Palytoxin disrupts cardiac excitation-contraction coupling through interactions with P-type ion pumps

Jens Kockskämper, Gias U. Ahmmed, Aleksey V. Zima, Katherine A. Sheehan, Helfried G. Glitsch, and Lothar A. Blatter

Palytoxin disrupts cardiac excitation-contraction coupling through interactions with P-type ion pumps. Am J Physiol Cell Physiol 287: C527–C538, 2004. First published April 14, 2004; 10.1152/ajpcell.00541.2003.—Palytoxin is a coral toxin that seriously impairs heart function, but its effects on excitation-contraction (E-C) coupling have remained elusive. Therefore, we studied the effects of palytoxin on mechanisms involved in atrial E-C coupling. In field-stimulated cat atrial myocytes, palytoxin caused elevation of diastolic intracellular 

Ca2+ concentration ([Ca2+]i) transient, which underlies contraction, have not been however, have shown unambiguously that palytoxin indeed targets the Na+-K+ pump, converting the enzyme into a nonselective cation channel (summarized in Ref. 44). Furthermore, elegant kinetic studies of palytoxin-induced channels in cardiac myocytes and HEK-293 cells strongly support the notion that the toxin binds to the pump molecule and locks it in a channel-like open state (2).

In the heart, palytoxin leads to myocardial ischemia, ventricular fibrillation, and cardiac failure. These effects have been attributed in part to intense constriction of the coronary vasculature (25, 50). In addition, palytoxin exerts direct effects on the heart, including depolarization of resting membrane potential, changes of action potential (AP) configuration, generation of afterpotentials and arrhythmias, reduction of phasic tension, and contracture (23, 24, 38, 41, 42, 51). Studies of the underlying mechanisms, however, are sparse and controversial (11, 12, 41, 42). Given the pronounced effects on ion homeostasis, membrane potential, and tension, palytoxin is expected to markedly alter cardiac excitation-contraction (E-C) coupling. However, palytoxin effects on Ca2+-induced Ca2+ release (CICR) and the resulting intracellular Ca2+ concentration ([Ca2+]i) transient, which underlies contraction, have not been investigated so far. Furthermore, most previous studies have focused on ventricular preparations, whereas palytoxin effects on the atrium have gained much less attention. Atrial physiology differs in several respects from ventricular physiology. For example, AP duration is much shorter in the atrium than in the ventricle. Atrial myocytes are smaller than ventricular cells. They lack a regular T-tubular system and contain peripheral junctional and central nonjunctional sarcoplasmic reticulum (SR) (30). Thus, unlike ventricular cells, atrial myocytes exhibit characteristic spatiotemporal inhomogeneities in AP-induced SR Ca2+ release (for review, see Ref. 7). Ca2+ release starts in the peripheral junctional SR, triggered by Ca2+ influx via L-type Ca2+ channels during the AP, and then spreads actively in a wavelike fashion to the central nonjunctional SR via CICR. In addition, Na+/Ca2+ exchange plays a prominent role in atrial Ca2+ homeostasis, particularly during diastole, resulting in a decrease of SR Ca2+ content after a rest period, which is in clear contrast to the postrest potentiation observed in the ventricle (32). Finally, the atrium also performs endocrine functions by secreting atrial natriuretic peptide (ANP) in response to stretch. ANP secretion has a Ca2+-dependent component and is likely to be affected by changes in Ca2+ homeostasis.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Because of the paucity of mechanistic insight into palytoxin action on cardiac tissue in general and the distinct differences between the atrium and the ventricle in particular with respect to E-C and excitation-secretion coupling, we set out to characterize palytoxin effects on the various steps of E-C coupling in atrial myocytes. We investigated the effect of palytoxin on membrane potential and AP configuration, voltage-activated Ca\(^{2+}\) current (\(I_{\text{Ca}}\)), SR function, and the AP-induced [Ca\(^{2+}\)] transient. The results revealed that palytoxin interferes with the sarcoplaemmal (SL) Na\(^+\)-K\(^+\) pump and the SR Ca\(^{2+}\) pump (SERCA), two major P-type ion pumps in cardiac myocytes, and thereby disrupts atrial E-C coupling. A preliminary account of this work was published previously in abstract form (29).

METHODS

Cell isolation. Atrial myocytes from cat heart were isolated by using an enzymatic procedure described previously (52). The procedures for myocyte isolation from cat hearts and SR vesicle preparation from rat ventricles were approved by the Institutional Animal Care and Use Committee of Loyola University Chicago, Stritch School of Medicine. Briefly, cats were anesthetized with pentobarbital sodium (50 mg/kg body wt ip). After thoracotomy, hearts were excised, mounted on a Langendorff apparatus, and perfused via the aorta with collagenase-based oxygenated solutions (37°C). Freshly isolated myocytes were plated on glass coverslips or culture dishes and allowed to settle for \(>30\) min until they were used for experimentation. All experiments were conducted at room temperature (22–25°C).

Imaging of [Ca\(^{2+}\)]\(_i\), transients. Cells were loaded with the fluorescent Ca\(^{2+}\) indicator fluo 3-acetoxymethyl ester (fluo 3-AM; Molecular Probes, Eugene, OR) in Tyrode solution (in mM: 140 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, and 10 d-glucose, pH 7.35 adjusted with NaOH) for 20–30 min at room temperature. A coverslip with fluo 3-AM-loaded myocytes was placed on the stage of an inverted microscope (Axiovert 100; Carl Zeiss, Oberkochen, Germany). Cells were superfused continuously with Tyrode solution containing (in mM) 140 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, and 10 d-glucose, pH 7.35 adjusted with NaOH) for 20–30 min at room temperature. A coverslip with fluo 3-AM-loaded myocytes was placed on the stage of an inverted microscope (Axiovert 100; Carl Zeiss, Oberkochen, Germany). Cells were superfused continuously with Tyrode solution containing (in mM) 140 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, and 10 d-glucose, pH 7.35 adjusted with NaOH) for 20–30 min at room temperature. A coverslip with fluo 3-AM-loaded myocytes was placed on the stage of an inverted microscope (Axiovert 100; Carl Zeiss, Oberkochen, Germany). Cells were superfused continuously with Tyrode solution containing (in mM) 140 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, and 10 d-glucose, pH 7.35 adjusted with NaOH) for 20–30 min at room temperature. A coverslip with fluo 3-AM-loaded myocytes was placed on the stage of an inverted microscope (Axiovert 100; Carl Zeiss, Oberkochen, Germany). Cells were superfused continuously with Tyrode solution containing (in mM) 140 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, and 10 d-glucose, pH 7.35 adjusted with NaOH) for 20–30 min at room temperature. A coverslip with fluo 3-AM-loaded myocytes was placed on the stage of an inverted microscope (Axiovert 100; Carl Zeiss, Oberkochen, Germany).

Electrophysiology. Currents and membrane voltage were recorded in the whole cell (ruptured patch) configuration of the patch-clamp technique (15) with the use of an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA). After backfilling with pipette solution (see below), patch pipettes had resistances of 2–3 MΩ. After establishment of the whole cell configuration, at least 3–5 min were allowed for equilibration between the pipette solution and the cell interior.

For recording of \(I_{\text{Ca}}\), cells were superfused with a modified Tyrode solution composed of (in mM) 135 NaCl, 4 CsCl, 5 CaCl\(_2\), 1 MgCl\(_2\), 0.1 ouabain, 10 HEPES, and 10 d-glucose, pH 7.3 (adjusted with NaOH). The pipette solution contained (in mM) 130 cesium glutamate, 20 CsCl, 0.33 MgCl\(_2\), 4 Na\(_2\)ATP, 5 EGTA, and 10 HEPES, pH 7.4 (adjusted with CsOH). The cell interior was filled with 0.1M sodium acetate. Oscillation was used to prevent palytoxin effects on the Na\(^+\)-K\(^+\) pump. Myocytes were voltage clamped at a holding potential of \(-40\) mV to inactivate Na\(^+\) channels. \(I_{\text{Ca}}\) was elicited by 200-ms rectangular voltage steps to +10 mV every 5 s. Current amplitude was measured as the difference between the peak inward current and the steady-state current at the end of the depolarization step. Current-voltage (\(I-V\)) relationships were obtained by step depolarizations to potentials between \(-30\) and +50 mV in 10-mV increments. To allow comparison between cells, \(I_{\text{Ca}}\) was normalized to cell membrane capacitance (\(C_{\text{m}}\)), estimated by integration of the capacitative current transient during hyperpolarizing voltage steps. The average \(C_{\text{m}}\) of the atrial myocytes used for \(I_{\text{Ca}}\) recordings was 48 ± 2 pF (\(n = 14\)).

AP were recorded in the current-clamp mode. Cells were superfused continuously with Tyrode solution containing (in mM) 140 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 0.33 Na\(_2\)HPO\(_4\), 10 HEPES, and 10 d-glucose, pH 7.4 (adjusted with NaOH). The pipette solution was composed of (in mM) 100 potassium glutamate, 40 KCl, 1 MgCl\(_2\), 4 Na\(_2\)ATP, 10 HEPES, and 2 EGTA, pH 7.2 (adjusted with KOH).

Planar lipid bilayer recordings of ryanodine receptor channel activity. Rat ventricular SR vesicles were obtained as described previously (54). Planar lipid bilayers were formed from a lipid mixture containing phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (ratio 5:4:1) dissolved in n-decane at a final lipid concentration of 45 mg/ml. SR vesicles were added to the cis-chamber corresponding to the cytosolic side of the ryanodine receptor (RyR) channel. The trans-chamber (luminal side of the RyR) was connected to the virtual ground of the amplifier. During fusion, the cis- and trans-chambers contained the following solutions (in mM): 400 CsCH\(_3\)SO\(_3\) (cis), 400 (trans); 0.1 CaCl\(_2\); and 20 HEPES, pH 7.3 (CsOH). After channel incorporation, the concentration of CsCH\(_3\)SO\(_3\) in the trans-chamber was increased to 400 mM and free [Ca\(^{2+}\)] in the cis-chamber was adjusted to 3 \(\mu\)M by addition of EGTA. Free [Ca\(^{2+}\)] in the experimental solutions was verified with a Ca\(^{2+}\)-sensitive minielectrode (4). Single-channel currents were recorded with the use of an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). All recordings were made at a holding potential of \(-15\) mV. Currents were filtered at 1 kHz and digitized at 5 kHz.

Ca\(^{2+}\) uptake by SR microsomes. SR vesicles (25–50 \(\mu\)g) prepared from rat ventricle were added to a cuvette containing 1 ml of buffered phosphate medium comprising (in mM) 100 KH\(_2\)PO\(_4\), 3 MgCl\(_2\), 2 ATP, 0.01 ruthenium red, and 0.2 antipyrlylazo III (APIII; Sigma), pH 7.0. Changes in Ca\(^{2+}\) were measured as changes in absorbance between 710 and 790 nm of the Ca\(^{2+}\)-sensitive dye APIII by means of an ultraviolet-visible diode array spectrophotometer (Cory 50; Varian). Ca\(^{2+}\) uptake was initiated by the addition of Ca\(^{2+}\) aliquots (CaCl\(_2\), 10 \(\mu\)M) to the cuvette. The rapid rise in Ca\(^{2+}\)-dependent APIII absorbance was followed by a slower absorbance decrease due to ATP-dependent Ca\(^{2+}\) uptake by the SR vesicles. The rate of Ca\(^{2+}\) loading of the vesicles, i.e., the net Ca\(^{2+}\) uptake, equals the SR pumping rate minus the Ca\(^{2+}\) leak rate. The latter reflects the activity of the RyRs, which were blocked by ruthenium red. Thus, under our experimental conditions, net Ca\(^{2+}\) uptake by the vesicles was equal to the experimental Ca\(^{2+}\) pumping rate.

Drugs. Palytoxin was isolated (6) and generously provided by Dr. L. Bérès (Universität Kiel, Germany) or was purchased from Sigma-RBI. Palytoxin from both sources was prepared as 10\(^{-5}\) M aqueous.
stock solution and stored in aliquots at −20°C. Solutions containing 1–10 nM palytoxin were used throughout this study. The respective solutions were prepared freshly immediately before the experiments by diluting the desired amount of stock solution in the experimental solution. The cardiac glycoside ouabain (Alexis Biochemicals, San Diego, CA) was used as a specific inhibitor of the Na⁺-K⁺-ATPase (43). Ouabain was directly dissolved in the extracellular solution at concentrations (10–50 μM) indicated elsewhere in the text.

Statistics. Data from n cells are presented as means ± SE. In bilayer single-channel recordings and SR microsome Ca²⁺ uptake experiments, n refers to the number of individual measurements obtained from different SR preparations. Statistical differences between data sets were evaluated by performing Student’s t-test and were considered significant at P < 0.05.

RESULTS

Palytoxin induces a ouabain-sensitive inward current in cat atrial myocytes. In mammalian ventricular myocytes, palytoxin induces opening of nonselective cation channels permeable to Na⁺ and K⁺ by converting the Na⁺-K⁺ pump into a channel (2, 20, 27, 35). At negative holding potentials, the palytoxin-induced inward current is carried mainly by Na⁺ and can be inhibited by cardiac glycosides (27). Thus, in an initial set of experiments, we examined whether palytoxin was able to induce an inward current with similar characteristics in cat atrial myocytes. Figure 1 shows original recordings of the membrane current of two atrial myocytes that were whole cell voltage clamped at −40 mV. Current deflections (thin vertical lines in Fig. 1) are due to short step depolarizations to various test potentials (−30 to +50 mV) to elicite voltage-dependent Ca²⁺ currents (see Fig. 7). Palytoxin (2 nM) was applied either in the absence (Fig. 1A) or in the presence (Fig. 1B) of ouabain. In the absence of ouabain, palytoxin induced an inward current (Fig. 1A). This inward current started after ~20 s and reached an apparent maximum of ~500 pA after 6–7 min. Withdrawal of the coral toxin slightly diminished the inward current. After washout, steady-state membrane current amounted to −327 pA compared with −16 pA before palytoxin. In a total of six cells, the maximum magnitude of the palytoxin-induced inward current was −346 ± 52 pA (Fig. 1C). To further characterize the palytoxin-induced current, I-V relationships were obtained from the steady-state currents of the step depolarizations. Figure 1D illustrates average I-V curves (n = 4) obtained before and during palytoxin application. The difference current, i.e., the palytoxin-induced membrane current, exhibited almost linear dependence on membrane voltage with a reversal potential between 0 and 10 mV.

Figure 1B shows an experiment conducted in the presence of ouabain (100 μM). When palytoxin was applied under these conditions, there was no inward current shift during ≥15 min. In nine cells, the coral toxin caused virtually no change in the holding current in the presence of ouabain (0 ± 2 pA; Fig. 1C). Thus, in cat atrial myocytes, palytoxin induced a current displaying a linear I-V relationship with a reversal potential of ~5 mV that could be blocked by ouabain. The results resemble previous findings from ventricular myocytes (27) and are in line with the notion that the toxin targets the Na⁺-K⁺ pump, converting a fraction of pumps into nonselective cation channels.

Palytoxin effects on electrically evoked atrial [Ca²⁺]i transients. Figure 2 shows examples of palytoxin-induced changes of electrically evoked [Ca²⁺]i transients in single atrial myocytes. When an atrial myocyte was challenged with palytoxin (Fig. 2A), there was a quickly developing, monotonic reduction in the amplitude of the [Ca²⁺]i transient. Peak systolic [Ca²⁺]i, decreased from 650 nM under control conditions to ~200 nM in the presence of the coral toxin (Fig. 2A, left). The reduction in the amplitude of the [Ca²⁺]i transient was not accompanied by a reduction of SR Ca²⁺ content. Application of 10 mM caffeine (to assess SR Ca²⁺ content) induced a large increase in [Ca²⁺]i, indicating that, although electrically evoked [Ca²⁺]i, transients were small, a large amount of releasable Ca²⁺ was stored in the SR (Fig. 2A, middle). Moreover, propagating waves of [Ca²⁺]i were observed shortly after the caffeine application (Fig. 2A, right). Thus palytoxin reduced the amplitude of the electrically evoked [Ca²⁺]i transient and, at the same time, induced [Ca²⁺]i waves, most likely by causing SR Ca²⁺ overload.

Figure 2B shows two other effects of palytoxin on [Ca²⁺]i. Exposure of an atrial myocyte to the coral toxin caused alternating large and small amplitude [Ca²⁺]i transients (Ca²⁺ alternans). Furthermore, in the continued presence of palytoxin, electrical stimulation of the myocyte failed to elicit [Ca²⁺]i transients. Transient increases of [Ca²⁺]i occurred at irregular intervals and were no longer correlated with the stimulus. The results presented in Fig. 2, A and B, demonstrate that palytoxin seriously impairs atrial E-C coupling, causing a decrease in [Ca²⁺]i transient amplitude, Ca²⁺ alternans, and [Ca²⁺]i waves as well as failures of Ca²⁺ release. In all of the 23 atrial cells studied, palytoxin induced at least one of these abnormalities in Ca²⁺ signaling, in most cases two or more (see Fig. 2, A and B). A reduction of the [Ca²⁺]i transient amplitude was observed in 21 cells (91%), Ca²⁺ alternans in 12 cells (52%), failure of electrically evoked Ca²⁺ release in 6 cells (26%), and propagating [Ca²⁺]i waves in 8 cells (35%). Typically, decreases in [Ca²⁺]i transient amplitude and/or Ca²⁺ alternans were elicited first, within seconds after application of palytoxin (Fig. 2, A and B; see also Fig. 4A). Failures of stimulation-induced Ca²⁺ release and [Ca²⁺]i waves occurred later, after tens of seconds (Fig. 2, A and B). In addition to the changes in systolic [Ca²⁺]i, palytoxin also altered diastolic [Ca²⁺]i levels. As is evident in Fig. 2, A and B, resting [Ca²⁺]i increased in the presence of the coral toxin. Elevated diastolic [Ca²⁺]i levels under palytoxin treatment were observed in all but 2 of the 23 atrial myocytes studied. On average, palytoxin increased diastolic [Ca²⁺]i, from 100 nM (assumed [Ca²⁺]i) to 191 ± 20 nM (n = 23) and decreased peak systolic [Ca²⁺]i from 786 ± 91 to 341 ± 57 nM (n = 23; P < 0.001) or by 55 ± 5%. The palytoxin-mediated alterations of atrial [Ca²⁺]i signaling were poorly reversible, which is in line with previous observations in various preparations (1, 2, 9, 21, 22).

Palytoxin causes subcellular alterations of Ca²⁺ release. Examples of the subcellular alterations of Ca²⁺ release induced by palytoxin are presented in Fig. 3. An atrial myocyte and the position of the scan line are shown in the center of Fig. 3Ab (same cell and recording as in Fig. 2A). Subsarcolemmal [Ca²⁺]i transients from both edges of the cell (black and red traces as marked in Fig. 3Ab) are displayed in Fig. 3, Aa and Ac. Figure 3Ab illustrates linescan images obtained before (left) and during (right) exposure of the cell to palytoxin as indicated by the horizontal bars under the top trace. Before palytoxin exposure, both subsarcolemmal regions exhibited
large $[\text{Ca}^{2+}]$, transients with amplitudes >1 μM. The corresponding linescan image revealed U-shaped $[\text{Ca}^{2+}]$ signals, indicating that the $[\text{Ca}^{2+}]$ increase started at the subsarcolemmal space before spreading to the center of the myocyte. Furthermore, regions of high and low $[\text{Ca}^{2+}]$, could be distinguished. These regions of high $[\text{Ca}^{2+}]$, occurred at regular intervals of ~2–3 μm. They were recently identified as individual Ca$^{2+}$ release sites from the subsarcolemmal junctional
SR (j-SR) and the central nonjunctional SR (nj-SR) (7, 30). Their successive recruitment from the periphery to the center indicates that the centripetal propagation of \([\text{Ca}^{2+}]\), occurred via active CICR from j-SR and nj-SR. During palytoxin treatment, \([\text{Ca}^{2+}]\), transients were depressed. Generally, the amplitudes of the subsarcolemmal \([\text{Ca}^{2+}]\), transients were reduced to <300 nM (Fig. 3, Aa and Ac), and central \([\text{Ca}^{2+}]\) release was entirely absent (Fig. 3Ab, right). Whereas subsarcolemmal \([\text{Ca}^{2+}]\), transients from one side of the cell (Fig. 3Aa, black trace) were uniformly suppressed, their counterparts from the other side exhibited \([\text{Ca}^{2+}]\) alternans (Fig. 3Ac, red trace). During the small amplitude \([\text{Ca}^{2+}]\), transient, \([\text{Ca}^{2+}]\) release from j-SR and nj-SR appeared to be absent. The small \([\text{Ca}^{2+}]\), signal observed under these conditions (signal 4 in Fig. 3Ab) was most likely caused by SL \([\text{Ca}^{2+}]\) influx only. By contrast, during the large-amplitude \([\text{Ca}^{2+}]\), transients (signals 2 and 6 in Fig. 3Ab), some \([\text{Ca}^{2+}]\) release from j-SR and nj-SR (arrows) was detectable. Centripetal propagation of \([\text{Ca}^{2+}]\) was graded with the amplitude of the initial \([\text{Ca}^{2+}]\) release from j-SR. When subsarcolemmal release was comparably large, \([\text{Ca}^{2+}]\) propagated over two more central sites (signal 6). When subsarcolemmal release was somewhat smaller, only one more central site was activated (signal 2), and when subsarcolemmal release was even smaller or absent, no additional central release was triggered (signal 4). The results show that the palytoxin-induced \([\text{Ca}^{2+}]\) alternans was characterized by subcellular inhomogeneities reminiscent of those described previously for pyruvate- and pacing-induced \([\text{Ca}^{2+}]\) alternans in these cells (7, 28).

Figure 3B illustrates the subcellular \([\text{Ca}^{2+}]\) release patterns of another cell treated with palytoxin. The atrial myocyte and the position of the scan line are illustrated in Fig. 3Bb, right. Figure 3Ba shows the transverse linescan image, and Fig. 3Bb demonstrates the corresponding \([\text{Ca}^{2+}]\), signals from the right and left sides of the cell. Dashed vertical lines indicate the time of electrical stimulations [stimuli 1–5 (S1–S5)]. Except for S3 and S5, SR \([\text{Ca}^{2+}]\) release was spatially inhomogeneous and not directly correlated with the stimulus. During S1, for example, SR \([\text{Ca}^{2+}]\) release was greatly reduced on the left side and completely absent on the right side of the myocyte. Shortly after the stimulus, however, a large \([\text{Ca}^{2+}]\), signal was generated, apparently by spontaneous release of \([\text{Ca}^{2+}]\), from the central nj-SR (asterisk). Before the next stimulus (S2), spontaneous propagating \([\text{Ca}^{2+}]\) release occurred, first on the left side and then on the right side of the cell. The latter event is marked by a black arrow. It took place immediately (~120 ms) before the electrical stimulation. Thus SR \([\text{Ca}^{2+}]\) release is still refractory at this time, and no further release is triggered by S2. Instead, the spontaneous \([\text{Ca}^{2+}]\) release propagated in a wave-like fashion from the right to the left side of the cell. Similarly, spontaneous \([\text{Ca}^{2+}]\) release from the subsarcolemmal j-SR on the left side of the myocyte (arrow) preceded S4 (by ~120 ms), rendering AP-induced release at this site refractory. However, S4 was able to trigger SR \([\text{Ca}^{2+}]\) release on the right side of the myocyte, which then traveled back as a \([\text{Ca}^{2+}]\), wave across the cell center to the left side. These results demonstrate that the palytoxin-induced alterations of E-C coupling can exhibit significant subcellular variations, including subcellular \([\text{Ca}^{2+}]\) alternans (Fig. 3A), and illustrate how uncoordinated, spatially restricted \([\text{Ca}^{2+}]\) release can generate local failure of AP-evoked \([\text{Ca}^{2+}]\) release and arrhythmogenic \([\text{Ca}^{2+}]\), waves. 

Atrial \([\text{Ca}^{2+}]\), transients in the presence of ouabain and palytoxin. Because ouabain was able to completely block the palytoxin-induced inward current (Fig. 1), we tested whether it could also attenuate or abolish the palytoxin-mediated changes of E-C coupling. Exposure of an atrial myocyte to 10 µM

---

**Fig. 2.** Palytoxin alters atrial excitation-contraction (E-C) coupling. A: action potential (AP)-induced intracellular \([\text{Ca}^{2+}]\) transients ([\(\text{Ca}^{2+}\)_i]) transients in an atrial myocyte. Application of 1 nM palytoxin reduces the amplitude of the \([\text{Ca}^{2+}]\), transient (left) and causes \([\text{Ca}^{2+}]\), waves (right). Assessment of sarcoplasmic reticulum (SR) \([\text{Ca}^{2+}]\) content by 10 nM caffeine (middle) reveals high SR \([\text{Ca}^{2+}]\), load despite greatly reduced \([\text{Ca}^{2+}]\), transient amplitudes. B: same measurements as described in A in a different cell. Palytoxin (2 nM) reduces \([\text{Ca}^{2+}]\), transient amplitude and causes \([\text{Ca}^{2+}]\) alternans and failures of \([\text{Ca}^{2+}]\), release. Electrical stimulations are indicated by filled circles under the trace. Dashed lines in A and B mark diastolic \([\text{Ca}^{2+}]\), levels (100 nM) at the beginning of the recordings. Palytoxin elevated diastolic \([\text{Ca}^{2+}]\), in both cells.
Fig. 3. Subcellular changes of atrial Ca\(^{2+}\) signaling induced by palytoxin. A: same cell and recording as shown in Fig. 2A. Atrial myocyte and position of the scan line are shown schematically in b (middle), as well as linescan images of \([\text{Ca}\^{2+}]_i\) transients obtained before (left) and during (right) palytoxin treatment. Subsarcolemmal \([\text{Ca}\^{2+}]_i\) transients (spatial width 2.8 \(\mu\)m) from the edges of the cell as indicated in the drawing of the cell and the linescan images are shown in a and c, respectively. B: a different palytoxin-treated atrial myocyte is shown schematically with the scan line in b (right). Transverse linescan image (a) and corresponding \([\text{Ca}\^{2+}]_i\) traces (b, spatial width 1.0 \(\mu\)m) are shown from the right (red) and left (black) sides of the cell. Electrical stimulations S1–S5 are indicated by dashed vertical lines. Various events of spontaneous Ca\(^{2+}\) release are evident. Arrows mark spontaneous Ca\(^{2+}\) release from the peripheral junctional SR immediately preceding stimulation and thus causing local failures of AP-induced Ca\(^{2+}\) release.
ouabain resulted in a large increase of systolic [Ca\(^{2+}\)], from 400 to 730 nM (Fig. 4A), consistent with the well-known positive inotropic effect of the cardiac glycoside. In five myocytes, ouabain (10–25 μM) increased systolic [Ca\(^{2+}\)], from 643 ± 178 to 807 ± 177 nM (n = 5; P < 0.05). When palytoxin was applied in the continued presence of ouabain (Fig. 4A), the amplitude of the [Ca\(^{2+}\)], transient started to decline immediately, and, after ~30 s, Ca\(^{2+}\) alternans developed. The decrease in [Ca\(^{2+}\)], transient amplitude occurred despite high SR Ca\(^{2+}\) load, as assessed by caffeine. In additional experiments, palytoxin was also able to cause failures of Ca\(^{2+}\) release and [Ca\(^{2+}\)], waves in the presence of ouabain (data not shown). In all seven atrial myocytes pretreated with 10–25 μM ouabain, palytoxin (2 nM) elicited a reduction of the [Ca\(^{2+}\)], transient amplitude [from 812 ± 128 to 431 ± 91 nM (n = 7; P < 0.01), or by 45 ± 9%], Ca\(^{2+}\) alternans in six cells (86%), failure of Ca\(^{2+}\) release in one cell (14%), and [Ca\(^{2+}\)], waves in two cells (29%). These changes qualitatively and quantitatively resemble the changes in E-C coupling in the absence of ouabain and indicate that micromolar concentrations of ouabain were unable to prevent palytoxin effects on atrial SR Ca\(^{2+}\) release. We therefore set out to study palytoxin effects in the presence of higher concentrations of ouabain. Figure 4B shows electrically evoked [Ca\(^{2+}\)], transients of an atrial myocyte challenged with 500 μM ouabain. In sharp contrast to the effects of lower concentrations of the cardiac glycoside (Fig. 4A), this high concentration of ouabain elicited a decrease in [Ca\(^{2+}\)], transient amplitude. Furthermore, Ca\(^{2+}\) alternans developed within less than 15 s. Similar results were obtained in five more cells. At 500 μM, ouabain never led to an increase of the [Ca\(^{2+}\)], transient amplitude (0 of 6 cells) but instead induced Ca\(^{2+}\) alternans in four (67%) and [Ca\(^{2+}\)], waves in one of these cells (17%). Thus high concentrations of ouabain induced changes in SR Ca\(^{2+}\) release comparable to those evoked by palytoxin. This finding rendered irrelevant any further studies of palytoxin actions in the presence of high concentrations of ouabain. It suggested that serious impairment of Na\(^+\)-K\(^+\) pump function by either palytoxin or high concentrations of ouabain may result in similar defects in E-C coupling.

An alternative explanation for the palytoxin-mediated disruption of E-C coupling might be a direct action of the coral toxin on the CICR mechanism. Therefore, we examined whether palytoxin modulated the activity of the RyR Ca\(^{2+}\) release channel, Ca\(^{2+}\) uptake by the SR, and/or voltage-dependent Ca\(^{2+}\) influx (i.e., I\(_{\text{Ca}}\)).

Palytoxin does not affect RyR channel activity. Activity of single RyR channels reconstituted into planar lipid bilayers was recorded before (control) and after the addition of 3 and 10 nM palytoxin (Fig. 5). Examples of original current traces are presented in Fig. 5A. Statistical analysis of single-channel currents from a total of six RyRs (Fig. 5B) revealed that open probability (P\(_{\text{o}}\)), mean open time (t\(_{\text{op}}\)), and current amplitude (I) were unaffected by the toxin. Mean values for the three parameters under control conditions and after the addition of 3 and 10 nM palytoxin were P\(_{\text{o}}\), 0.076 ± 0.029, 0.078 ± 0.027, and 0.070 ± 0.020, respectively; t\(_{\text{op}}\), 2.52 ± 0.69, 2.38 ± 0.74, and 2.27 ± 0.65 ms, respectively; and I, −7.84 ± 0.37, −7.84 ± 0.41, and −7.78 ± 0.33 pA, respectively (n = 6 channels for all data; all differences not statistically significant). Thus palytoxin did not affect cardiac RyR Ca\(^{2+}\) release channels.

Palytoxin inhibits SR Ca\(^{2+}\) uptake. Figure 6A shows original recordings of Ca\(^{2+}\) uptake by SR vesicles obtained in the
absence and presence of 3 nM palytoxin. The addition of Ca\(^{2+}\) (10 \(\mu\)M) resulted in a rapid rise of the APIII absorbance signal, followed by a slower decay toward baseline values. The absorbance decay reflects the net Ca\(^{2+}\) uptake by the SR vesicles. With the RyRs blocked by ruthenium red (10 \(\mu\)M), this net Ca\(^{2+}\) uptake is a measure of the ability of the SR Ca\(^{2+}\)-ATPase to pump Ca\(^{2+}\) into the vesicles (see METHODS). The experiments revealed that SR Ca\(^{2+}\) pumping was slowed by palytoxin (3 nM) compared with control conditions (Fig. 6A). For a quantitative comparison, the time constant, \(\tau\), of the Ca\(^{2+}\) decay was assessed under each condition. Average \(\tau\) values for a total of four experiments are shown in Fig. 6B. Palytoxin increased \(\tau\) from 0.78 ± 0.11 min (control) to 1.24 ± 0.19 min (palytoxin, \(P < 0.05\), or on average by 58%, demonstrating that the coral toxin inhibited the SERCA.

Palytoxin does not affect \(I_{Ca}\). Figure 7A shows a series of Ca\(^{2+}\) currents (\(a\)–\(g\)) recorded from an atrial myocyte whole cell voltage-clamped at −40 mV. The extracellular solution contained 100 \(\mu\)M ouabain to block palytoxin effects on the Na\(^{+}\)-K\(^{+}\) pump (see Fig. 1). \(I_{Ca}\) was elicited by 200-ms step depolarizations to 10 mV. The first \(I_{Ca}\) (\(a\)) was recorded 3 min after the whole cell configuration was established (time 0). \(I_{Ca}\) in control conditions revealed stable amplitudes and inactivation kinetics. Exposure of the cell to palytoxin did not affect \(I_{Ca}\) amplitude and kinetics (cf. Fig. 7A, \(e\)–\(g\)). I-V relationships of the Ca\(^{2+}\) current were obtained before (\(Ad\)) and after (\(Af\)) 2-min exposure to palytoxin. The voltage protocol and the corresponding current traces are displayed in Fig. 7B. Data from six atrial myocytes are summarized in Fig. 7C. The mean \(I_{Ca}\)-V relationships recorded before and during palytoxin treatment were almost identical. Thus, in cat atrial myocytes, palytoxin did not directly affect voltage-dependent Ca\(^{2+}\) influx.

Palytoxin depolarizes the resting membrane potential and induces delayed afterdepolarizations. In a final step, palytoxin effects on atrial AP were studied. AP were elicited by extracellular field stimulation (1 Hz), and membrane potential was measured by means of whole cell current clamp. Figure 8A displays a recording of the membrane potential of an atrial myocyte before and during exposure to palytoxin (2 nM). The baseline indicates resting membrane potential (RP), and upward deflections are due to evoked AP. The parts of the recording marked \(a\)–\(e\) (horizontal lines under the voltage trace) are shown on an expanded scale in Fig. 8B. Under control conditions, RP was fairly stable at about −68 mV (dashed line in Fig. 8A). Palytoxin caused an initial depolarization of RP to −50 mV (next to \(trace b\)) followed by a short repolarization to −75 mV (\(trace c\)). Finally, a large and sustained depolarization to values close to 0 mV occurred. During the transient repolarization (Fig. 8Bc), RP was stable, similar to control conditions (Fig. 8Ba). By contrast, during both the initial (Fig. 8Bb) and the final depolarizations (Fig. 8Bd), significant fluctuations of RP were observed, including first occasional (Fig. 8Bb) and later regular (Fig. 8Bd) delayed afterdepolarizations (DADs, arrows in \(b\) and \(d\)), presumably because of spontaneous SR Ca\(^{2+}\) release and [Ca\(^{2+}\)] waves. AP marked \(i\)–\(iv\) are shown superimposed in Fig. 8C. Comparison of AP \(i\)–\(iv\) revealed that, except for a broader shoulder in \(iv\), AP configuration remained rather constant. At the end of the recording, when RP was close to 0 mV, AP no longer could be evoked and only a stimulus artifact remained [Fig. 8, \(v\) in \(Be\) and \(C\)]. In a total of three cells, RP was initially depolarized from −66.3 ± 2.7 to −40.3 ± 7.7 mV (\(P < 0.05\)); after transient repolarization to
−64.7 ± 5.8 mV (P < 0.05), sustained depolarization to −17.0 ± 6.6 mV occurred (P = 0.05). In all cells, palytoxin induced frequent DADs with amplitudes up to 12 mV. Changes in AP configuration were minor, however, and no trend toward longer or shorter AP was evident.

DISCUSSION

Primary actions of palytoxin: modulation of Na⁺-K⁺ pump and SERCA, two closely related P-type ion pumps. Previous studies have indicated that the Na⁺-K⁺ pump is a molecular target of palytoxin (14, 18, 26, 39, 46). Palytoxin binds to the Na⁺-K⁺ pump and converts it into a nonselective cation channel (26). The palytoxin-induced current through this channel was characterized previously in ventricular myocytes (20, 27, 35). In addition, a more recent study revealed detailed insights into the molecular mechanism of palytoxin action on the Na⁺-K⁺ pump (2, 17). According to these findings, the Na⁺-K⁺ pump can be regarded as an unselective cation channel with two gates. During the physiological transport cycle of the Na⁺-K⁺ pump, the two gates are never open at the same time. Rather, binding of ligands (Na⁺, K⁺, ATP) causes sequential opening and closing of the two gates and a change in ion selectivity, allowing for directed transport of Na⁺ and K⁺ against their respective electrochemical gradients. Palytoxin, by binding to the pump molecule, induces a unique conformational state in which both gates are open at the same time and in which the pump molecule is thus converted to an unselective cation channel permeable to both Na⁺ and K⁺. Palytoxin action is modulated by the physiological ligands of the pump (Na⁺, K⁺, ATP) and can be explained well by the known partial reactions of the pump cycle (2, 17).

In line with the observations in ventricular myocytes (2, 20, 27, 35), we found that palytoxin generated a very similar current in atrial myocytes. In particular, the palytoxin-induced current displayed a linear I-V relationship that was reversed at −5 mV, was comparable in magnitude, and could be antagonized by cardiac glycosides. From these results, we conclude that palytoxin induced an inward current in cat atrial myocytes through conversion of Na⁺-K⁺ pumps into nonselective cation channels. Assuming a single-channel conductance of ~10 pS (2, 17), a specific membrane capacitance of 1 μF/cm², and a Na⁺-K⁺ pump density of 1,000–2,500 μm⁻² (13), we estimate that far less than 1 of every 1,000 Na⁺-K⁺ pumps was converted into a channel by palytoxin.

One of the novel findings of this study is that in addition to its action on the Na⁺-K⁺ pump, palytoxin also inhibited the
function of the SERCA in isolated SR vesicles. The fact that palytoxin interferes with both the Na\textsuperscript{+}/H\textsuperscript{+}-K\textsuperscript{+}/H\textsuperscript{+} pump and the SERCA might be explained by structural similarities and the high degree of homology between the two ion pumps (48). In line with this notion, Scheiner-Bobis et al. (45) suggested in a recent report that palytoxin can convert the colonic H\textsuperscript{+}/K\textsuperscript{+} pump, another closely related P-type ion pump, into a cation channel. While our experiments on isolated SR vesicles clearly establish an inhibitory action of palytoxin on the SERCA, they do not provide any insights into the mechanism of inhibition. Furthermore, it is not known whether the toxin is able to enter the cells and reach the SERCA. Thus a possible contribution of SERCA inhibition to the palytoxin effects in intact cells remains elusive. Nonetheless, the results regarding SERCA lend further support to the notion that, from a biochemical point of view, palytoxin acts on P-type ion pump structures. Likewise, it is not known whether the SL Ca\textsuperscript{2+} pump, another related P-type ion pump involved in Ca\textsuperscript{2+} regulation, could also be a target for palytoxin. While we cannot rule out this possibility, putative inhibition of the SL Ca\textsuperscript{2+} pump is unlikely to contribute significantly to palytoxin effects on atrial Ca\textsuperscript{2+} signaling, given that Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (NCX) and SERCA are the dominating Ca\textsuperscript{2+} transport systems in cardiac myocytes, accounting for 98% of Ca\textsuperscript{2+} elimination during relaxation (37; see also Ref. 47). Most important, however, is that conversion of SL Na\textsuperscript{+}/K\textsuperscript{+} pumps into nonselective cation channels is sufficient to explain the observed palytoxin actions and thus might be the major mechanism underlying palytoxin effects on atrial E-C coupling, as discussed below.

Secondary effects of palytoxin: disruption of atrial E-C coupling. To the best of our knowledge, this is the first report of palytoxin effects on atrial Ca\textsuperscript{2+} signaling. It indicates that the coral toxin elevated diastolic [Ca\textsuperscript{2+}], decreased the amplitude of the AP-induced [Ca\textsuperscript{2+}], transient, and caused Ca\textsuperscript{2+} alternans and [Ca\textsuperscript{2+}] waves as well as failures of Ca\textsuperscript{2+} release (Figs. 2–4). These alterations of atrial [Ca\textsuperscript{2+}] signaling may well explain the negative inotropic and arrhythmogenic effects of palytoxin noted in earlier studies (23, 38, 41). Further examination of the subcellular patterns of Ca\textsuperscript{2+} signaling revealed spatial inhomogeneities in the palytoxin effects and identified spontaneous release of Ca\textsuperscript{2+}, predominantly from the subsarcolemmal j-SR, as the underlying mechanism for the
palytoxin-induced \([\text{Ca}^{2+}]\) waves and failures of SR Ca\(^{2+}\) release.

Because palytoxin seriously impairs atrial Ca\(^{2+}\) signaling, it follows that the mechanism of CICR is somehow affected by the toxin, either directly or indirectly. Previous voltage clamp studies of atrial trabeculae and isolated ventricular myocytes found palytoxin-mediated changes of \(I_{\text{Ca}}\) (27, 41). In both cases, however, these changes were attributed to secondary effects caused mainly by a shift of the reversal potential of \(I_{\text{Ca}}\) due to intracellular Ca\(^{2+}\) accumulation. Consistent with these results, we did not observe any effect of the toxin on \(I_{\text{Ca}}\) in \([\text{Ca}^{2+}]_i\)-buffered atrial myocytes. Furthermore, RyR activity remained completely unaffected by palytoxin, suggesting that the coral toxin does not exhibit any direct effects on CICR but rather impairs atrial E-C coupling via indirect mechanisms. Recordings of membrane potential indicated that AP configuration and the development of DADs (5). Similarly, Na\(^{+}\)/H\(^{+}\) extrusion (via reverse mode NCX) and spontaneous SR Ca\(^{2+}\) release as visualized in the Ca\(^{2+}\) imaging studies.

**Involvement of Na\(^{+}\)/Ca\(^{2+}\) and Na\(^{+}\)/H\(^{+}\) exchange.** Palytoxin-induced Na\(^{+}\) loading of the atrial myocytes affects the activities of Na\(^{+}\)-dependent ion transporters. In this regard, NCX and Na\(^{+}\)/H\(^{+}\) exchange (NHE) are of particular importance because of their involvement in the regulation of Ca\(^{2+}\) and H\(^{+}\) homeostasis and thus atrial contraction. Previous studies of palytoxin cardiotoxicity have implicated either NCX (5). Similarly, Na\(^{+}\) loading is expected to reduce H\(^{+}\) extrusion via the NHE and to decrease intracellular pH (pH\(_i\)).

However, it remains unclear why high and low concentrations of ouabain might have effects other than inhibiting the Na\(^{+}\)-K\(^{+}\) pump. For example, at higher concentrations, ouabain could increase the SR Ca\(^{2+}\) leak through the RYR (33, 40), thereby causing decreased SR Ca\(^{2+}\) content and reduced [Ca\(^{2+}\)]\(_i\) transients. **Proposed mechanism of palytoxin action in atrium.** According to our results, we propose the following mechanism of palytoxin action in atrial myocytes. The primary molecular target of the toxin is the SL Na\(^{+}\)-K\(^{+}\) pump. Palytoxin binding converts a small fraction of pumps into nonselective cation channels, resulting in K\(^{+}\) efflux, Na\(^{+}\) influx, and concomitant depolarization. Intracellular Na\(^{+}\) loading and depolarization reduce the driving force for Ca\(^{2+}\) extrusion by NCX and thus promote intracellular Ca\(^{2+}\) loading. Elevated diastolic [Ca\(^{2+}\)]\(_i\) and depolarization may decrease \(I_{\text{Ca}}\) and the AP-induced [Ca\(^{2+}\)]\(_i\) transient. Furthermore, sufficiently large depolarizations lead to failures of stimulation-induced Ca\(^{2+}\) release. The increased SR Ca\(^{2+}\) content, on the other hand, causes spontaneous Ca\(^{2+}\) release, [Ca\(^{2+}\)]\(_i\) waves, DADs, and, ultimately, arrhythmias and contracture.

**ACKNOWLEDGMENTS**

We are grateful to Dr. L. Béress (Universität Kiel, Germany) for a generous gift of palytoxin. The technical assistance of A. Balzer-Ferrai, U. Müller, and H. R. Gray is gratefully acknowledged.


**GRANTS**

This work was supported by National Heart, Lung, and Blood Institute Grants HL-51941 and HL-62231 (to L. A. Blatter), American Heart Association National Center Grants 95002520 and 9905448N (to L. A. Blatter), and the Deutsche Forschungsgemeinschaft (grant Gl 72/3-7 to H. G. Glitsch, fellowship to J. Kockskämper). J. Kockskämper also was a recipient of a postdoctoral fellowship from the Falk Foundation (Loyola University Chicago, IL). K. A. Sheehan was supported by an Arthur J. Schmitt Dissertation Fellowship (Loyola University Chicago) and a Lilly Graduate Student Fellowship in Cardiovascular Research (Loyola University Chicago and Eli Lilly).

**REFERENCES**

Palytoxin and Cardiac E-C Coupling

8. Cannell MB, Cheng H, and Lederer WJ. Spatial non-uniformities in [Ca\(^{2+}\)] during excitation-contraction coupling in cardiac myocytes. Bio-


10. Frelin C and Van Renterghem C. Palytoxin. Recent electrophysiological and pharmacological evidence for several mechanisms of action. Gen


13. Glütsch HG. Electrophysiology of the sodium-potassium-ATPase in car-

14. Habermann E. Palytoxin acts through Na\(^+\),K\(^+\)-ATPase. Toxicon 27:

15. Hamill OP, Marty A, Neher E, Sakmann B, and Sigworth FJ. Im-
proved patch-clamp techniques for high-resolution current recording from

16. Harkins AB, Kurebayashi N, and Baylor SM. Depolarization induced by palytoxin and grayanotoxin-I in isolated cardiac
components of Ca\(^{2+}\)–Ca\(^{2+}\) exchange current reveals two components of Ca\(^{2+}\) release from sarcoplasmic reticulum of cardiac atrial myocytes. FEBs Lett 275: 181–184, 1990.

17. Ikeda M, Mitani K, and Ito K. Palytoxin induces a nonselective cation
channel in single ventricular cells of rat. Naunyn Schmiedebergs Arch

currents and Ca\(^{2+}\) mobilization in smooth muscle cells of rabbit portal

19. Ishii K, Ito KM, Uemura D, and Ito K. Possible mechanism of paly-
toxin-induced Ca\(^{2+}\) mobilization in porcine coronary artery. J Pharma-

20. Ito K, Karaki H, and Urakawa N. Effects of palytoxin on mechanical
and electrical activities of guinea pig papillary muscle. Jpn J Pharma-

21. Ito K, Saruwatari N, Mitani K, and Enomoto Y. Characterization of
depolarization induced by palytoxin and grayanotoxin-1 in isolated cardiac
tissue from dogs and guinea pigs. Naunyn Schmiedebergs Arch Phar-

22. Ito K, Urakawa N, and Koike H. Cardiovascular toxicity of palytoxin in

23. Kim SY, Marx KA, and Wu CH. Involvement of the Na,K-ATPase in the
induction of ion channels by palytoxin. Naunyn Schmiedebergs Arch Phar-

24. Kinosita K, Ikeda M, and Ito K. Properties of palytoxin-induced whole
cell current in single rat ventricular myocytes. Naunyn Schmiedebergs

25. Kochskämper J and Blatter LA. Subcellular Ca\(^{2+}\) alternans represents a
novel mechanism for the generation of arrhythmogenic Ca\(^{2+}\) waves in

26. Kochskämper J, Hüscher J, Glütsch HG, and Blatter LA. Palytoxin-
induced alterations in E-C coupling in isolated cat atrial myocytes (Ab-

27. Kochskämper J, Sheehan KA, Bare DJ, Lipsius SL, Mignery GA, and
Blatter LA. Activation and propagation of Ca\(^{2+}\) release during excitation-