Role of the Ryanodine Receptor of Skeletal Muscle in Excitation-Contraction Coupling

MICHAEL FILL,[a] JIANJIE MA,[a] C. MICHAEL KNUDSON,[b]
TOSHIAKI IMAGAWA,[b] KEVIN P. CAMPBELL,[b]
AND ROBERTO CORONADO[a]

[a] *Department of Physiology and Molecular Biophysics*
Baylor College of Medicine
Houston, Texas 77030

[b] *Department of Physiology and Biophysics*
The University of Iowa School of Medicine
Iowa City, Iowa 52242

In skeletal muscle, contraction is initiated by a depolarization of the transverse tubular membrane (t-tubule), which in turn signals the release of Ca from the sarcoplasmic reticulum (SR). A key protein involved in this process is the ryanodine receptor, an SR membrane protein of MW 450,000 that binds the alkaloid ryanodine with nanomolar affinity and is present exclusively at the junction between t-tubule and SR membranes.[1-4] The ryanodine receptor plays a dual role: Functionally, it is the putative Ca-release channel of the SR,[4] and structurally, it is the major protein responsible for forming "bridges" or "feet" that anatomically connect t-tubule and SR.[2,5] Here we demonstrate that the ryanodine receptor is steeply gated by both voltage and protons, and for the first time *in vitro*, we measured nonlinear capacitance (charge movement) that may be involved in the gating of this channel protein.

We recently identified the 450,000-Da ryanodine receptor-feet protein (FIG. 1A) as the Ca-release channel of native SR.[4,9,10] This was achieved using the planar bilayer recording technique and by comparing ligand-dependent gating, ionic selectivity, and pharmacology of purified ryanodine receptors to that of native Ca-release channels.[11,22] Voltage dependence was a gating property notoriously absent in our study and in studies by others that followed.[12,13] Its inconspicuousness in our earlier work is related to the effect of protons. At pH 7.4 (FIG. 1B) the channel dwells in a fast gating mode (p = 0.38). A drop to pH 7.2 drives the channel into an almost closed condition (p = 0.08) and at pH 7.0 the channel never opens ($p < 0.01$). Reversibility is shown in the last record of FIGURE 1 where alkalinization from pH 7.0 to pH 7.6 reopens the channel, resulting in a higher level of activity (p = 0.72) than seen at pH 7.4. Over this narrow range of pH, slope conductance is not affected (FIG. 2C, inset), and the kinetics remain fast, with a mean open event duration of approximately 100 μsec. Thus for all practical purposes, a change of 0.6 unit in solution pH from 7.6 to 7.0 units is sufficient to make the channel switch from an almost all-open to an almost all-closed conformation. The fitted Hill coefficient for data in FIGURE 1 was n = 6.8 and the apparent pK_a was 7.5. FIGURE 2 describes the ensuing changes in voltage

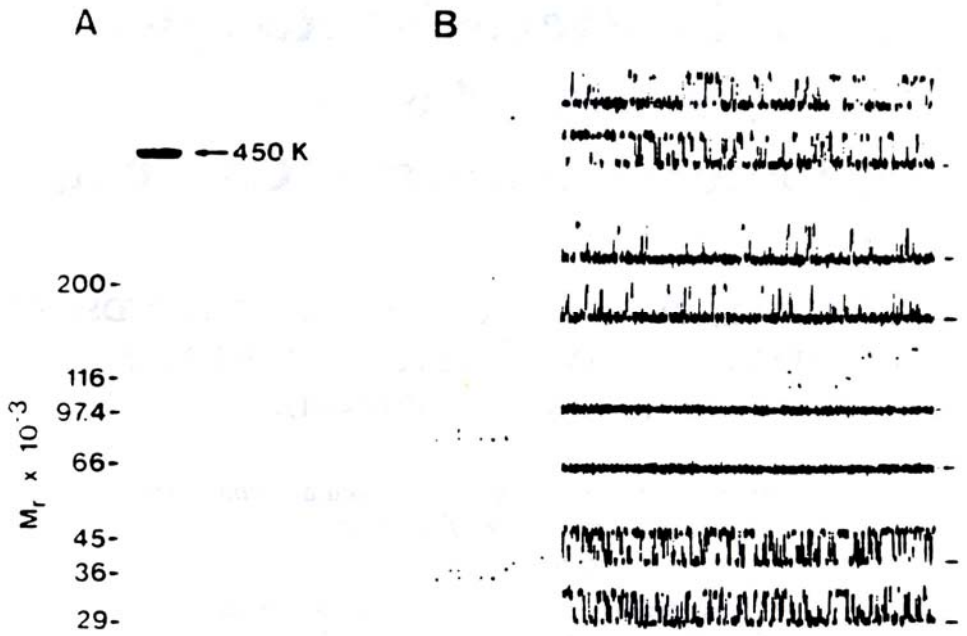

FIGURE 1. Ryanodine receptor polypeptide composition and pH dependence of purified receptor channels. (**A**) SDS-PAGE analysis of 3 μg of purified ryanodine receptor on 3-12% gradient gel and stained with Coomassie blue. The 450,000-Da receptor, indicated by the arrow (450K), migrated as a single band. Standards are indicated at the left. Purity of the 450,000-Da protein determined by gel scans was greater than 95%. Scatchard analysis of [^{3}H]ryanodine binding to the purified receptor (detailed in Smith *et al.*[10]) yielded a straight line with an apparent $B_{max.}$ and K_d of 490 pmoles/mg and 7.0 n*M*, respectively. (**B**) Single-channel activity mediated by the purified receptor in solutions of different pH. All records from the same experiment, HP +60 mV (380 pS open-channel conductance), 3-kHz cutoff frequency. Baseline current is indicated next to each record. Solutions in both chambers were 0.25 *M* KCl, 0.5 m*M* EGTA (pCa 7). 25 m*M* MOPS (3-[*n*-morpholino]propanesulfonic add) pH 7.4. pH (indicated next to each record) was adjusted with calibrated aliquots of MOPS (free acid) or KOH. To increase frequency response, K ions (instead of Ca or Ba) were used as current carriers. At low Ca (pCa > 6), K conductance is 6-10-fold larger than Ca conductance (detailed in Smith *et al.*[10]).

Ryanodine receptor was purified from isolated adult rabbit skeletal muscle triads by immunoaffinity chromatograpby (as in Imagawa *et al.*[4] with modifications introduced in Smith *et al.*[10]). Isolated triads were solubilizcd in 1% CHAPS in 0.5 *M* NCl and buffer A (0.5 *M* sucrose, 0.75 m*M* benzamidine, 0.1 m*M* PMSF, and 50 m*M* Tris-HCl at pH 7.4) at the protein concentration 1 mg/ml in the presence of several protease inhibitors. Solubilized triads were applied to a MAb-XA7-Sepharose column (20 ml) and recycled overnight. Receptor was eluted in 0.3% CHAPS, 0.15% asolecithin, and 0.5 *M* KSCN in buffer A. KSCN was exchanged for KC1 using a Pharmada PD-10 column. Planar bilayers were cast from 20 mg/ml brain phospholipids in decane (PE/PS = 1/1 wt/wt). The two chambers in contact with the bilayer were denned as *cis* (side of receptor addition, connected to head-stage amplifier) and *trans* (protein-free side, connected to ground potential). Solutions in *cis* and *trans* chambers were the same, 250 m*M* KCl, 1 m*M* EGTA (pCa 7), 25 m*M* MOPS pH 7.0, 7.2, 7.4. or 7.6. For capadtance measurements we used 50 m*M* NaCl and 10 m*M* HEPES-TRIS, pH 7.2. Receptor concentration in solutions was 0.5-1 μg/ml for single-channel measurements and 80-100 μg/ml from capacitance measurements. An equal amount of receptor buffer (0.3% CHAPS, 0.15% asoledthin, 0.5 *M* KCl; 50 m*M* Tris pH 7.2) was always present in the *trans* chamber. At the concentrations used, CHAPS detergent (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) had no effect on conductance or mechanical stability. Head-stage amplifier was a List EPC7 (List-Electronic, DA-Eberstadt, West-Germany) and pulse protocols were constructed using Basic-Fastlab software (Indec System, Sunnyvale, CA, USA). Current records were filtered at 3-10-kHz corner frequency on an eight-pole Bessel (Frequency Devices, Haverhill, MA, USA) and digitized at 14-30 μsec/point.

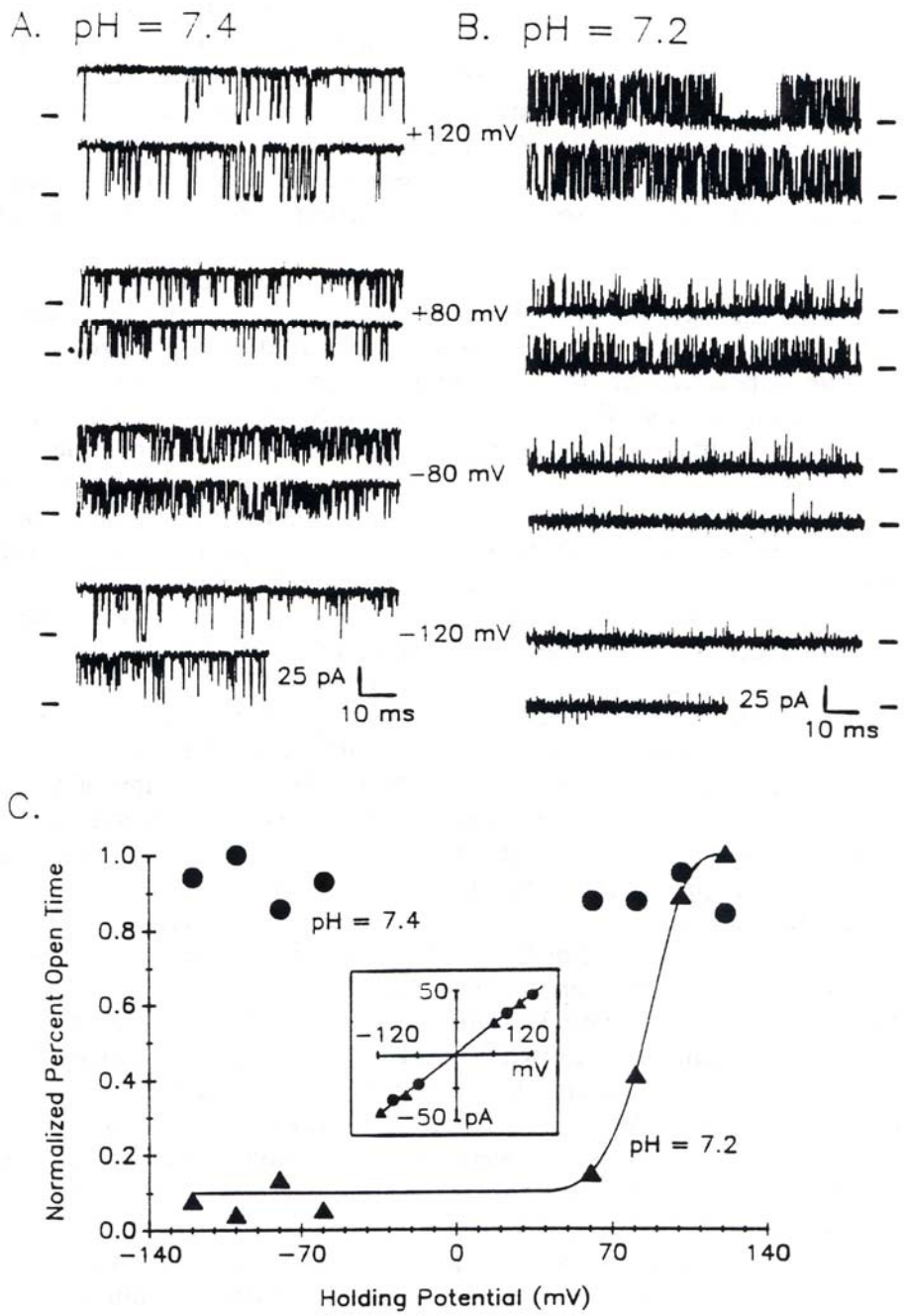

FIGURE 2. Voltage-dependence of ryanodine receptor channel. (**A,B**) Channel activity in *cis* solution pH 7.4 and 7.2 at the indicated holding potentials. Openings at every voltage are shown as deflecting the trace upward. Baseline is given for each trace. (**C**) The fraction of time spent open during a representative 10-sec interval (*p*, open probability) is plotted against holding potential at pH 7.2 (triangles) and pH 7.4 (circles), *p* at HP +100 mV was given a value of unity at both pHs. Actual values are $p = 0.82$ (pH 7.4) and $p = 0.41$ (pH 7.2). The current voltage curve (inset) at each pH was adequately fitted by the same line with a slope of 380 pS.

dependence. At pH 7.4 (FIG. 2A), the open probability remains fairly constant over the complete range of test potential, +120 mV to –120 mV. In this experiment open probability averaged 0.87 (FIG. 2C, circles). At pH 7.2 (FIG. 2B), a similar level of activity was present at positive potentials, and it dropped to 0.02 and less at potentials below +50 mV (FIG. 2C, triangles). Transition from open to closed was steep, with a midpoint at +85.7 mV (9.3 mV standard deviation, n = 8) and an e-fold increase in open probability every 3.5 mV. At pH 7.0 no openings were observed at any test potential. Thus, the receptor channel is steeply dependent on both membrane potential and pH with the switching from closed to open occurring over an unusually narrow range.

A demonstration of voltage dependence prompted us to search for charge movement associated with this protein. In skeletal muscle, nonlinear charge movements are argued to be a manifestation of some molecular event that is involved in transduction of t-tubule depolarization to SR calcium release. Experimentally, charge movement was looked for as a small voltage-dependent component of the total membrane capacitance. To ensure a large density of receptors in the planar membrane, we used 100-200 times more protein (80 μg/ml) than used in single-channel experiments. To avoid trivial artifacts related to adding receptor to only one chamber, we added protein-free receptor buffer (see legend FIG. 1) to the opposite chamber. Hence, the only asymmetry in the system was the protein itself. We measured the voltage-dependent component of membrane capacitance, C(V), as a function of holding potential, V. Capacitance was determined in the range of –150 mV to +150 mV by integrating of the "on" or "off" charging current in response to a constant test pulse. The test pulse was applied midway during a long holding pulse. In order to compare the shape of the voltage-dependent component, we subtracted the capacitance at V = 0 mV from each curve and plotted C(V) – C(V = Ø) as a function of V^2 (FIG. 3). Bare bilayers and bilayers with protein-free buffer displayed a characteristic minimum at V = 0 mV and a quadratic increase in C(V) as a function of V, symmetric with respect to zero. This phenomenon has been well described and is due to electrostriction.[14,15] Capacitance of bare bilayers was typically 12% higher at +150 mV (or –150 mV) than at 0 mV. A plot of C(V) versus V^2 (FIG. 3, circles) shows that in the absence of receptor each arm of the curve, the one at positive and the one at negative potentials, varies linearly with V^2 with a slope of approximately 4.9 pF/V^2. Receptor protein (FIG. 3, triangles) had two effects on this relationship. It significantly increased the steepness at positive potentials, and it decreased the steepness at negative potentials. The effects are complex with no less than three slopes clearly different from the slope expected on the basis of electrostriction alone. One component at negative potentials is weakly voltage dependent; a second component around 0 mV is steeply voltage dependent, and a third component at large positive potentials tends to saturate with a limiting slope of about 20 pF/V^2, six times larger than without receptor. Thus clearly, the ryanodine receptor *in vitro* mediates nonlinear charge movement of several kinds.

Although we could not specify components responsible for gating, the bulk of the charge correlated well with open-channel probabilities. FIGURE 4A shows receptor charge movement (protein minus protein-free capacitance) as a function of holding voltage. More charge is moved at progressively positive potentials with the largest change occurring between –60 mV and +60 mV. Under similar conditions, records of single channels are shown in FIGURE 4B. Activation occurs at positive potentials with few openings below +60 mV. Thus the two processes have voltage dependencies that increase in the same direction. This is plotted in FIGURE 4C where charge movement during either the "on" transient (squares) or "off" transient (circles) was found to be superimposable with open-channel probabilities (diamonds). The latter

measured independently from single-channel records obtained at a low concentration of receptor. The equivalence of charge moved during the "on" and "off" of the test pulse is given in the inset of FIGURE 4. One followed the other in the protein-free control (lower curves) and in the presence of receptor (upper curves).

Four hallmarks of Ca release studied in skinned fibers and SR vesicles,[16-25] namely, activation of release by micromolar Ca and millimolar adenine nucleotide, and inhibition by micromolar ruthenium red and millimolar Mg, can be traced to the channel formed by the 450,000-Da ryanodine receptor.[10] The purified polypeptide alone contains the binding site for ryanodine and the regulatory sites for Ca, ATP, and ruthenium

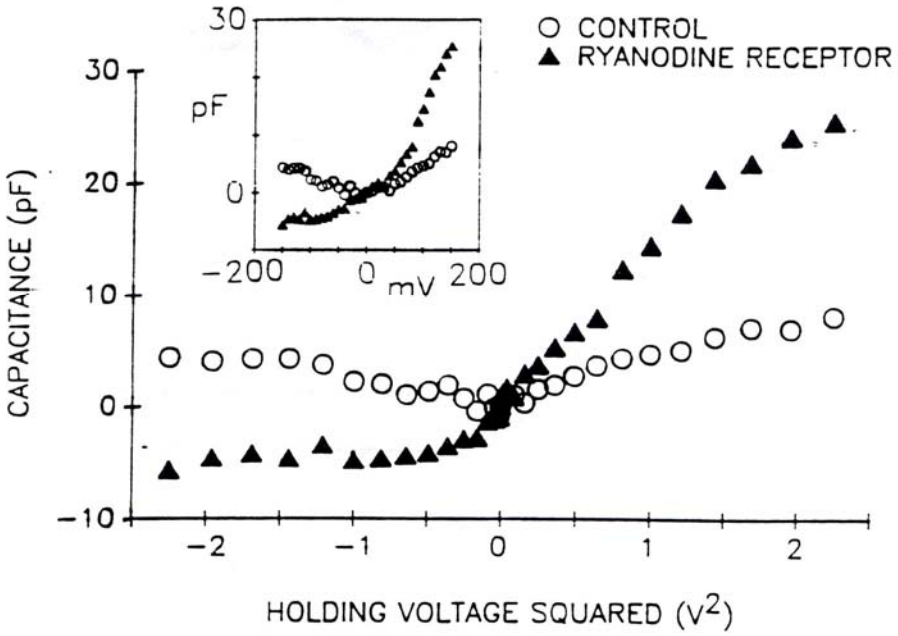

FIGURE 3. Voltage-dependent capacitance mediated by ryanodine receptor. Capacitance as a function of voltage C(V), was measured using a two-pulse protocol. A square voltage pulse of 10-mscc duration and constant +10 mV amplitude (test pulse) was added to square voltage pulse of 200-mscc duration and variable amplitude (holding voltage pulse). Test pulse was applied 100 msec into the holding pulse. Holding voltage was varied between –150 mV to +150 mV. Capacitance was measured by integrating the first 8 msec of the "on" transient of the test pulse at each holding voltage. Curves are the average of four separate experiments. Circles correspond to bilayers with receptor protein and receptor buffer on the *cis* and *trans* sides, respectively. Capacitance at 0 mV holding voltage was subtracted from each curve (control C(V = 0) = 314 pF; ryanodine receptor C(V = 0) = 347 pF. C(V) – C(V = 0) is plotted against holding voltage, mV (inset) or V^2 (center).

red.[4] The identified receptor channel is novel in several respects. It has a large conductance for K and Ca ions and a fast kinetics composed of open times in the range of 60-100 μsec.[10] These properties are expected of a channel that mediates SR Ca release. The ryanodine receptor *in vivo* forms part of a large oligomeric structure that spans the 100-Å gap between t-tubules and SR membranes.[2,5,12,13] Recent evidence suggests that the attachment to the t-membrane may contain the dihydropyridine (DHP) receptor.[26] The DHP receptor has been identified as the t-tubule voltage sensor of excitation-contraction coupling.[8] Thus, our observation of nonlinear capacitance may lead to the possibility that the ryanodine receptor protein may sense and convey

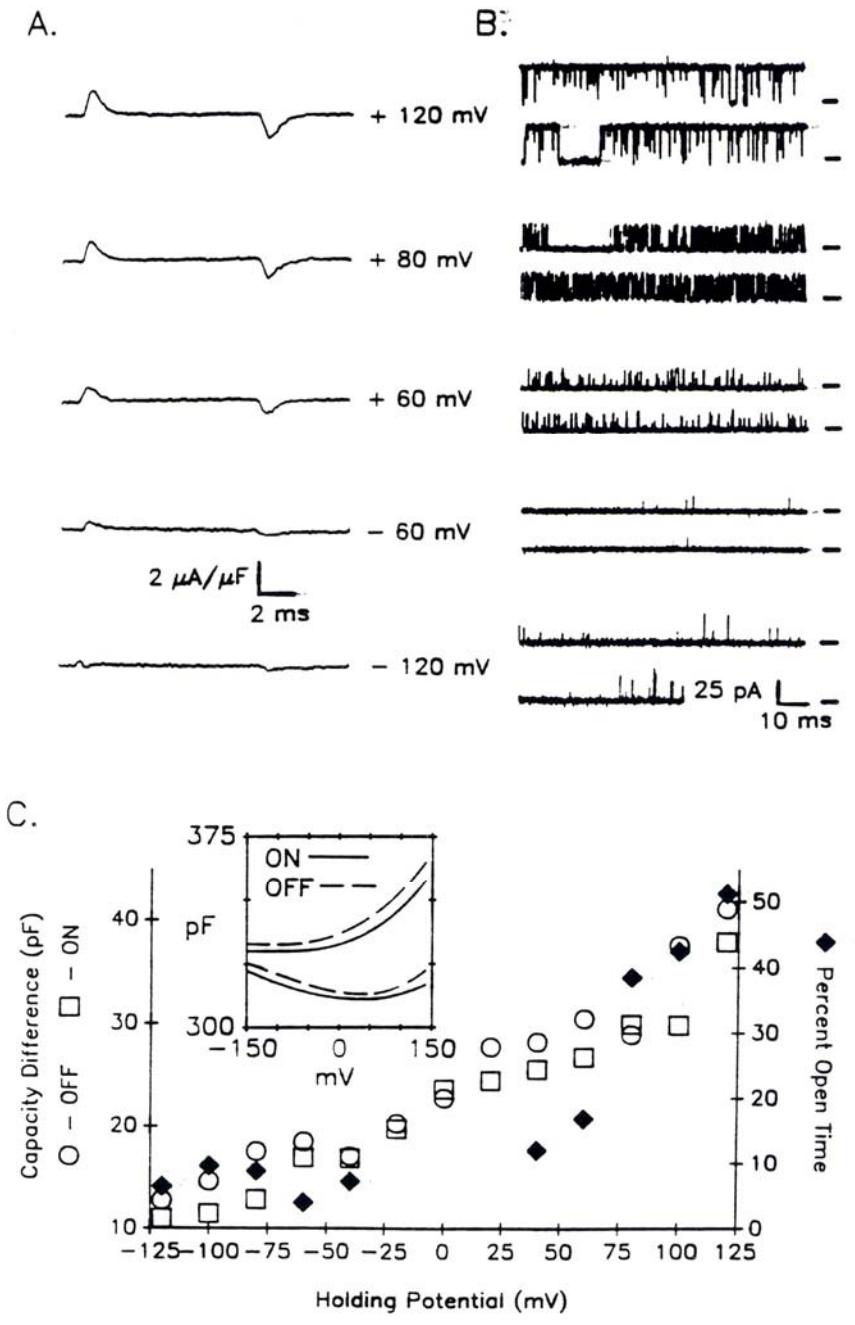

FIGURE 4. Correlation charge movement and channel opening in the ryanodine receptor. (**A**) Charge movement mediated by the receptor protein obtained by subtracting pairs of test pulses (protein minus protein free) at each indicated holding potential. (**B**) Single-channel activity at the corresponding holding voltages measured in a separate experiment. Channel opening is always shown as upward deflections from the baseline which is indicated for each record. (**C**) Capacitance difference after subtraction of protein minus protein-free bilayers (open symbols) and open-channel probability (filled diamonds) are plotted against holding voltage. Inset shows C(V) versus V separately in protein-free (lower curves in inset) and protein-containing (upper curves in inset) bilayers. The "on" and "off" transients have the same voltage dependence.

t-tubule voltage changes to the SR. Further experiments, however, emphasizing the pharmacology and specificity of the nonlinear capacitance are required to assess its significance *in vivo*.

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