 ± 47 (8)
	ATP (5)	44 ± 16 (4)
	Caffeine (10)	68 ± 16 (4)
	IP_3 (0.01)	83 ± 34 (5)
10^{-6}	—	40 ± 10 (3)
10^{-5}	—	34 ± 10 (6)
	AMP-PCP (5)	30 ± 5 (3)
	Caffeine (10)	27 ± 3 (3)
	IP_3 (0.01)	46 ± 13 (3)
	Mg^{2+} (5)	76 ± 19 (4)
10^{-4}	—	21 ± 11 (5)
10^{-3}	—	19 ± 6 (5)

The half-times ($t_{1/2}$) of $^{45}Ca^{2+}$ efflux were determined after subtracting the amounts of $^{45}Ca^{2+}$ retained by the microsomes at 150 s in 1 mM Ca^{2+} efflux medium (0.5–1.0 nmol/mg of protein; see Fig. 2). Addition of 10 mM Mg^{2+} and 20 μM ruthenium red to $<10^{-8}$ M medium fully inhibited the Ca^{2+}-release channel in single-channel measurements—i.e., $^{45}Ca^{2+}$ efflux in medium containing 10 mM Mg^{2+} and 20 μM ruthenium red represents baseline Ca^{2+} permeability of microsomal membranes. Number in parentheses indicates the number (*n*) of experiments carried out in duplicate. AMP-PCP, adenosine 5′-[α,β-methylene]triphosphate.

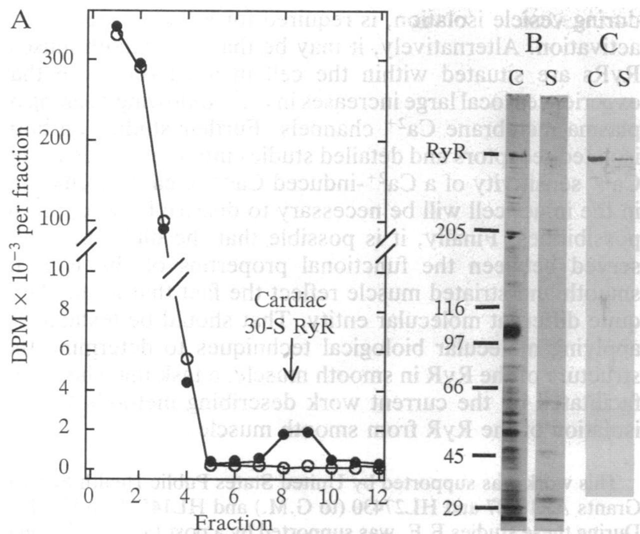

FIG. 3. Sedimentation, SDS gel, and immunoblot analysis of CHAPS-solubilized toad RyR. (*A*) Toad stomach muscle microsomes (1.5 mg of protein per ml) were solubilized with 1.6% CHAPS in the presence of 4 nM [^{3}H]ryanodine (●) or 4 nM [^{3}H]ryanodine plus 4 μM unlabeled ryanodine (○) and were centrifuged through a 5–20% linear sucrose gradient in a Beckman SW 41 rotor. Twelve fractions of 1 ml each were collected and analyzed for tritium radioactivity. The arrow indicates the position of the [^{3}H]ryanodine peak fraction of a parallel gradient containing the CHAPS-solubilized, [^{3}H]ryanodine-labeled 30-S canine cardiac muscle RyR. (*B*) Coomassie blue-stained SDS/polyacrylamide 5–20% gradient gels (25) of canine cardiac SR membranes (27) (lane C) and toad sucrose gradient fraction 9 of *A* (lane S). Positions of the cardiac RyR polypeptide of $M_r \approx 565{,}000$ and molecular weight standards ($\times 10^{-3}$) are indicated on the left. (*C*) Canine cardiac SR proteins (lane C) and toad sucrose gradient fraction 9 of A (lane S) were electrophoresed through SDS/polyacrylamide 3–12% gradient gels, electrophoretically transferred onto Immobilon polyvinylidene difluoride membranes, incubated with a canine cardiac muscle RyR monoclonal antibody (C3-33) (28) and a peroxidase-conjugated secondary antibody as described (28), and developed with 3,3′-diaminobenzidine and H_2O_2.

fractions contained a high molecular weight protein band that comigrated with the cardiac RyR polypeptide of $M_r \approx 565{,}000$ (Fig. 3*B*). The high molecular weight toad protein band reacted with a monoclonal antibody to the canine cardiac RyR (Fig. 3*C*). No immunoreactivity was observed with a rat skeletal RyR antiserum (29) (not shown).

The presence of channel activity in the 30-S toad smooth muscle RyR complex was tested by incorporating the CHAPS-solubilized RyR, partially purified on a single sucrose gradient in the absence of [^{3}H]ryanodine, into Mueller–Rudin planar lipid bilayers. Channels were reconstituted in a symmetric 250 mM KCl/20 mM Pipes potassium salt (pH 7) medium, since the reconstituted skeletal and cardiac muscle Ca^{2+} release channels have been shown to be impermeant to Cl^- and to conduct monovalent cations more efficiently than Ca^{2+} (25, 30, 31). In Fig. 4*A* (top trace), a single K^+-conducting channel was nearly completely activated with 50 μM Ca^{2+} in the cis chamber (the side of the bilayer to which the sample was added; cis and trans chambers correspond to the SR cytoplasmic and lumenal sides, respectively; refs. 25 and 30). A decrease in channel activity after the addition of 1 mM EGTA cis (resulting in 60 nM free Ca^{2+}) and a subsequent increase to near the original level after the addition of 0.95 mM Ca^{2+} cis (resulting in 20 μM free Ca^{2+}) indicated that the channel could be reversibly activated by micromolar concentrations of cis Ca^{2+} (Fig. 4*A*, middle and bottom traces, respectively).

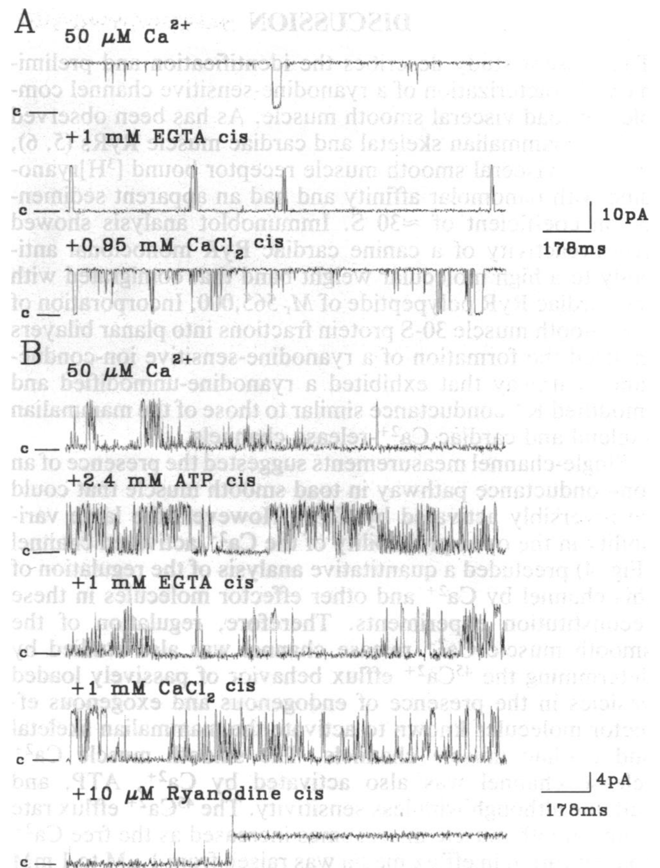

FIG. 4. Single-channel recordings of 30-S toad stomach sucrose gradient fractions reconstituted into planar lipid bilayers. (*A*) Single-channel currents, shown as upward deflections, were recorded in symmetric 0.25 M KCl/20 mM Pipes potassium salt, pH 7.0, medium containing 50 μM free Ca^{2+} cis (top trace, $P_o = 0.98$) and after the successive addition of 1 mM EGTA cis (resulting in 60 nM free Ca^{2+}) (middle trace, $P_o = 0.002$) and 0.95 mM Ca^{2+} cis (resulting in 20 μM free Ca^{2+}) (bottom trace, $P_o = 0.97$). Bars beside each trace indicate the current level of the closed (c) state. Holding potential (H.P.) = −20 mV. (*B*) A separate single channel was recorded as in *A* with 50 μM free Ca^{2+} cis [top (first) trace, $P_o = 0.05$] and after the successive addition of 2.4 mM ATP cis (9 μM free Ca^{2+}) (second trace, $P_o = 0.33$), 1 mM EGTA cis (60 nM free Ca^{2+}) (third trace, $P_o = 0.06$), 1 mM Ca^{2+} cis (13 μM free Ca^{2+}) (fourth trace, $P_o = 0.22$), and 10 μM ryanodine cis (bottom trace). Appearance of subconductance state (bottom trace) was observed about 10 min after the addition of 10 μM ryanodine cis. H.P. = −10 mV.

The open probability (P_o) of the Ca^{2+}-activated channel (at 50 μM Ca^{2+} cis) varied widely among the single-channel experiments with a mean $P_o = 0.44 \pm 0.48$ ($n = 6$). Fig. 4*B* [top (or first) trace] shows, as an example, the current traces of a single channel that was minimally activated ($P_o = 0.05$) with 50 μM Ca^{2+} in the cis chamber. Addition of 2.4 mM ATP to the cis chamber resulted in a marked increase in channel activity (second trace of Fig. 4*B*). The ATP-activated channel could be inactivated and again activated by the successive addition of 1 mM EGTA and 1 mM Ca^{2+} (third and fourth traces of Fig. 4*B*). The bottom trace of Fig. 4*B* shows that the K^+-conducting toad channel could be modified by ryanodine. Addition of 10 μM ryanodine cis induced the formation of an open-channel state of reduced conductance, as observed for the mammalian skeletal (25, 30) and cardiac (31) Ca^{2+}-release channels. With 250 mM K^+ as the current carrier, the unitary conductances of the ryanodine-unmodified and -modified channel states were 826 ± 53 ($n = 6$) and 450 ± 10 ($n = 2$) pS, respectively (not shown). Under the same conditions, channel conductances of the skeletal muscle RyR were 750 and 400 pS, respectively (32).

DISCUSSION

The present study describes the identification and preliminary characterization of a ryanodine-sensitive channel complex of toad visceral smooth muscle. As has been observed for the mammalian skeletal and cardiac muscle RyRs (5, 6), the toad visceral smooth muscle receptor bound [^{3}H]ryanodine with nanomolar affinity and had an apparent sedimentation coefficient of ≈30 S. Immunoblot analysis showed cross-reactivity of a canine cardiac RyR monoclonal antibody to a high molecular weight band that comigrated with the cardiac RyR polypeptide of M_r 565,000. Incorporation of the smooth muscle 30-S protein fractions into planar bilayers induced the formation of a ryanodine-sensitive ion-conductance pathway that exhibited a ryanodine-unmodified and -modified K^+ conductance similar to those of the mammalian skeletal and cardiac Ca^{2+}-release channels.

Single-channel measurements suggested the presence of an ion-conductance pathway in toad smooth muscle that could be reversibly activated by Ca^{2+}. However, the large variability in the open probability of the Ca^{2+}-activated channel (Fig. 4) precluded a quantitative analysis of the regulation of this channel by Ca^{2+} and other effector molecules in these reconstitution experiments. Therefore, regulation of the smooth muscle Ca^{2+} release channel was also studied by determining the $^{45}Ca^{2+}$ efflux behavior of passively loaded vesicles in the presence of endogenous and exogenous effector molecules known to activate the mammalian skeletal and cardiac muscle channels. The smooth muscle Ca^{2+} release channel was also activated by Ca^{2+}, ATP, and caffeine although with less sensitivity. The $^{45}Ca^{2+}$ efflux rate from smooth muscle microsomes increased as the free Ca^{2+} concentration in efflux media was raised from 1 μM to 1 mM Ca^{2+}, whereas $^{45}Ca^{2+}$ efflux from skeletal and cardiac muscle SR vesicles is maximal in the micromolar Ca^{2+} concentration range. In skeletal and cardiac preparations, the presence of 5 mM ATP or 10 mM caffeine in the efflux media resulted in a pronounced activation (27). By comparison, neither compound was effective in increasing by >2-fold the $^{45}Ca^{2+}$ efflux rate from toad microsomes. Results of vesicle $^{45}Ca^{2+}$ flux measurements suggest that the toad smooth muscle and mammalian muscle Ca^{2+} channels are related but functionally distinct protein complexes.

Results from the intact cells indicate that a ryanodine-sensitive Ca^{2+}-release mechanism that can be activated by caffeine exists in these cells. The magnitude of the transient Ca^{2+} level induced by caffeine in intact cells was comparable to that induced by acetylcholine (data not shown), which causes intracellular Ca^{2+} to increase almost entirely by the release of Ca^{2+} from internal stores (33), presumably mediated at least in part by IP_3. Thus, the RyR in these cells appears to exist on stores capable of producing Ca^{2+} transients as large and as fast as those induced by a natural neurotransmitter. Thus, it is surprising that caffeine proved to be a rather weak stimulant of Ca^{2+} release from the vesicle preparations. This difference may reflect the loss during vesicle preparation of an important soluble factor required for the normal activity of the toad muscle RyR *in vivo* or alternatively may reflect disruption during vesicle preparation of functionally important structural relations within the sarcoplasmic reticulum of smooth muscle. Such alterations may also account for the lack of response of the microsomal preparations to IP_3 and the relatively lower Ca^{2+} sensitivity of Ca^{2+} release observed for smooth muscle SR vesicles versus those from skeletal and cardiac SR. Given that the RyR in the smooth muscle cells appears to be more similar to the RyR of cardiac than skeletal muscle, it may be that, like the cardiac RyR (34), the smooth muscle receptor is activated following its phosphorylation by calmodulin-dependent protein kinase II and that this protein kinase, which may be lost during vesicle isolation, is required for full Ca^{2+}-dependent activation. Alternatively, it may be that the smooth muscle RyRs are situated within the cell in a microdomain that experiences local large increases in Ca^{2+} following opening of plasma membrane Ca^{2+} channels. Further studies on both isolated receptors and detailed studies into the existence and Ca^{2+} sensitivity of a Ca^{2+}-induced Ca^{2+} release mechanism in the intact cell will be necessary to discern between these possibilities. Finally, it is possible that the differences observed between the functional properties of the RyR in smooth and striated muscle reflect the fact that it may be a quite different molecular entity. That should be testable by applying molecular biological techniques to determine the structure of the RyR in smooth muscle, a task that should be facilitated by the current work describing methods for the isolation of the RyR from smooth muscle.

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