A new scorpion toxin (BmK-PL) stimulates Ca^{2+}-release channel activity of the skeletal-muscle ryanodine receptor by an indirect mechanism

Akihiko KUNIYASU*, Seiko KAWANO†, Yoshiyuki HIRAYAMA†, Yong-Hua JI‡, Ke XU‡, Masamichi OHKURA§, Ken-Ichi FURUKAWA§[1], Yasushi OHIZUMI§, Masayasu HIRAOKA† and Hitoshi NAKAYAMA*[2]

*Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Ohe-Honmachi, Kumamoto 862-0973, Japan, †Department of Cardiovascular Diseases, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 113-0034, Japan, ‡Shanghai Institute of Physiology, Academia Sinica, Shanghai, The People's Republic of China, and §Department of Pharmaceutical Molecular Biology, Faculty of Pharmaceutical Sciences, Tohoku University, Sendai 980-0845, Japan

A peptide toxin isolated from the Chinese scorpion *Buthus martensi* Karsch (BmK-PL) stimulated Ca^{2+}-release channel activity in both triad membranes and reconstituted ryanodine receptors partially purified from rabbit skeletal muscle. In [^{3}H]ryanodine binding experiments, the toxin increased the affinity of ryanodine for the receptor, from a K_d of 24.3 nM to 2.9 nM, which is an enhancement similar to that seen with known receptor activators, such as ATP and high concentrations of KCl. In contrast, toxin enhancement was not observed with purified receptors, although intrinsic binding activity and stimulation by the conventional receptor activators were retained. In single channel recordings of Ca^{2+}-release activity, the toxin increased the open channel probability (P_o) from 0.019 to 0.043 (226 % of control) in triad preparations. Further toxin enhancement of P_o from 0.07 to 0.37 (529 % of control) was observed using partially-purified receptors in the presence of ATP. When purified receptors were assayed in the presence of ATP, however, they showed a high value of P_o (0.33) and no further increase was observed following application of the toxin. Results derived from two different experimental methods consistently suggest that a molecule(s) required for toxin-induced enhancement is absent from the purified receptor preparation. Western blot analysis of receptors prepared using three different protocols showed that triadin was missing from the purified receptor preparation. The scorpion toxin minimally enhanced Ca^{2+}-release channel activity of cardiac preparations. From these results, we conclude that the toxin preferentially increases the activity of skeletal-muscle ryanodine receptors by an indirect mechanism, possibly binding to associated protein molecule(s). Triadin is a strong candidate for such a molecule.

Key words: open channel probability, ryanodine binding, single channel recording, triadin.

INTRODUCTION

There are approximately 800 species of scorpions in the world. They have been classified into six families [1] including two major ones, namely Buthidae and Chactidae. Most scorpions including Buthidae produce peptide neurotoxins. These toxins have been shown to be a family of structurally and functionally related basic peptides composed of a single chain of 60–70 amino acid residues [2]. Neurotoxicity has been correlated mainly with a toxin's action on voltage-dependent sodium channels [3]. Recently, however, several scorpion toxins have been shown to modulate voltage-dependent potassium channels [4–6] and ryanodine receptor/Ca^{2+}-release channels [7,8].

The ryanodine receptor, which plays a key role in excitation–contraction coupling of muscle tissues [9,10], is the Ca^{2+}-release channel found in sarcoplasmic reticulum. Ca^{2+} release in skeletal and cardiac muscle is regulated by similar but distinct mechanisms [11,12]. In cardiac muscles, depolarization leads to opening of voltage-gated L-type Ca^{2+} channels (dihydropyridine receptors). Ca^{2+} influx through the Ca^{2+} channels triggers opening of the ryanodine receptor/Ca^{2+}-release channel in the sarcoplasmic reticulum. However, in skeletal muscle, entry of external Ca^{2+} is not required for this signal transduction process. Identifying protein components and determining how they function in the junctional sarcoplasmic reticulum are fundamental to our understanding of the mechanisms of Ca^{2+} storage and release in muscle cells. Calsequestrin [13–15], triadin [16,17] and junctin [15,18] have been identified as proteins that form a complex with the ryanodine receptor/Ca^{2+}-release channel [19] and modulate its function.

Molecular probes that specifically interact with ryanodine receptors provide useful tools to analyse how the receptors function in excitation–contraction coupling. Ryanodine, a plant alkaloid, is widely used for structural and functional studies of ryanodine receptors, since its binding can be correlated with the number of receptors in the open conformational state. Ryanodine, however, cannot discriminate between receptors expressed on skeletal and cardiac muscle. Recently, probes that preferentially modulate skeletal-muscle ryanodine receptors were shown to enhance ryanodine receptor/Ca^{2+}-release channel activities in preparations using purified ryanodine receptors [20].

We have investigated a peptide toxin newly isolated and purified [21] from the scorpion *Buthus martensi* Karsch (BmK-PL). In the present work, we demonstrate that BmK-PL preferentially enhances the function of the ryanodine receptor/Ca^{2+}-release channel from skeletal-muscle sarcoplasmic reticulum by [^{3}H]-ryanodine binding and single channel recording of Ca^{2+}-release activity. In contrast to imperatoxin A, BmK-PL-induced enhancement is not observed when highly-purified ryanodine receptors are used. Our results suggest that BmK-PL enhance-

Abbreviations used: BmK-PL, peptide toxin isolated from a Chinese scorpion *Buthus martensi* Karsch; B_{max}, maximum binding capacity; P_o, open channel probability; RP, reversed phase.

[1] Present address: Department of Pharmacology, Hirosaki University School of Medicine, Zaifu-cho 5-1, Hirosaki 036-8216, Japan.

[2] To whom correspondence should be addressed (e-mail jin@gpo.kumamoto-u.ac.jp).

ment of Ca^{2+}-release activity does not involve direct interaction with the Ca^{2+}-release channel (ryanodine receptor) itself, but is mediated by an associated protein(s). We show that triadin is a candidate for that protein.

MATERIALS AND METHODS

Materials

Ryanodine was purchased from Wako Pure Chemicals (Osaka, Japan). The following chemicals were obtained from the companies indicated: [^{3}H]ryanodine (60 Ci/mmol) from Du Pont NEN Life Science Products (Boston, MA, U.S.A.); heparin–agarose and phosphatidylcholine (from soybean, type II-S) from Sigma Chemical Co. (St. Louis, MO, U.S.A.); peroxidase-conjugated anti-(rabbit IgG) from Zymet (San Francisco, CA, U.S.A.); and SuperSignal Chemiluminescent Substrate from Pierce (Rockford, IL, U.S.A.). Phosphatidylethanolamine and phosphatidylserine (from brain) were purchased from Avanti Polar Lipids, (Birmingham, AL, U.S.A.).

Purification of BmK-PL from *Buthus martensi* Karsch

Crude venom, which was obtained by electrical stimulation of the scorpion *Buthus martensi* Karsch and lyophilized, was purchased from an individual culture farm in Zhengzhou, Hunan province, China. Extraction of the venom and purification was performed as described previously [21]. The crude venom (10 g) was dissolved in distilled water, centrifuged (9000 ***g***, 10 min at 4 °C), and the supernatant used as the extract. The extract was first separated by reversed phase (RP)-HPLC using a YWG-CH C_{18} column (0.9 cm × 30 cm). Solvents used were, solvent A (0.1 % trifluoroacetic acid/water) and solvent B (0.1 % trifluoroacetic acid/60 % acetonitrile/water), and elution was performed with two successive linear gradients: 0–43 % of solvent B for 5 min followed by 43–100 % of solvent B for the next 60 min at a flow rate of 1 ml/min.

A peak eluting between 57–58 min, just after the largest peak, was collected as an active fraction, as determined by [^{3}H]-ryanodine-binding assays using skeletal-muscle triad membranes. The peak fraction was further purified by RP-HPLC using a Lichrosorb RP-18 column (7 μm, 0.47 cm × 25 cm) and a linear gradient elution of 30–60 % acetonitrile with solvents A and B at a flow rate of 1 ml/min. Purified BmK-PL from peak 5 in the second chromatography was shown to contain a single peptide of 8.7 kDa by SDS/PAGE. The purified toxin was further subjected to amino acid analysis and sequencing after reductive carboxymethylation.

Preparation of membranes

Triad membranes were prepared from rabbit back and leg white muscle as described by Mitchell et al. [22]. Heavy sarcoplasmic reticulum membranes were prepared from bovine ventricles according to the method of Lindsay and Williams [23]. All membrane fractions were prepared in the presence of protease inhibitors (0.1 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml pepstatin A, 0.5 mM iodoacetamide) and stored at −80 °C in 0.25 M sucrose, 0.2 M NaCl, 25 mM Hepes/Tris, pH 7.2.

Preparation of purified and reconstituted ryanodine receptors

Ryanodine receptors were purified from rabbit skeletal triads by a modification of the methods reported by Meissner's [24] and Fleischer's [25] groups. Rabbit skeletal triads (2 mg/ml) were solubilized with 1 % (w/w) CHAPS, 0.5 % phosphatidylcholine in 0.5 M NaCl, 50 μM $CaCl_2$, 25 mM Hepes/Tris, pH 7.2, containing protease inhibitors (0.1 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml pepstatin A), and then centrifuged for 30 min at 100000 ***g***. The supernatant was layered on to 10–30 % linear sucrose gradients in buffer A (0.5 % CHAPS, 0.25 % phosphatidylcholine, 50 μM $CaCl_2$, 25 mM Hepes/Tris, pH7.2, 0.1 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml pepstatin A) and 0.5 M NaCl. The gradients were centrifuged at 2 °C in a Beckman SW28 rotor at 122000 ***g*** for 16 h and fractionated. After locating ryanodine receptor bands by SDS/PAGE analysis, ryanodine-receptor fractions were collected and diluted with buffer A to a concentration of 0.2 M NaCl. The diluted fractions were then applied to a small heparin–agarose column equilibrated in buffer A containing 0.2 M NaCl and 0.25 M sucrose. After washing with the equilibration buffer, the ryanodine receptor was eluted with buffer A containing 0.8 M NaCl and 0.25 M sucrose. The purified receptor was reconstituted into phosphatidylcholine vesicles by dialysis to remove the detergent. The heparin–agarose pools (3 ml) were dialysed at 4 °C against 1 litre of dialysis buffer (0.3 M sucrose, 0.1 M KCl, 50 μM $CaCl_2$, 10 mM Hepes/Tris, pH 7.2). The buffer was changed every 6 h for 24 h. The dialysed sample was quickly frozen in liquid N_2 and stored at −80 °C.

Partial purification of ryanodine receptors was achieved using heparin–agarose chromatography. Briefly, the solubilized triads were diluted with buffer A to 0.2 M NaCl and applied to a heparin–agarose column. The ryanodine receptor was eluted and reconstituted into phosphatidylcholine vesicles as described above.

[^{3}H]Ryanodine binding assay

Rabbit skeletal triads (30 μg) or bovine cardiac heavy sarcoplasmic reticulum membranes (50 μg) were incubated at 30 °C for 2 h in 100 μl of buffer B (0.15 M KCl, 1.01 mM $CaCl_2$, 1 mM EGTA, 25 mM Hepes/Tris, pH 7.2) and 2 or 10 nM [^{3}H]ryanodine with various concentrations of BmK-PL. For the saturation assay, skeletal triads were incubated with various concentrations of [^{3}H]ryanodine in buffer B alone or with 100 μg/ml purified BmK-PL, 5 mM ATP or 1 M KCl. When purified ryanodine receptors (2 μg) were assayed, the receptors were incubated with 10 nM [^{3}H]ryanodine in 100 μl of buffer B alone or with 5 mM ATP, 10 mM caffeine, 100 μg/ml purified BmK-PL or a combination of the above. Some assays of the purified receptor were carried out by the addition of KCl to 1 M or the depletion of $CaCl_2$.

The amount of bound [^{3}H]ryanodine was determined by the method described previously for the inositol trisphosphate receptor [26]. For assaying membrane fractions, the incubation mixture was centrifuged at 10000 ***g*** for 10 min at 4 °C and the pellet was dissolved in 1 % (w/v) SDS. The sample was mixed with ACS II scintillation cocktail (Amersham, Little Chalfont, Bucks., U.K.) and the radioactivity was measured by a scintillation counter. For the purified ryanodine receptor, the sample was mixed with 4 μl of 50 mg/ml γ-globulin and 100 μl of a solution containing 30 % (w/v) poly(ethylene glycol) 6000, 25 mM Hepes/Tris, pH 7.2. After incubation for 5 min on ice, the protein–poly(ethylene glycol) complex was precipitated by centrifugation at 10000 ***g*** for 5 min at 2 °C. The pellet was dissolved in water and the radioactivity was measured as described above. Non-specific binding was determined using excess unlabelled ryanodine (10 μM) and was subtracted from total bound [^{3}H]ryanodine.

SDS/PAGE

Standard SDS/PAGE was performed using Laemmli's buffer system with 6 % separating gels and 3 % stacking gels [27]. Gels were stained with Coomassie Blue.

Protein assay

Protein concentrations were determined by the method of Bradford [28] using BSA as the calibration standard.

Planar lipid bilayer methods

Planar lipid bilayers (20 mg/ml) were composed of brain phosphatidylethanolamine and brain phosphatidylserine (1:1, w/w) dissolved in decane. Triad preparations or purified ryanodine receptors were added to the cis chamber and fused to lipid bilayers which formed across a 0.2 mm diameter hole in a Lexan polycarbonate partition. In the present experiments the cis chamber is defined as the side to which proteins were added and the opposite side is referred to as the trans chamber. Passing currents were applied to the cis chamber and the voltage was defined with respect to the trans chamber held at ground.

Solutions

The composition of the solution in the cis chamber (cis solution) was 500 mM CsCl, 1 mM EGTA, 10 mM Hepes, pH 7.4 adjusted with Tris base, and that of the trans solution was 50 mM CsCl, 5.4 mM $CaCl_2$, 10 mM Hepes, pH 7.4. The free Ca^{2+} concentration of the cis solution was 1 μM, as calculated by Fabiato's program [29]. Assays of ATP effects were performed in the presence of 5 mM Na_2ATP in the cis solution.

Data acquisition and analysis

The channel currents were recorded at room temperature (22 ± 1 °C), amplified by a patch–clamp amplifier (Axopatch 1C, Axon Instruments Inc., Foster City, CA, U.S.A.) and stored on a videocassette tape recorder through a PCB converter system (RP-880, NF Instruments, Yokohama, Japan) digitized at 10 kHz. Data were reproduced and low-pass filtered at 1 kHz by a filter with Bessel characteristics (octave attenuation, 48 dB), sampled at 10 kHz and analysed off-line on a computer (Compaq DESKPRO EX466, Compaq Computer Corp., Reston, VA, U.S.A.) using the P Clamp program (Axon Instruments, Inc.). For single channel analysis, the threshold used to judge the channel open state was set at half of the unit current amplitude [30]. To analyse the lifetime of the channel state, data were sampled at 10 kHz and filtered at 1 kHz.

Western blot analysis using anti-triadin antibody

The anti-triadin antibody was generated as described previously [31]. Western blot analysis was performed as described previously [31], except that 6 % polyacrylamide gels were used. Triad membranes (30 μg), partially-purified receptor (9 μg), and highly purified receptor (6 μg) were first subjected to SDS/PAGE. The separated polypeptides were electrophoretically transferred on to polyvinylidene difluoride membranes, Immobilon P (Millipore, Bedford, MA, U.S.A.), at 20 V for 30 min. The membrane was incubated with 600-fold diluted anti-triadin antibody and the bound anti-triadin antibody was detected by peroxidase-conjugated anti-rabbit IgG and visualized using SuperSignal Chemiluminescent Substrate. Control blotting in the presence of 10 μM antigen peptide was used to evaluate non-specific immunoreactions of the anti-triadin antibody.

RESULTS

Purification of BmK-PL

BmK-PL was purified from crude venom by two successive rounds of RP-HPLC. In the first HPLC (Figure 1A), toxin activity was eluted between 57–58 min as an active peak (peak 13), as indicated by [^{3}H]ryanodine binding assays using skeletal-muscle triad membranes. The peak fraction was further purified by RP-HPLC using a Lichrosorb RP-18 column under different elution conditions (Figure 1B). Peak 5 in the second chromatograph produced the greatest enhancement of [^{3}H]ryanodine binding, and contained a single peptide of 8.7 kDa as determined by SDS/PAGE (Figure 1C). The size is consistent with an amino acid composition of 75–76 residues. The amino acid sequence of BmK-PL was analysed up to 30 amino acid residues and shown to be: DNGYLLDKYTGCKVWCVINNESCNSECKIR.

Effects of BmK-PL on [^{3}H]ryanodine binding to triad fractions

The effect of purified BmK-PL on sarcoplasmic-reticulum Ca^{2+}-release channels was first analysed using assays of [^{3}H]ryanodine

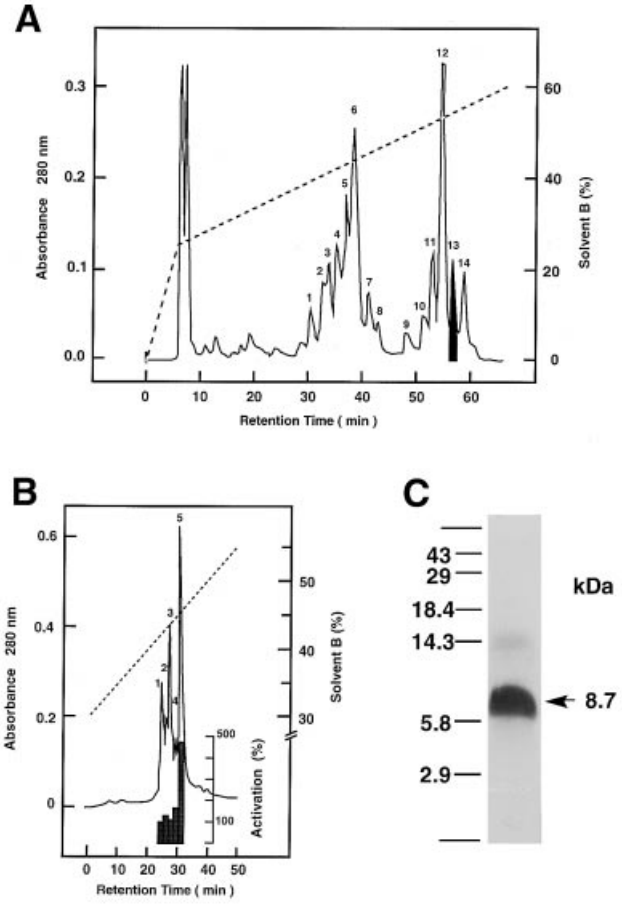

Figure 1 Purification of BmK-PL

(**A**) A water-extract from crude venom was first separated by RP-HPLC on a C_{18} column. Elution, at a flow rate of 1 ml/min, was performed with two successive linear gradients (dashed line) using solvents A and B as described in the Materials and methods section. A peak eluting between 57–58 min (filled area) was collected as an active fraction, as determined by a [^{3}H]ryanodine binding assay. (**B**) The peak fraction was further purified by RP-HPLC using a Lichrosorb RP-18 column by gradient elution (dashed line) with solvents A and B. Activity profiles determined by a [^{3}H]ryanodine binding assay are also shown (filled bars). (**C**) The purity of BmK-PL (peak 5 in Figure 1B) was determined by SDS/PAGE.

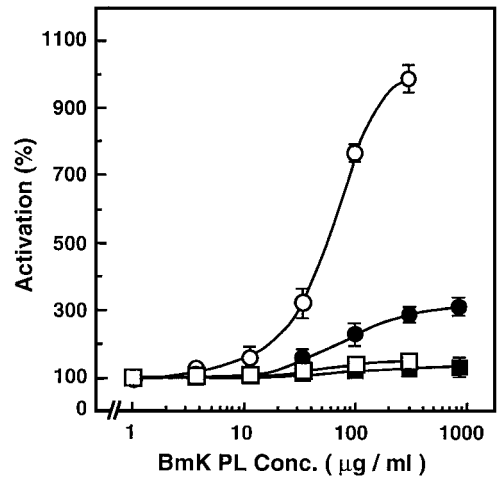

Figure 2 BmK-PL stimulation of [^{3}H]ryanodine binding

Rabbit skeletal triads (30 μg, circle) or bovine cardiac heavy sarcoplasmic reticulum membranes (50 μg, square) were incubated at 30 °C with various concentrations of BmK-PL in the presence of 2 nM (open symbol) or 10 nM (filled symbol) [^{3}H]ryanodine.

binding to triad fractions from skeletal muscle and cardiac heavy sarcoplasmic-reticulum fractions. In low ionic strength (0.15 M KCl) buffer, the increase in [^{3}H]ryanodine binding to the skeletal-muscle preparations was progressively enhanced by increasing BmK-PL concentrations (Figure 2). The maximum enhancements were dependent on the concentration of [^{3}H]ryanodine. In the presence of 10 nM [^{3}H]ryanodine, the binding activity was $310 \pm 60\,\%$ of the control at a BmK-PL concentration of 1000 μg/ml. Since the binding enhancement reached a plateau at this concentration, the EC_{50} of BmK-PL was calculated to be $8 \pm 1.6\,\mu$M, based on a molecular mass of 8.7 kDa. In the presence of 2 nM [^{3}H]ryanodine, however, the binding enhancement was $950 \pm 80\,\%$ of the control even at 300 μg/ml BmK-PL, but did not reach a maximum level. In contrast to skeletal-muscle preparations, binding enhancements were only $125 \pm 30\,\%$ and $150 \pm 20\,\%$ of the control in cardiac preparations in the presence of 10 nM and 2 nM [^{3}H]ryanodine respectively.

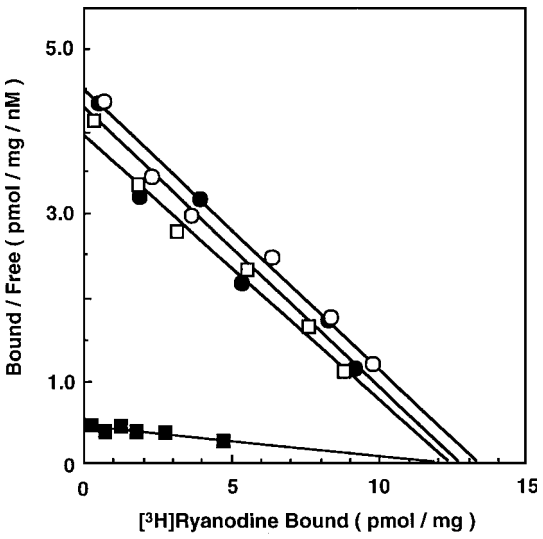

Figure 3 Scatchard analysis of [^{3}H]ryanodine binding to skeletal triads in the presence or absence of BmK-PL, ATP or KCl

Skeletal triads (30 μg) were incubated at 30 °C in buffer B (containing 0.15 M KCl) with various concentrations of [^{3}H]ryanodine alone (■) or with either 100 μg/ml BmK-PL (●), 5 mM ATP (○) or 1 M KCl (□), as described in the Materials and methods section. Non-specific binding, determined using excess unlabelled ryanodine (10 μM), accounting for $< 9\%$ of the total binding, was subtracted from total binding to yield specific binding. Data are means $\pm$ S.E.M. ($n = 3$).

Since activation by BmK-PL was dependent on [^{3}H]ryanodine concentration, we used Scatchard analysis to determine the binding parameters of [^{3}H]ryanodine in skeletal-muscle preparations, with or without BmK-PL (Figure 3). In the absence of BmK-PL, [^{3}H]ryanodine bound to a single class of receptor sites with a K_d of 24.3 ± 0.5 nM and a maximum binding capacity (B_{max}) of 11.6 ± 1.8 pmol/mg protein. In the presence of BmK-PL, however, the K_d decreased to 2.9 ± 0.2 nM, but B_{max} was unchanged at 12.5 ± 2.0 pmol/mg protein.

We also compared the effect of BmK-PL with that of known activators of ryanodine receptors, ATP and KCl (Figure 3) [32–34]. Similar decreases in K_d were observed in the presence of 5 mM ATP (2.9 ± 0.2 nM) or 1M KCl (3.1 ± 0.4 nM), while the B_{max} values were almost unchanged at 13.1 ± 1.5 and 12.2 ± 2.0 pmol/mg protein respectively.

Stimulation of Ca^{2+}-release channel activity by BmK-PL in triads or partially-purified receptors reconstituted into planar lipid bilayers

We used single channel recording to examine the effects of BmK-PL on ryanodine-receptor Ca^{2+}-release channels incorporated into planar lipid bilayers. Figure 4 shows records of Ca^{2+}-release channel activities before and during application of BmK-PL using triad preparations. The application of BmK-PL to the cis solution increased the open channel probability (P_o) from 0.019 to 0.043. However, channel conductance and kinetic properties were not changed by the addition of BmK-PL. In the control, open time histograms were fitted by a single exponential with a time constant of 0.6 ms and closed time histograms were fitted by double exponentials with time constants of 2.3 and 9.4 ms. After application of BmK-PL, these values were similar, namely 0.6 ms and 2.3 and 9.1 ms as the open and closed time constants, respectively.

A pronounced effect of BmK-PL was obtained using partially-purified ryanodine receptors (Figure 5). In the presence of 5 mM ATP, the P_o of the receptor showed a rather high value of 0.07. Application of BmK-PL to the cis solution markedly increased the P_o to 0.37, however, the channel conductance remained unchanged.

Effects of BmK-PL on purified ryanodine receptors

To determine whether BmK-PL affects the ryanodine receptor directly or indirectly, we tested the effects using highly-purified ryanodine-receptor preparations. In [^{3}H]ryanodine binding experiments, purified receptors showed typical activation in response to known modulators of the release channels, as shown in Figure 6. Their binding activity increased 1.9-fold by the application of ATP (5 mM) or caffeine (10 mM), and by 2.5–2.6-fold by the application of 1 M KCl or ATP plus caffeine, while activity was blocked by the depletion of Ca^{2+}. Addition of BmK-PL, however, did not change [^{3}H]ryanodine binding activity relative to controls, in clear contrast to the results obtained using triad membrane preparations. Furthermore, no additional effect was seen following the application of BmK-PL in the presence of ATP, caffeine, or ATP plus caffeine. This result

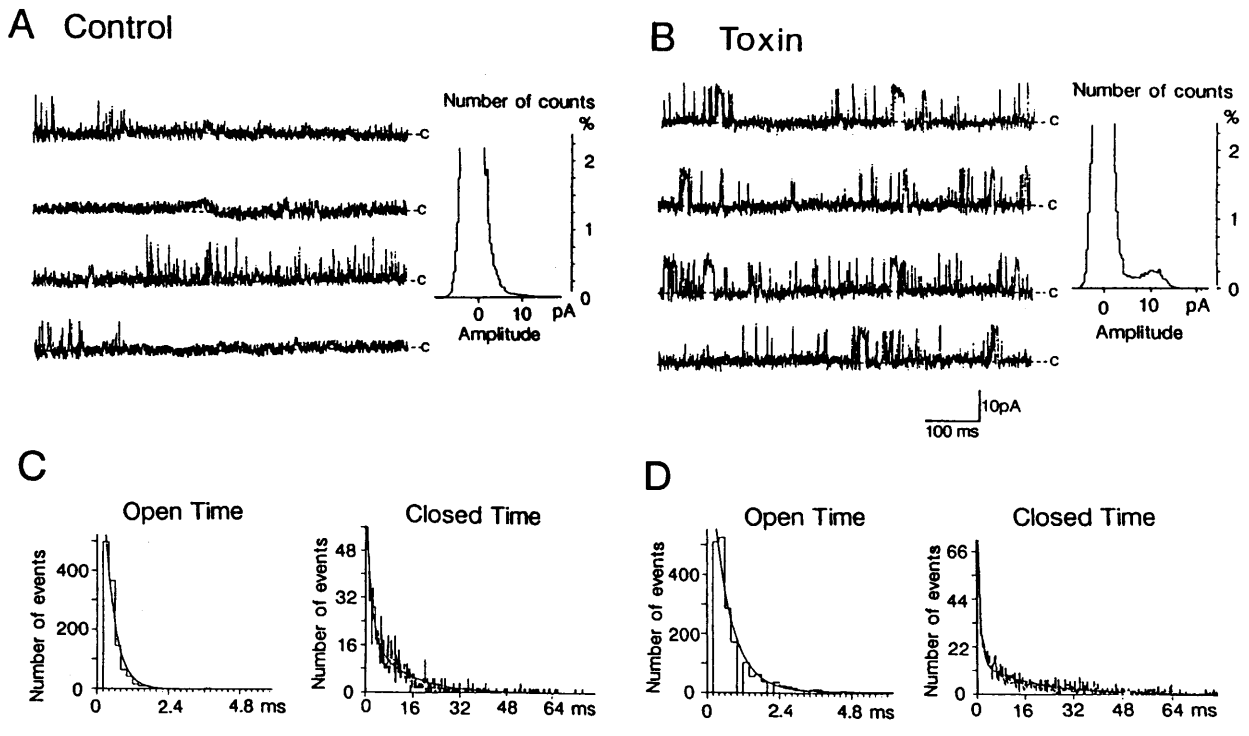

Figure 4 Single channel recordings of ryanodine receptor Ca^{2+}-release channel of skeletal triads

Continuous recordings of single channel activities in the control (**A**) and after application of the toxin, BmK-PL (**B**), are shown. Data were obtained from a single experiment. Upward direction indicates current flow from cis to trans. 'c', indicates closed level of the channel. Amplitude histograms are indicated at right side of each trace. Unitary current amplitude was 12 pA at a membrane potential of −20 mV. Histograms of open and closed time in control (**C**) and in the presence of BmK-PL (**D**) are shown. The solution contained 500 mM Cs^+ in the cis solution and 50 mM Cs^+ in the trans solution and the free Ca^{2+} concentration was 1 μM in both solutions.

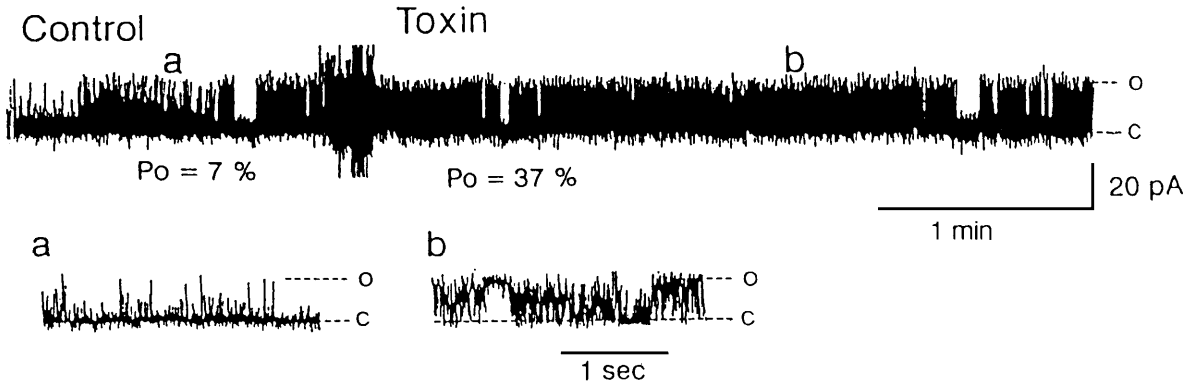

Figure 5 Single channel recordings of partially-purified ryanodine receptor Ca^{2+}-release channel

Current traces show continuous recordings of single channel activities using partially purified ryanodine receptor Ca^{2+}-release channel before (a) and after (b) application of BmK-PL in the presence of 5 mM ATP. The conditions of the solutions were same as those used in Figure 4. Tracings at the points indicated by a and b are expanded in the lower panels. 'o', indicates open level of the channel.

suggests that BmK-PL modulates the ryanodine receptor by a different mechanism from that of ATP, caffeine or KCl.

In single channel recordings made in the presence of 5 mM ATP, the P_o was 0.33 with the purified receptor, which is 4–5 times higher than that of partially-purified receptors before the application of BmK-PL. Application of BmK-PL did not increase the P_o (Figure 7A). Kinetic studies also gave similar parameters: a single open time constant of 0.8 versus 0.6 ms and two closed time constants of 2.3 and 9.4 ms versus 2.9 and 9.1 ms, before and after application of BmK-PL, respectively (results not shown). We also studied the effects of BmK-PL on the purified ryanodine receptor in the absence of ATP. As shown in Figure

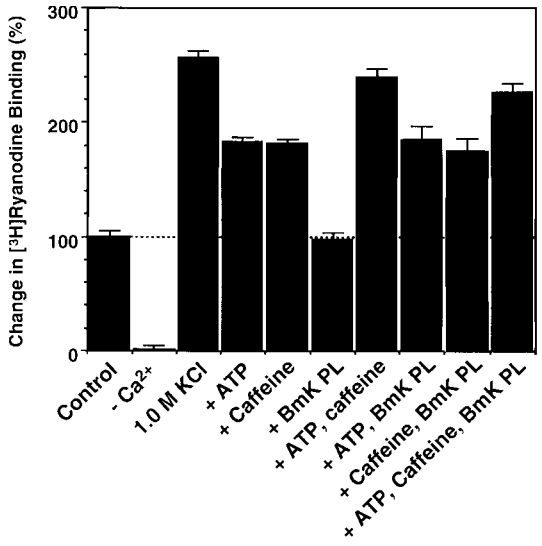

Figure 6 Effects of BmK-PL on [^{3}H]ryanodine binding to the purified ryanodine receptor in the presence and absence of various modulators

Purified ryanodine receptor (2 μg) was incubated with 10 nM [^{3}H]ryanodine at 30 °C in buffer B as a control. The addition or depletion of receptor modulators was also examined. $-Ca^{2+}$, omission of $CaCl_2$ from buffer B; 1.0 M KCl, KCl added to buffer B to a final concentration of 1.0 M; + ATP, 5 mM ATP; + caffeine, 10 mM caffeine; + BmK-PL, 100 μg/ml; + ATP, caffeine, 5 mM ATP plus 10 mM caffeine. BmK-PL (100 μg/ml, final concentration) was added to all other samples as indicated. Data are means ± S.E.M. ($n = 3$) expressed as a percentage of the control.

7(B), the P_o (0.03) was low, and the value was virtually unchanged (0.032) after the application of BmK-PL. These results indicate that ATP apparently increases the P_o, but purified ryanodine receptors are not affected by the application of BmK-PL either in the presence or in the absence of ATP. This is consistent with data obtained in the [^{3}H]ryanodine binding studies described above.

Polypeptide components of the ryanodine receptor preparations and Western blot analysis

In this study, we investigated the effect of BmK-PL on ryanodine receptors obtained from three different purification protocols: triad membranes, preparations partially purified by heparin–agarose columns after solubilization, and highly-purified preparations obtained by sucrose-gradient sedimentation followed by heparin–agarose columns. Polypeptide components of the three different preparations were analysed by SDS/PAGE. As shown in Figure 8(A), the triad membrane contained a ryanodine receptor of 360 kDa as well as several smaller polypeptides (Figure 8A, lane 1), while the partially-purified sample was composed mainly of the ryanodine receptor plus a 95 kDa polypeptide (Figure 8A, lane 2). The 95 kDa component is totally excluded from the highly-purified sample (Figure 8A, lane 3).

Western blot analysis was employed to identify the 95 kDa component using an anti-triadin antibody. Although highly non-specific immunoreactions interfered with analysis of the triad preparation (Figure 8B, lanes 1 and 4), the antibody clearly reacted with a 95 kDa band in the partially-purified preparations (Figure 8B, lane 2), and did not react at all with the purified-receptor preparations (Figure 8B, lane 3).

DISCUSSION

The scorpion toxin, BmK-PL, enhanced [^{3}H]ryanodine binding to triad preparations from skeletal muscles; apparent maximum enhancements reached 310% at 10 nM [^{3}H]ryanodine, with an EC_{50} of 70 μg/ml (8 μM), and > 950% at 2 nM [^{3}H]ryanodine. The data suggest that BmK-PL activates ryanodine binding to the receptor by modulating K_d or B_{max}. Scatchard analysis revealed that the K_d increased 8.4-fold but B_{max} was unchanged (Figure 3). Similar results were obtained by the addition of 5 mM ATP and 1 M KCl (Figure 3). Previous studies have shown that ryanodine preferentially binds to the open channel conformation of the Ca^{2+}-release channel (ryanodine receptor), and that chemicals such as ATP, caffeine and high concentrations of salts (e.g., 1 M KCl and 1 M NaCl) stimulate [^{3}H]ryanodine binding to the open-state of the release channels [32–34]. Therefore the present data strongly suggest that BmK-PL also activates the Ca^{2+}-release channel by opening the channels.

The electrophysiological studies confirmed that the application of BmK-PL increased the Ca^{2+}-release channel openings in both triad preparations and in partially-purified ryanodine receptors which were incorporated into planar-lipid bilayers (Figures 4 and 5). The increased openings were due to an increase in the P_o with no change in the single channel conductance and kinetic parameters.

In contrast, BmK-PL did not enhance the [^{3}H]ryanodine-binding activity when purified receptors were used (Figure 6). The purified receptors retained their intrinsic activity; they were activated by ATP, caffeine, or 1 M KCl, and inhibited by the depletion of Ca^{2+}. Therefore the lack of sensitivity to BmK-PL of purified receptors did not result from loss of receptor activity during purification. This was also confirmed by studies of single channel activities (Figure 7). The P_o was low (0.03) in the absence of ATP but increased to 0.33 following the addition of 5 mM ATP. However, the addition of BmK-PL did not affect the P_o in either the presence or absence of ATP. These results are consistent with those obtained from [^{3}H]ryanodine binding assays. They also exclude the possibility that the BmK-PL effect was masked in highly-purified receptor preparations by an increased sensitivity to ATP.

Our data show that BmK-PL enhances release-channel activity in triad or partially-purified preparations, but has little or no effect on purified receptors. These results suggest that BmK-PL modulates ryanodine receptors indirectly, by binding to molecule(s) other than the receptor (channel) protein itself. If so, BmK-PL functions differently from direct activators, such as ATP, caffeine and 1 M KCl [32–34]. It follows that putative acceptors of BmK-PL could be expressed in muscle cells, are present in partially-purified preparations, and absent from highly purified ones. A polypeptide band of 95 kDa was observed in triad and partially-purified preparations, but was absent from purified receptor preparations (Figure 8A). This polypeptide is a candidate for the BmK-PL acceptor and could mediate toxin activity. Western blot analysis identified the 95 kDa polypeptide as triadin (Figure 8B), a membrane protein localized to the junctional sarcoplasmic reticulum of skeletal muscle and thought to play an important role in muscle excitation–contraction coupling [16,17]. Recently, we demonstrated [31] that triadin inhibited ryanodine binding and Ca^{2+}-release channel opening in purified ryanodine receptors.

Here, we propose that BmK-PL can restore the activity of ryanodine-receptor–triadin complexes in which Ca^{2+}-release activity is inhibited. When BmK-PL is applied, it may bind to triadin and eliminate the release channel inhibition. This

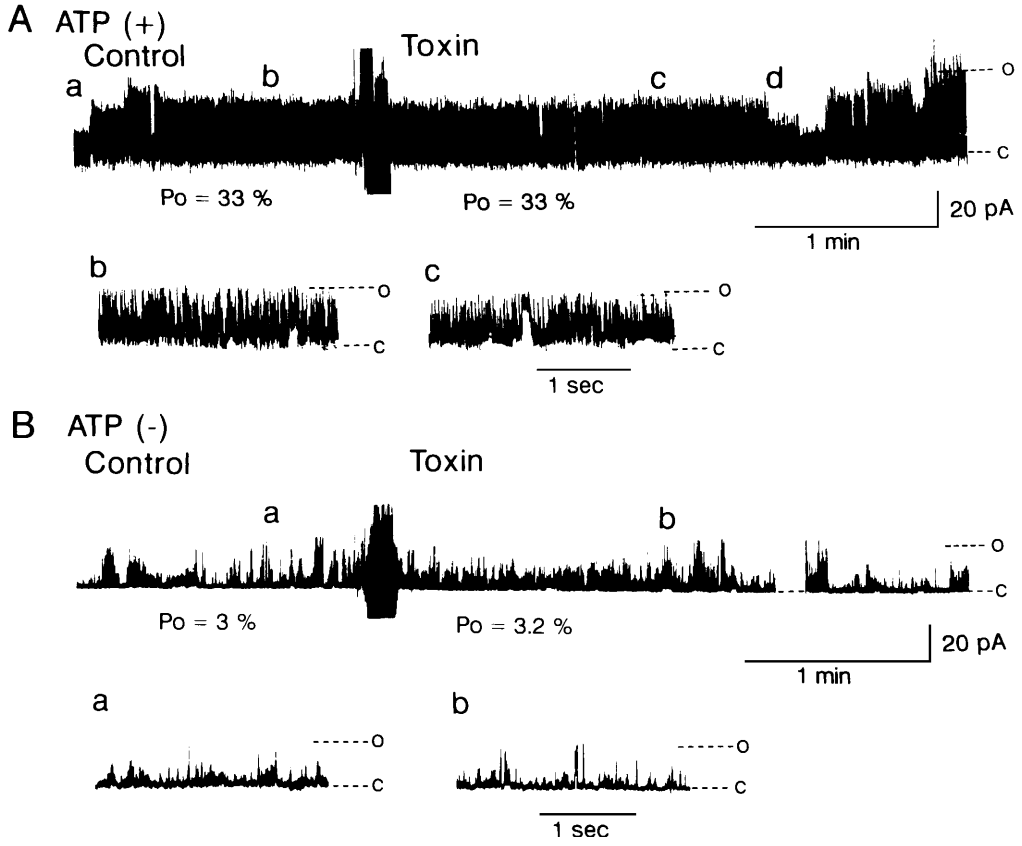

Figure 7 Single channel recordings of the purified ryanodine receptor Ca^{2+}-release channel

Continuous recordings of single channel activities of the purified ryanodine receptor before and after the application of BmK-PL (100 μg/ml), in the presence (**A**) or absence (**B**) of 5 mM ATP, are shown. The solutions were same as those used in Figures 4 and 5. (**A**) The membrane potential was held at +40 mV as indicated by b and c in the upper panel. Membrane potentials were changed between 0 to +60 mV in the traces indicated by a and d. Tracings at the points indicated by b and c are shown with an expanded time scale in the lower panels. (**B**) Membrane potential was held at +20 mV. Tracings at the points indicated by a and b are shown with an expanded time scale in the lower panels.

hypothesis is supported by the finding that the P_o of the triad or partially-purified preparations is low (0.019 and 0.07, respectively) when BmK-PL is absent, while the P_o of the purified receptor is high (0.33) without BmK-PL, a value equivalent to that (0.37) obtained in partially-purified receptors plus BmK-PL. In purified receptors in which triadin was excluded, no enhancement of the activities was observed following the addition of BmK-PL, suggesting that BmK-PL does not bind to the ryanodine receptor.

Several scorpion toxins have been isolated from different species. Most of them act on sodium channels [3] and have 60–70 amino acid residues (molecular mass approx. 7 kDa) with disulphide bonds in conserved positions. Some toxins, such as charybdotoxin and agitoxin, inhibit potassium channels [4–6] and have only 35–40 amino acid residues (molecular mass approx. 4 kDa). With respect to the defined 30 amino acids of BmK-PL, this toxin has no sequence similarity to scorpion toxins affecting potassium channels. Valdivia et al. [7,8,20] reported that a toxin from the African scorpion *Buthotus hottentota* (molecular mass 5–8 kDa), and imperatoxin A (activator) and I (inhibitor) from *Pandinus imperator* (molecular mass of 5 and 10.5 kDa, respectively) modified ryanodine receptors. Therefore these toxins and BmK-PL constitute a new family of scorpion toxins with similar function. However, Valdivia et al. [7,8,20] showed that venom from *Buthutus hottentota* and imperatoxin A increased the B_{max} of ryanodine binding, while BmK-PL increased the K_d but did not change the B_{max} value. In addition, the amino acid sequence of imperatoxin A [35] shows no similarity to BmK-PL. Consequently, BmK-PL is different from *Buthutus hottentota* toxin and imperatoxin A not only in structure but also in activation mechanism.

Ohizumi and collaborators have observed that myotoxin α, a snake venom, stimulates skeletal-muscle ryanodine receptors [36]. They also found that calsequestrin is essential for receptor activation by myotoxin α [37]. Myotoxin α has no apparent sequence similarity to BmK-PL [38]. Therefore, animal toxins modulating ryanodine receptors can be divided into subclasses with regard to both structure and mechanism of action.

It is worth noting that BmK-PL activates the ryanodine receptor from skeletal muscles efficiently, while it has little effect on cardiac muscle. It is thought that excitation–contraction coupling occurs differently in skeletal and cardiac muscles [11,12], suggesting that ryanodine-receptor function may also differ in these muscle types. Triadin was first identified in skeletal-muscle junctional sarcoplasmic reticulum [16,17] but has also been found in cardiac muscle, in which three isoforms with different C-terminal sequences have been characterized [39]. However, functional differences in triadins found in skeletal and cardiac muscle

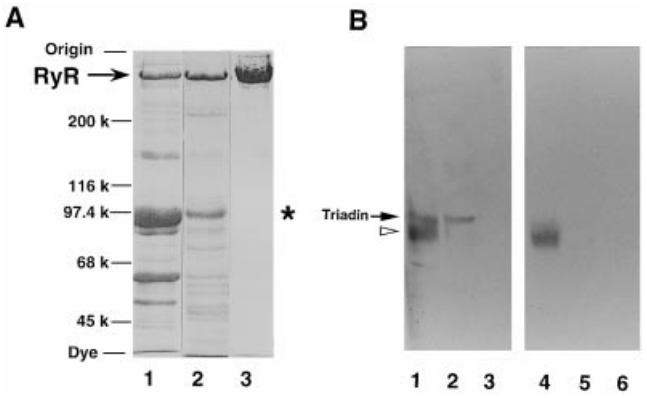

Figure 8 Polypeptide components (A) and Western blot analysis (B) of skeletal-muscle preparations from different purification protocols

(**A**) SDS/PAGE was used to analyse skeletal-muscle preparations as described in the Materials and methods section. Lane 1, triad membranes (40 μg); lane 2, sample partially-purified using a heparin–agarose column (0.3 μg); lane 3, highly-purified sample (0.3 μg) obtained by sucrose gradient sedimentation followed by heparin–agarose. Polypeptide bands were observed after Coomassie Brilliant Blue staining. Size-marker proteins used are myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), and ovalbumin (45 kDa). RyR, ryanodine receptor. The asterisk indicates a 95 kDa polypeptide. (**B**) Western blot analysis was carried out using an anti-triadin antibody (lanes 1–3) as described in the Materials and methods section. Lanes 1 and 4, triad membranes (30 μg); lanes 2 and 5, partially-purified receptor (9 μg); lanes 3 and 6, highly purified receptor (6 μg). Lanes 4–6, control blots using 10 μM antigen peptide to evaluate non-specific immunoreactions of the anti-triadin antibody. White arrowhead shows a non-specifically blotted band of 90 kDa (possibly Ca^{2+}-ATPase; lanes 1 and 4).

have not been demonstrated. Therefore BmK-PL will provide a new probe to analyse the mechanism of ryanodine receptor action associated with excitation–contraction coupling in both muscle types.

This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas 'Natural Supramolecules: Chemistry and Function' No. 07229102, 08219233 (H. N.), on General Subject No. 07457543 (H. N.), and 05044151 (M. H.) from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

1 Keegan, H. L. (1980) Scorpions of Medical Importance, The University Press of Mississippi, Mississippi
2 Rochat, H., Bernard, P. and Couraud, F. (1979) Adv. Cytopharmacol. **3**, 325–334
3 Catterall, W. A. (1980) Annu. Rev. Pharmacol. Toxicol. **20**, 15–43
4 Gimenez-Gallego, G., Navia, M. L., Reuben, J. P., Katz, G. M., Kaczorowski, G. J. and Garcia, M. L. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 3329–3333
5 Garcia-Caivo, M., Leonard, R. J., Novick, J., Stevens, S. P., Schmalhofer, W., Kaczorowski, G. J. and Garcia, M. L. (1993) J. Biol. Chem. **268**, 18866–18874
6 Garcia, M. L., Garcia-Caivo, M., Hidalgo, P., Lee, A. and MacKinnon, R. (1994) Biochemistry **33**, 6834–6839
7 Valdivia, H. H., Fuentes, O., El-Hayek, R., Morrissette, J. and Coronado, R. (1991) J. Biol. Chem. **266**, 19135–19138
8 Valdivia, H. H., Kirby, M. S., Lederer, W. J. and Coronado, R. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 12185–12189
9 Schneider, M. F. and Chandler, W. K. (1973) Nature (London) **242**, 244–246
10 Block, B. A., Imagawa, T., Campbell, K. P. and Franzini-Armstrong, C. (1988) J. Cell Biol. **107**, 2587–2600
11 Rios, E. and Pizzaro, G. (1991) Physiol. Rev. **71**, 849–908
12 Näbauer, M., Callewaert, G., Cleemann, L. and Morad, M. (1989) Science **244**, 800–803
13 Yano, K. and Zarain-Herzberg, A. (1994) Mol. Cell. Biochem. **135**, 61–70
14 Fliegel, L., Ohnishi, M., Carpenter, M. R., Khanna, V. K., Reithmeiner, R. A. and MacLennan, D. H. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 1167–1171
15 Mitchell, R. D., Simmerman, H. K. and Jones, L. R. (1988) J. Biol. Chem. **263**, 1376–1381
16 Caswell, A. H., Brandt, N. R., Brunschwig, J. P. and Purkerson, S. (1991) Biochemistry **30**, 7507–7513
17 Knudson, C. M., Stang, K. K., Jorgensen, A. O. and Campbell, K. P. (1993) J. Biol. Chem. **268**, 12637–12645
18 Jones, L. R., Zhang, L., Sanborn, K., Jorgensen, A. O. and Kelly, J. (1995) J. Biol. Chem. **270**, 30787–30796
19 Zhang, L., Kelly, J., Schmeisser, G., Kobayashi, Y. M. and Jones, L. R. (1997) J. Biol. Chem. **272**, 23389–23397
20 El-Hayek, R., Lokuta, A. J., Arevalo, C. and Valdivia, H. H. (1995) J. Biol. Chem. **270**, 28696–28704
21 Yong-Hua Ji (1994) Ph.D. Thesis, Shizuoka Prefectural University, Shizuoka, Japan
22 Mitchell, R. D., Palade, P. and Fleischer, S. (1983) J. Cell Biol. **96**, 1008–1016
23 Lindsay, A. R. G. and Williams, A. J. (1991) Biochim. Biophys. Acta **1064**, 89–102
24 Lai, F. A., Erickson, H. P., Rousseau, E., Liu, Q.-Y. and Meissner, G. (1988) Nature (London) **331**, 315–319
25 Inui, M., Saito, A. and Fleischer, S. (1987) J. Biol. Chem. **262**, 15637–15642
26 Miyawaki, A., Furuichi, T., Ryou, Y., Yoshikawa, S., Nakagawa, T., Saito, T. and Mikoshiba, K. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 4911–4915
27 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
28 Bradford, M. M. (1970) Anal. Biochem. **72**, 248–254
29 Fabiato, A. (1988) Methods Enzymol. **157**, 378–417
30 Colquhorn, O. and Sigworth, F. J. (1983) in Single Channel Recording (Sackman, B. and Neher, E., eds.), pp. 191–263, Plenum Publishing Co., New York
31 Ohkura, M., Furukawa, K.-I., Kuruma, A., Nomura, A., Kawano, S., Hiraoka, M., Kuniyasu, A., Nakayama, H. and Ohizumi, Y. (1998) Biochemistry **37**, 12987–12993
32 Imagawa, T., Smith, J. S., Coronado, R. and Campbell, K. P. (1987) J. Biol. Chem. **262**, 16636–16643
33 Chu, A., Diaz-Munnoz, M., Hawkies, M. J., Btush, K. and Hamilton, S. L. (1990) Mol. Pharmacol. **37**, 735–741
34 Imagawa, T., Takasago, T. and Shigekawa, M. (1989) J. Biochem. (Tokyo) **106**, 342–348
35 Zamudio, F. Z., Gurrola, G. B., Arevalo, C., Areekumar, R., Walker, J. W., Valdivia, H. H. and Possani, L. D. (1997) FEBS Lett. **405**, 385–389
36 Furukawa, K., Funayama, K., Ohkura, M., Shima, Y., Tu, A. T. and Ohizumi, Y. (1994) Br. J. Pharmacol. **113**, 233–239
37 Ohkura, M., Ide, T., Furukawa, K., Kawasaki, T., Kasai, M. and Ohizumi, Y. (1995) Can. J. Physiol. Pharmacol. **73**, 1181–1185
38 Fox, J. W., Elzinga, M. and Tu, A. T. (1979) Biochemistry **18**, 678–684
39 Guo, W., Jorgensen, A. O., Jones, L. R. and Campbell, K. (1996) J. Biol. Chem. **271**, 458–465

Received 4 August 1998/11 December 1998; accepted 1 February 1999