Inhibition of the Inositol Trisphosphate Receptor of Mouse Eggs and A7r5 Cells by KN-93 via a Mechanism Unrelated to Ca\(^{2+}\)/Calmodulin-dependent Protein Kinase II Antagonism*

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KN-93, a Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMII) inhibitor, concentration-dependently and reversibly inhibited inositol 1,4,5-trisphosphate receptor (IP\(_3\)R)-mediated [Ca\(^{2+}\)]\(_{i}\) signaling in mouse eggs and permeabilized A7r5 smooth muscle cells, two cell types predominantly expressing type-1 IP\(_3\)R (IP\(_3\)R-1). KN-92, an inactive analog, was ineffective. The inhibitory action of KN-93 on Ca\(^{2+}\) signaling depended neither on effects on IP\(_3\) metabolism nor on the filling grade of Ca\(^{2+}\) stores, suggesting a direct action on the IP\(_3\)R. Inhibition was independent of CaMII, since in identical conditions other CaMII inhibitors (KN-62, peptide 281–309, and autocamtide-related inhibitory peptide) were ineffective and since CaMII activation was precluded in permeabilized cells. Moreover, KN-93 was most effective in the absence of Ca\(^{2+}\). Analysis of Ca\(^{2+}\) release in A7r5 cells at varying [IP\(_3\)], of IP\(_3\)R-1 degradation in eggs, and of [\(^{3}H\)]IP\(_3\) binding in Sf9 microsomes all indicated that KN-93 did not affect IP\(_3\)_R binding. Comparison of the inhibition of Ca\(^{2+}\) release and of [\(^{3}H\)]IP\(_3\) binding by KN-93 and calmodulin (CaM), either separately or combined, was compatible with a specific interaction between KN-93 and a CaM-binding site on IP\(_3\)R-1. This was also consistent with the much smaller effect of KN-93 in permeabilized 16HBE14o– cells that predominantly express type 3 IP\(_3\)R, which lacks the high affinity CaM-binding site. These findings indicate that KN-93 inhibits IP\(_3\)R-1 directly and may therefore be a useful tool in the study of IP\(_3\)R functional regulation.

Ca\(^{2+}\) acts as a ubiquitous second messenger that mediates a wide array of cellular functions, including muscle contraction, neurotransmitter release, and egg activation (reviewed in Ref. 1). The spatial and temporal dynamics of Ca\(^{2+}\) release are complex and may range from localized, brief “puffs” of Ca\(^{2+}\) to regenerative oscillations of the global cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) that may last from several minutes to several hours. The nature of the Ca\(^{2+}\) response elicited by a specific cell stimulus is highly regulated; thus, the specificity of the cellular response to a stimulus may be dictated by the precise dynamics of the Ca\(^{2+}\) signal (2, 3).

The complexity of Ca\(^{2+}\) signaling mandates that the regulation of Ca\(^{2+}\) release dynamics must be very intricate and precise. The phosphoinositide pathway is a major intracellular signaling pathway that is involved in the regulation of Ca\(^{2+}\) homeostasis (1). The hydrolysis of phosphatidylinositol 4,5-bisphosphate by a phospholipase C (PLC) leads to the formation of diacylglycerol and inositol 1,4,5-trisphosphate (IP\(_3\)). IP\(_3\) then induces Ca\(^{2+}\) release from the endoplasmic reticulum by binding to and activating the IP\(_3\) receptor (IP\(_3\)R), which acts as a ligand-gated Ca\(^{2+}\) channel. Three isoforms of the IP\(_3\)R, IP\(_3\)R-1, IP\(_3\)R-2, and IP\(_3\)R-3, have been characterized thus far (4). The convergence of multiple regulatory pathways at the IP\(_3\)R is likely to directly influence the characteristic properties of various Ca\(^{2+}\) signals (5). For example, Ca\(^{2+}\) itself is known to regulate its own release through the IP\(_3\)R, giving rise to a bell-shaped relationship between [Ca\(^{2+}\)] and IP\(_3\)-induced Ca\(^{2+}\) release, whereby low [Ca\(^{2+}\)] potentiates Ca\(^{2+}\) release and high [Ca\(^{2+}\)] inhibits it. Ca\(^{2+}\) may influence IP\(_3\)R function by directly binding to the receptor, resulting in conformational changes to the receptor, or it may act through Ca\(^{2+}\)-binding proteins such as calmodulin (CaM) (reviewed in Refs. 6 and 7). Three CaM-binding sites have been described on the IP\(_3\)R-1: a low affinity site near the N terminus of the IP\(_3\)R-1 that essentially binds Ca\(^{2+}\)-free CaM (apo-CaM) (8, 9), a high affinity site in the central portion of the regulatory domain that mainly binds Ca\(^{2+}\)/CaM (9, 10), and a third site that only appears after splicing out of S2 (i.e. in peripheral tissues) and is antagonized by CaM-dependent phosphorylation of IP\(_3\)R-1 (11). The consequences of CaM binding to IP\(_3\)R-1 are inhibitory; apo-CaM

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1 The abbreviations used are: PLC, phospholipase C; AIP, autocamtide-related inhibitory peptide; [Ca\(^{2+}\)]\(_{i}\), ionized cytoplasmic calcium concentration; CaM, calmodulin; CaMII, Ca\(^{2+}\)/CaM-dependent protein kinase II; DTT, dithiothreitol; IP\(_3\), inositol 1,4,5-trisphosphate; IP\(_3\)R, IP\(_3\) receptor; MII, metaphase II; pSF, porcine sperm factor.
Inhibits IP₃ binding to its receptor (8, 12), whereas the binding of Ca²⁺/CaM inhibits Ca²⁺ release through the channel (13, 14). The relation, however, between the nature of the CaM-binding sites and the functional effects of CaM remains to be established.

Phosphorylation of the IP₃R provides another means of functional regulation. Phosphorylation of the IP₃R by protein kinases A, C, and G as well as by Ca²⁺/CaM-dependent protein kinase II (CaMIIKII) and Src family tyrosine kinases has been demonstrated (reviewed in Ref. 4), but the functional consequences of these phosphorylations are poorly understood. Cyclic phosphorylation and dephosphorylation of the IP₃R, particularly by CaMIIKII, have been proposed as mechanisms by which complex Ca²⁺-signaling patterns, such as repetitive [Ca²⁺], oscillations, may be regulated (15, 16). In any case, modifications of the IP₃R by Ca²⁺, whether through accessory proteins such as CaM or via direct binding to the IP₃R, contribute to the stringent control of various Ca²⁺ release patterns (6). Thus, further characterization of the intricate regulatory mechanisms of IP₃R function is essential to advance our understanding of Ca²⁺ signaling.

We now report that KN-93, a pharmacological inhibitor of CaMIIKII (17), inhibits IP₃-induced Ca²⁺ release in both permeabilized A7r5 smooth muscle cells, which respond to IP₃ with a single [Ca²⁺], rise, and in mouse metaphase II (MII) stage eggs, which exhibit repetitive [Ca²⁺], oscillations, proportional to IP₃ response in stimulation by IP₃. Both of these cell types express predominantly the IP₃R-1 subtype (18–20), and the Ca²⁺ release properties of A7r5 cells mainly reflect the characteristics of IP₃R-1 (21, 22). Importantly, our data suggest that the inhibition of IP₃-induced Ca²⁺ release by KN-93 is independent of the compound’s ability to antagonize CaMIIKII activity in both cell systems. Furthermore, we show that the site of inhibition occurs at the IP₃R itself. Thus, KN-93 may prove to be a useful tool in the study of IP₃R function and regulation. In addition, our study suggests that caution must be exerted in the use of KN-93 as a CaMIIKII antagonist, given the propensity of the compound to block the Ca²⁺ signaling needed for eventual CaMIIKII activation.

**EXPERIMENTAL PROCEDURES**

**Mouse Egg Recovery and Culture**—Female CD-1 mice were superovulated by sequential injection of 5 IU of equine chorionic gonadotropin (Sigma) followed 48 h later by injection of 5 IU of human chorionic gonadotropin (Chugai Pharmaceutical). MII stage eggs were recovered from the oviducts of stimulated mice 12–15 h following human chorionic gonadotropin injection into a HEPES-buffered solution (TL-Hepes; Sigma) containing 120 mM KCl, 30 mM imidazole-HCl, pH 6.8, and 1 mM EGTA. The eggs were then incubated in a humidified atmosphere containing 7% CO₂.

**Inhibition Preparations and Loading—** KN-93, KN-92, and KN-62 (Calbiochem) were dissolved in MeSO-W (7:1) (Calbiochem) was dissolved in H₂O. For MII egg experiments, the compounds were further diluted in KSOl to working concentrations (1–25 μM), and eggs were loaded by 30-min incubations. For [Ca²⁺]ᵢ fluorescence experiments in permeabilized cells, the water-soluble and the MeSO-soluble forms of KN-93 (Calbiochem) were used with identical results. [²⁵³H]IP₃ binding experiments were all performed with the water-soluble form. Inhibitory peptides 281–309 (Calbiochem) and autoantibody-related inhibitory peptide (AIP; Calbiochem) were dissolved in H₂O and were introduced into MII eggs by microinjection.

**Parotis Sperm Factor (pSF) Preparation—** Cytosolic pSF was prepared as described (29). Briefly, the semen was washed, the sperm suspension was sonicated, and the lysate was ultra-centrifuged. The supernatant was concentrated using ultrafiltration membranes (Centriprep-30; Amicon, Beverly, MA) to final concentrations of 20–30 μg/μl protein. Protein extracts were then mixed with ammonium sulfate at 50% final saturation for 30 min at 4 °C, the precipitates were collected by centrifugation (10,000 × g, 15 min, 4 °C), and the pellets were resuspended in injection buffer (75 mM KCl, 20 mM HEPES, pH 7.0), washed in the same buffer to remove the ammonium sulfate, and concentrated with ultrafiltration membranes. Samples were aliquoted and stored at −80 °C.

**Expression and Purification of CaM—** A pE4D4 expression vector containing human CaM (24) was transformed into BL21 Escherichia coli, grown to midlog phase, and induced with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h at 37 °C. The cultures were lysed by three rapid cycles of freeze/thawing between liquid nitrogen and 37 °C in a buffer containing 50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1 mM dithiothreitol (DTT), 0.8 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, and 75 μg aprotinin. The lysate was cleared by ultracentrifugation at 140,000 × g for 30 min at 4 °C and NaCl and CaCl₂ were added to a final concentration of 500 and 50 mM, respectively. The lysate was subsequently heated to 70 °C, immediately cooled on ice, and centrifuged at 140,000 × g for 30 min at 4 °C. The supernatant was applied to a phenyl-Sepharose column in the presence of Ca²⁺ (50 mM Tris-HCl, pH 7.4, 5 mM CaCl₂, 1 mM DTT, 0.1 mM NaCl). The column was subsequently washed with low salt (50 mM Tris-HCl, pH 7.4, 0.1 mM CaCl₂, 1 mM DTT) and high salt buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM CaCl₂, 1 mM EDTA, 0.5 mM NaCl). Finally, CaM was eluted with 50 mM Tris-HCl, pH 7.4, 5 mM EGTA, 1 mM DTT. The purity of CaM was verified by SDS-PAGE, and the protein concentration was determined (25).

**Microinjection and [Ca²⁺]ᵢ, Monitoring in MII Eggs—** Microinjection techniques were performed as previously described (23). Glass micropipettes were filled with fura-2 dextran (fura-2D, dextran 10 kDa; Molecular Probes, Inc., Eugene, OR), pSF, IP₃ (Molecular Probes), adenophostin A (a generous gift from Dr. K. Tanzawa, Sankyo Co., Tokyo, Japan), peptide 281–309, or AIP, and the loaded solutions were expelled into the eggs’ cytoplasm by pneumatic pressure (FLL-100 picoinjector, Harvard Apparatus, Cambridge, MA). Injection volumes were ~5–10 pl, resulting in final intracellular concentrations of injected compounds of 1.5–5% of the concentration in the injection pipette. [Ca²⁺], monitoring of fura-2D-loaded eggs was performed exactly as previously described (23). [Ca²⁺], monitoring was initiated 30–45 min following fura-2D injection. Differences in basal [Ca²⁺], levels from egg to egg may be partly attributable to variability in the volume of fura-2D received by each egg.

**[Ca²⁺]ᵢ Fluxes on Permeabilized Cells—** Cell culture and [Ca²⁺]ᵢ fluxes on permeabilized A7r5 smooth muscle cells and 16HBE140 bronchial epithelial cells were essentially performed as previously described (21, 26). In brief, cells were permeabilized by treating them for 10 min with 20 μg/ml saponin at 25 °C in a medium containing 120 mM KCl, 30 mM imidazole-HCl, 1 mM EDTA, MgCl₂, 1 mM ATP, and 1 mM EGTA. The nonmitochondrial Ca²⁺ stores were loaded for 45 min in loading medium containing 120 mM KCl, 30 mM imidazole-HCl, pH 6.8, 5 mM MgCl₂, 5 mM ATP, 0.44 mM EGTA, 10 mM NaN₃, and 150 mM free [Ca²⁺] (23 μC/ml; Amersham Biosciences). Efflux was performed in a medium containing 120 mM KCl, 30 mM imidazole-HCl, pH 6.8, and 1 mM EGTA. Activation of the IP₃R was routinely performed with 1 μM IP₃ (Roche Diagnostics, Mannheim, Germany). Any further modifications to loading or to efflux media are indicated in the figure legends. Free [Ca²⁺] was calculated by the Cabup program (available on the Internet at ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/cabup.zip) and based on the stability constants given by Fabiato and Fabiato (27). The efflux medium was collected every 2 min for a 20-min time period. At the end of the experiment, the [Ca²⁺]ᵢ remaining in the stores was released by incubation with 1 ml of a 2% SDS solution for 30 min.

**[²⁵³H]IP₃ Binding to Microsomes of IP₃R-1-overexpressing SF9 Cells—** The heterologous expression of IP₃R-1 in SF9 insect cells, the preparation of microsomes, and the [²⁵³H]IP₃ binding assay were previously described in detail (12, 28). The binding buffer contained 50 mM Tris-HCl, pH 7.0, 10 mM EGTA, and 10 mM β-mercaptoethanol. [²⁵³H]IP₃ (Amersham Biosciences) was used at a concentration of 5 nm, and protein concentration amounted to 50 μg of protein/sample.

**Western Blots—** Western blots were performed as previously described (29). For each replicate, 15 control or KN-93-treated eggs were collected 2 h after injection of adenophostin A. Time-matched uninjected controls were analogously collected. Proteins were separated in SDS-polyacrylamide gels, followed by transfer to nitrocellulose membranes (Micron Separations, Westboro, MA) using a Mini Trans Blot Cell (Bio-Rad). After several washes, the membranes were incubated overnight with a rabbit polyclonal antibody raised against the C-terminal end of IP₃R-1 (Rb04) (30). Membranes were then incubated with a secondary antibody coupled to horseradish peroxidase and were developed using Western blot chemiluminescent reagents (PerkinElmer Life Sciences). The developed membranes were exposed.
Inhibition of IP₃-induced Ca²⁺ Release by KN-93

TABLE I

| Treatment | Egg activation | Number of eggs with \([\text{Ca}^{2+}]_i\) oscillations | \(n\) |
|-----------|----------------|---------------------------------|------|
| Untreated controls | Normal | 8 (100) | 8 |
| KN-93 (25 \(\mu\)M) | Inhibited | 0 (0)² | 10 |
| KN-92 (25 \(\mu\)M) | Normal | 5 (83) | 6 |
| KN-62 (15 \(\mu\)M) | Normal | 6 (86) | 7 |
| Peptide 281–309 (75 \(\mu\)M) | Normal | 5 (83) | 6 |
| AIP (100 \(\mu\)M) | Normal | 10 (100) | 10 |
| W-7 (15 \(\mu\)M) | Delayed | 5 (83) | 6 |

CaMKII antagonists other than KN-93 do not inhibit egg activation or pSF-induced \([\text{Ca}^{2+}]_i\) oscillations

Peptide inhibitors (peptide 281–309 and AIP) were introduced into eggs by microinjection; concentrations reported are approximate intracellular concentrations following microinjection. Eggs were treated with the other inhibitors for a 30-min preincubation. Egg activation was judged on extrusion of the second polar body, an indicator of meiosis resumption. “Normal” indicates that at least 50% of eggs extruded the second polar body by 1.5 h post-pSF injection; “delayed” indicates that at least 50% of eggs required greater than 2 h post-pSF injection to exhibit second PB extrusion; “inhibited” indicates that second PB extrusion was never seen. “[Ca²⁺]i” oscillations refers to eggs that exhibited ≥3 [Ca²⁺]i spikes in 10 min following the initial high amplitude rise. Statistically significant differences compared with untreated controls are indicated by an asterisk (\(\chi^2, p < 0.05\)).

to maximum sensitivity film (Eastman Kodak Co., Fisher), and quantification of IP₃-R-1 bands was performed using Adobe Photoshop (Mountain View, CA). Bands from uninjected control eggs (KN-93/H9262) and respective controls.

Inhibition of IP₃-induced Ca²⁺ Release by KN-93—The initial goal of this study was to evaluate the involvement of CaMKII in mouse egg activation in response to injection of pSF, a proteinaceous factor isolated from boar semen that induces \([Ca^{2+}]_i\) oscillations in mouse MII eggs by stimulating the phosphoinositide pathway (23, 31–33). We initially found that 25 \(\mu\)M KN-93 completely blocked pSF-induced meiotic resumption, which is an early step in egg activation program (see Table I). Since \([Ca^{2+}]_i\) oscillations are responsible for inducing egg activation and resumption of meiosis, we also monitored the \([Ca^{2+}]_i\) responses in KN-93-treated eggs. We were surprised to find that 25 \(\mu\)M KN-93 also completely suppressed pSF-induced \([Ca^{2+}]_i\) oscillations. As shown in Fig. 1A, injection of an untreated MII egg with pSF at a concentration of 0.5 \(\mu\)g/\(\mu\)l in the injection pipette induced an initial high amplitude \([Ca^{2+}]_i\) spike followed by lower amplitude oscillatory spikes in 8 of 8 control eggs tested. In contrast, preincubation of eggs for 30 min in 25 \(\mu\)M KN-93 abolished the ability of pSF injection to induce \([Ca^{2+}]_i\) oscillations in 19 of 19 eggs tested (Fig. 1B). Although the \([Ca^{2+}]_i\) profiles of some eggs treated with 25 \(\mu\)M KN-93, such as shown in Fig. 1B, exhibited the initial \([Ca^{2+}]_i\) spike, subsequent oscillations did not occur. Also, the long delay in the time from pSF injection to the initiation of this \([Ca^{2+}]_i\) rise notable in Fig. 1B was typical of eggs treated with 25 \(\mu\)M KN-93. We found that preincubation of eggs in 25 \(\mu\)M KN-93 for as short as 15 min is sufficient to achieve significant inhibition of \([Ca^{2+}]_i\) oscillations (data not shown); however, a 30-min preincubation was used in these and all subsequent experiments involving MII eggs, since, under these conditions, \([Ca^{2+}]_i\) oscillations were consistently suppressed. Treatment of eggs for 30 min with 25 \(\mu\)M KN-93, the inactive analog of KN-93, did not inhibit \([Ca^{2+}]_i\) oscillations in response to pSF injection (\(n = 5/6\) eggs with oscillations), nor did KN-92 delay the time of the initial \([Ca^{2+}]_i\) rise (Fig. 1C), indicating that KN-93 has a specific effect on the initiation of pSF-induced \([Ca^{2+}]_i\) oscillations. Inhibition of pSF-induced \([Ca^{2+}]_i\) oscillations by KN-93 was found to be concentration-dependent, with maximal inhibition at 25 \(\mu\)M KN-93 and a complete loss of inhibition at concentrations of ≤1.0 \(\mu\)M (Fig. 1D). To evaluate the reversibility of the inhibitory effects of KN-93 on pSF-induced \([Ca^{2+}]_i\) oscillations, MII eggs were incubated in 25 \(\mu\)M KN-93 for 30 min, followed by a 30-min wash in incubation medium lacking KN-93. As shown in Fig. 1E, pSF injection was able to induce \([Ca^{2+}]_i\) oscillations in washed eggs (\(n = 8/8\)), indicating that the inhibitory effects of KN-93 on \([Ca^{2+}]_i\) release are reversible.

Inhibitory Effects of KN-93 Are Downstream of IP₃ Production—There is evidence indicating that KN-93 and other CaMKII antagonists may abrogate IP₃ production by acting at the level of PLC (34, 35). Therefore, it is possible that our observed lack of oscillations in MII eggs in the presence of KN-93 reflects an inhibition of IP₃ production. Since direct measurements of IP₃ production are not yet possible in mammalian eggs, we directly assayed Ca²⁺ release in MII eggs in response to IP₃ injection in order to determine whether KN-93 affects IP₃ production. Fig. 2A indicates that injection of eggs with 100 \(\mu\)M IP₃, which results in an intracellular concentration of ∼1 \(\mu\)M, induced \([Ca^{2+}]_i\) oscillations with a frequency similar to that induced by pSF injection (\(n = 7/7\)). Pretreatment of eggs with 25 \(\mu\)M KN-93, however, significantly inhibited the occurrence of IP₃-induced \([Ca^{2+}]_i\) oscillations (Fig. 2B; \(n = 3/7\) with oscillations, \(\chi^2, p < 0.05\)). Further, the mean frequency of spikes exhibited by the three KN-93-treated eggs that still had oscillations was significantly reduced compared with the frequency exhibited by controls (6.86 ± 0.58 spikes in 10 min for controls versus 4.00 ± 0.89 for KN-93-treated eggs; one-way analysis of variance, \(p < 0.05\)). KN-92, at a concentration of 25 \(\mu\)M, caused no inhibition of IP₃-induced \([Ca^{2+}]_i\) oscillations (Fig. 2C; \(n = 4/4\), \(\chi^2, p > 0.05\)). KN-93 also significantly inhibited the single \([Ca^{2+}]_i\) rise that was induced by injection of 1.0 \(\mu\)M IP₃ (−10 \(\mu\)M intracellular), which is near the threshold IP₃ concentration required for Ca²⁺ release in mouse MII eggs (data not shown). We also evaluated the efficacy of KN-93 at antagonizing Ca²⁺ release induced by injection of adenosinophin A, a potent, nonhydrolyzable agonist of the IP₃-R (36). Injection of untreated control eggs with 10 \(\mu\)M adenosinophin A (−100 \(\mu\)M intracellular) induced high frequency \([Ca^{2+}]_i\) oscillations (Fig. 2D; \(n = 8/9\)). Pretreatment of eggs with 25 \(\mu\)M KN-93 had a similar effect on adenosinophin A-induced \([Ca^{2+}]_i\) oscillations, as was shown for pSF and IP₃; adenosinophin A-induced oscillations were completely inhibited in 13 of 17 KN-93-treated eggs (Fig. 2E; \(\chi^2, p < 0.05\)). Again, KN-92 had no effect (Fig. 2F; \(n = 6/6\)). Thus, the major effect of KN-93 appears to occur downstream of IP₃ production, although a small effect on IP₃ production itself still cannot be ruled out. The sensitivity of adenosinophin A-induced \([Ca^{2+}]_i\) oscillations to KN-93 rules out a possible effect on IP₃ degradation.

To further characterize the inhibitory mechanism of KN-93, we tested the effect of the compound on IP₃-induced Ca²⁺ release in permeabilized A7r5 smooth muscle cells, a somatic cell system in which the properties of the IP₃-R-1 have been studied extensively (e.g. see Refs. 13, 21, 26). In permeabilized A7r5 cells, the addition of 1 \(\mu\)M IP₃, a subsaturating concentration in this system, to the efflux medium induced a more than 3-fold increase in the fractional Ca²⁺-loss from the non-mitochondrial internal stores, whereas the additional inclusion of KN-93 in the efflux medium reduced this IP₃-induced Ca²⁺ release in a concentration-dependent way (Fig. 3A). A closer examination of the concentration dependence of KN-93 inhibition is presented in Fig. 3B. Under those conditions (1 \(\mu\)M IP₃, no Ca²⁺), KN-93 inhibited IP₃-induced Ca²⁺ release half-maximally at a concentration of 17 \(\mu\)M. Increasing the IP₃ concentration (see further) or the Ca²⁺ concentration (Fig. 3C), how-
ever, decreased the potency of KN-93. Since the largest inhibitory effect of KN-93 is observed at low Ca\textsuperscript{2+} concentrations, whereas it becomes nearly ineffective at the high Ca\textsuperscript{2+} concentrations, the bell-shaped regulation of the IP\textsubscript{3}R is maintained, although it is slightly shifted to the right (Fig. 3C). The fact that KN-93 is most effective during the Ca\textsuperscript{2+}-dependent activatory phase of IP\textsubscript{3}-induced Ca\textsuperscript{2+} release suggests that the action of KN-93 is conformation-dependent. Importantly, neither KN-92, the inactive analog of KN-93, nor the structurally unrelated KN-62, another pharmacological CaMKII inhibitor that reportedly acts by a mechanism similar to that of KN-93 (i.e. by inhibiting Ca\textsuperscript{2+}/CaM binding (37)) had any effect on the IP\textsubscript{3}-induced Ca\textsuperscript{2+} release in A7r5 cells up to concentrations as high as 50 \(\mu\)M (Fig. 3B). Moreover, KN-93 inhibited in permeabilized A7r5 cells adenophostin A-induced Ca\textsuperscript{2+} release similarly as IP\textsubscript{3}-induced Ca\textsuperscript{2+} release (data not shown). Close examination of the concentration-dependence properties in MII eggs and A7r5 cells reveals that the effective concentration range of KN-93 for inhibition of IP\textsubscript{3}-induced Ca\textsuperscript{2+} release is similar for both cell types. The effects of KN-93 on IP\textsubscript{3}-induced

**Fig. 1.** KN-93 dose-dependently and reversibly inhibits pSF-induced [Ca\textsuperscript{2+}]\textsubscript{i} oscillations in mouse MII eggs. A, control, untreated MII eggs exhibited an initial high amplitude [Ca\textsuperscript{2+}]\textsubscript{i} spike, followed by multiple, lower amplitude spikes upon injection of 0.5 \(\mu\)g/\(\mu\)l pSF (\(n = 8/8\) with oscillations). B, MII eggs preincubated in 25 \(\mu\)M KN-93 for 30 min and injected with 0.5 \(\mu\)g/\(\mu\)l pSF in the continued presence of 25 \(\mu\)M KN-93 exhibited no [Ca\textsuperscript{2+}]\textsubscript{i} oscillations. The first high amplitude spike was seen, but it was delayed by over 5 min following injection of pSF in the graph shown (\(n = 0/19\) with oscillations). C, pretreatment with 25 \(\mu\)M KN-92 for 30 min followed by injection of 0.5 \(\mu\)g/\(\mu\)l pSF in the presence of 25 \(\mu\)M KN-92 did not inhibit [Ca\textsuperscript{2+}]\textsubscript{i} oscillations (\(n = 5/6\) with oscillations). D, percentage of eggs in which [Ca\textsuperscript{2+}]\textsubscript{i} oscillations were inhibited at various concentrations of KN-93. The total numbers of eggs tested (means \pm S.E.) at each concentration are indicated above each bar, and statistically significant differences compared with untreated controls are indicated by an asterisk above the bar (\(\chi^2, p < 0.05\)). E, eggs incubated for 30 min in 25 \(\mu\)M KN-93 and then washed for 30 min in medium lacking KN-93 exhibited [Ca\textsuperscript{2+}]\textsubscript{i} oscillations upon injection of 0.5 \(\mu\)g/\(\mu\)l pSF (\(n = 8/8\) with oscillations), indicating that the effects of KN-93 on pSF-induced [Ca\textsuperscript{2+}]\textsubscript{i} oscillations can be reversed by a 30-min wash. The arrows indicate times of pSF injection.
Ca\(^{2+}\) release are therefore not unique to a single cell type and may represent a general effect of this compound.

**KN-93 Does Not Affect Ca\(^{2+}\) Store Loading**—Based on the previous findings, only two possible mechanisms of KN-93-mediated inhibition remain; the compound may significantly reduce the Ca\(^{2+}\) level in IP\(_3\)-sensitive stores, or it may directly inhibit Ca\(^{2+}\) release through the IP\(_3\)R. We tested the first of these hypotheses in A7r5 cells by assessing the Ca\(^{2+}\) content of the internal stores either after performing store loading in the absence or presence of the various KN-compounds at concentrations up to 50 \(\mu\)M or by measuring the effects of those same compounds on the passive Ca\(^{2+}\) leak. ATP-driven Ca\(^{2+}\) uptake in the nonmitochondrial stores amounted in control conditions to 1.6 \(\pm\) 0.2 nmol of Ca\(^{2+}\)/10\(^6\) cells (\(n = 12\)) taken up in 45 min. None of the compounds inhibited Ca\(^{2+}\) uptake (Ca\(^{2+}\) leak) in the presence of 50 \(\mu\)M KN-93, KN-92, and KN-62 amounted to 111 \(\pm\) 4, 113 \(\pm\) 3, and 110 \(\pm\) 12\%, respectively, of the control uptake; three independent experiments, each performed in triplicate). Furthermore, none of the compounds affected the passive Ca\(^{2+}\) leak from the internal stores (data not shown). Thus, it can be concluded that internal stores are filled to equal capacity in KN-93-treated and untreated A7r5 cells. We obtained similar results in mouse MII eggs, since neither amplitude nor duration of Ca\(^{2+}\) release induced by the Ca\(^{2+}\) chelator ionomycin was affected by KN-93 (data not shown). Further, basal [Ca\(^{2+}\)]\(_i\), did not increase upon exposure of eggs to 25 \(\mu\)M KN-93, indicating that the compound does not induce Ca\(^{2+}\) leakage and subsequent depletion from internal stores. However, the regulation of Ca\(^{2+}\) store dynamics in eggs is more complicated due to the oscillatory pattern of Ca\(^{2+}\) release that occurs after stimulation. Since the occurrence of persistent [Ca\(^{2+}\)]\(_i\), oscillations depends on the timely replenishment of the Ca\(^{2+}\) stores and is, therefore, ultimately dependent on Ca\(^{2+}\) influx into the cell, we had to ascertain that KN-93 was not inhibiting the [Ca\(^{2+}\)]\(_i\), oscillations primarily by blocking the so-called capacitative Ca\(^{2+}\) entry mechanism. The mechanisms underlying capacitative Ca\(^{2+}\) entry in eggs are very poorly understood, so direct measurements of the process are difficult. We therefore performed several indirect experiments to examine whether KN-93 could affect [Ca\(^{2+}\)]\(_i\), oscillations indirectly (i.e., by interfering with Ca\(^{2+}\) entry). We first reasoned that if inhibition of [Ca\(^{2+}\)]\(_i\), oscillations in eggs by KN-93 is due solely to antagonism of capacitative Ca\(^{2+}\) entry, then eggs held in Ca\(^{2+}\)-free medium should also be unable to mount oscillations under our conditions. However, we found that injection of 10 \(\mu\)M adenophostin A in eggs held in nominally Ca\(^{2+}\)-free me-
Inhibition of IP$_3$-induced Ca$^{2+}$ Release by KN-93

**Fig. 3.** Inhibition of IP$_3$-induced Ca$^{2+}$ release by KN-93 in permeabilized A7r5 cells. A, fractional Ca$^{2+}$ loss from nonmitochondrial Ca$^{2+}$ stores in saponin-permeabilized A7r5 smooth muscle cells was measured in the absence of IP$_3$ and KN-93 (open squares) or in the presence of 1 μM IP$_3$ in the absence (closed squares) or in the presence of KN-93 (25 μM open triangles) or 50 μM (closed triangles)). EGTA (1 mM) was present throughout the efflux. Fractional loss is defined as the amount of Ca$^{2+}$ released in 2 min divided by the total store Ca$^{2+}$ content at that time. IP$_3$ was added for 2 min, as indicated by the upper bar. When present, KN-93 was added to the medium from the start of the efflux on, as indicated by the lower bar. Results are representative of four experiments, each performed in duplicate. S.D. is not indicated when smaller than the symbol. B, concentration-response curves for KN-93 (inverted triangles), KN-92 (circles), and KN-62 (triangles) on Ca$^{2+}$ release induced by 1 μM IP$_3$. Measurement of IP$_3$-induced Ca$^{2+}$ release, incubation with the KN compounds, and the presence of EGTA in the efflux medium was as in A. IP$_3$-induced Ca$^{2+}$ release was defined as the increase in fractional loss over the basal leak observed after 2 min of incubation with IP$_3$. The IP$_3$-induced Ca$^{2+}$ release measured in the absence of KN compounds was taken as 100%. Me$_2$SO, used as a vehicle for KN-62, had no effect on its own. Each value represents the mean ± S.E. of 3–5 experiments, each performed in duplicate. C, effect of CaMKII concentration on IP$_3$-induced Ca$^{2+}$ release in the absence (squares) or presence of 25 μM KN-93 (triangles). The fractional loss induced by IP$_3$ (1 μM) over the basal leak was measured at varying free Ca$^{2+}$ concentrations. Ca$^{2+}$ was added to the efflux medium for 2 min, together with IP$_3$. Each value represents the mean ± S.D. of four measurements.

**Diagrams and Figures:**

- **A**: Fractional loss of Ca$^{2+}$ over time, showing the effect of IP$_3$ and KN-93 on Ca$^{2+}$ release.
- **B**: Concentration-response curves for different KN compounds on Ca$^{2+}$ release induced by 1 μM IP$_3$.
- **C**: Fractional loss of Ca$^{2+}$ at varying Ca$^{2+}$ concentrations, with and without 25 μM KN-93.

**Text:**

Inhibition of IP$_3$-induced Ca$^{2+}$ Release Is Independent of CaMKII Inhibition—The absence of an inhibitory effect of KN-62 on IP$_3$-induced Ca$^{2+}$ release in permeabilized A7r5 cells (Fig. 3B), the absence of ATP, Mg$^{2+}$, and CaM in our assay conditions (Fig. 3), and the fact that the inhibitory effect of KN-93 is reversed at higher Ca$^{2+}$ concentrations (Fig. 3C), do not point to an effect of KN-93 on CaMKII activity in those cells. To analyze the possibility that KN-93 abrogates, at least partially, IP$_3$R function in eggs by interfering with a regulatory effect of CaMKII on the IP$_3$R, we assessed the effects of a panel of other commercially available CaMKII inhibitors on pSF-induced Ca$^{2+}$ release in eggs (Table I). As observed in A7r5 smooth muscle cells, KN-62 had no effect on pSF-induced [Ca$^{2+}$], oscillations or on egg activation. We also tested two highly potent and specific CaMKII inhibitory peptides. Neither peptide 281–309, which inhibits CaMKII by mimicking the autoinhibitory domain (41), nor AIP, which is a nonphosphorylatable, competitive substrate for autophosphorylation of CaMKII (42), inhibited [Ca$^{2+}$], oscillations or egg activation. Finally, inhibition of Ca$^{2+}$/CaM, the upstream agonist of CaMKII, with W-7 (43) also failed to inhibit pSF-induced [Ca$^{2+}$], oscillations. W-7 did, however, significantly delay egg activation, as evidenced by a delay in extrusion of the second polar body in eggs treated with the compound, an effect that has been previously documented (44). This delay cannot necessarily be attributed to a CaMKII effect, since Ca$^{2+}$/CaM activates a wide range of target molecules. We also found that KN-93 (25 μM) did not significantly reduce the basal CaMKII activity in MII eggs compared with untreated or KN-92-treated controls (data not shown). This is important, since, if KN-93 were to inhibit IP$_3$-induced Ca$^{2+}$ release by reducing CaMKII activity, then the compound would be expected to lower the basal level of CaMKII activity prior to the induction of Ca$^{2+}$ release. Thus, the inability of KN-93 to reduce basal CaMKII levels in eggs, together with the failure of CaMKII inhibitors aside from KN-93 to antagonize Ca$^{2+}$ release in both eggs and A7r5 cells, leads to the conclusion that KN-93 inhibits IP$_3$-induced Ca$^{2+}$ release through a mechanism that does not involve inhibition of CaMKII activity.

**KN-93 Does Not Affect IP$_3$ Binding to the IP$_3$R—**Since the inhibition of IP$_3$-induced Ca$^{2+}$ release by KN-93 was counteracted at increasing IP$_3$ concentrations, we first determined whether KN-93 acts competitively or noncompetitively with respect to IP$_3$ binding to the IP$_3$R. A Lineweaver-Burk plot of the fractional IP$_3$-induced Ca$^{2+}$ release measured in permeabilized A7r5 cells versus the IP$_3$ concentration shows that the presence of KN-93 (25 μM) did not alter the $K_i$ for IP$_3$ compared with untreated control conditions (Fig. 5A). This provides evidence that KN-93 did not interfere with the IP$_3$-binding site of the IP$_3$R. Moreover, direct measurements of $[^{3}$H]IP$_3$ binding to the IP$_3$R-1 in S9-microsomes also showed that KN-93 (50 μM) does not reduce IP$_3$ binding (Table II). Finally, we analyzed in eggs the effects of KN-93 on IP$_3$ binding to the IP$_3$R-1 in vivo.
Inhibition of IP₃-induced Ca²⁺ Release by KN-93

**FIG. 4.** KN-93 does not affect Ca²⁺ store filling in mouse MII eggs. A, untreated eggs were injected with 10 μM adenophostin A in nominally Ca²⁺-free medium to prevent Ca²⁺ entry. The removal of extracellular Ca²⁺ did not prevent the initiation of [Ca²⁺]ᵢ oscillations by 10 μM adenophostin A (n = 6/7 with oscillations), indicating that the inhibitory effect of KN-93 under our conditions is not due to a mere prevention of Ca²⁺ entry (compare with Fig. 2E). B, following an initial adenophostin A (10 μM) injection in normal Ca²⁺-containing medium (∼2 mM), eggs were injected a second time with adenophostin A, in the continuous presence of 25 μM KN-93. In eight of eight eggs tested, the second adenophostin A injection induced a Ca²⁺ release that was equal to or greater in magnitude than the first release event, indicating that even in the continuous presence of KN-93, internal Ca²⁺ stores are still sufficiently filled to elicit a new release. The arrows indicate times of adenophostin A (Ad) injection.

In mouse MII eggs as well as in several other systems that have been studied, it is known that the binding of IP₃ to the IP₃R-1 induces degradation of the IP₃R-1 (29, 45–47). Since this IP₃R degradation absolutely depends on the binding of its ligand (48), analysis of IP₃R-1 levels following agonist stimulation can be used as a measure for the *in vivo* binding of IP₃ to its receptor. As shown in Fig. 5B, injection of 10 μM adenophostin A in untreated MII eggs caused a significant reduction in the amount of IP₃R-1 protein detectable by Western blot compared with un.injected controls. KN-93 treatment did not significantly alter this degradation of the IP₃R in response to adenophostin A injection. Quantification of three blasts from independent experiments indicates that both untreated and KN-93-treated eggs exhibited a reduction in IP₃R-1 levels of 40% in response to adenophostin A injection compared with un.injected controls (Fig. 5C). These results verify that KN-93 does not hinder the binding of IP₃ to the IP₃R-1 in *in vitro* or *in vivo*.

**KN-93 Directly Affects IP₃R-1 Function**—Given that KN-93 does not interfere with IP₃ binding, a distinct possibility is that KN-93 interferes with the function of the IP₃-R-1 Ca²⁺ release channel, preventing the passage of Ca²⁺ ions from the endoplasmic reticulum. Such a mechanism has been postulated for the inhibition of IP₃R-1 function by the binding of Ca²⁺/CaM to the receptor (9, 13, 14). Since KN-93 inhibits CaMII by competitively binding to the Ca²⁺/CaM-binding site of the enzyme, a tantalizing hypothesis is that KN-93 may bind to a Ca²⁺/CaM-binding site on the IP₃R-1 and, like Ca²⁺/CaM itself, inhibit the function of the Ca²⁺ release channel.

First, we explored the possibility that KN-93 is acting through the Ca²⁺/CaM-binding site of the IP₃R-1 by analyzing the combined effects of KN-93 and of Ca²⁺/CaM on IP₃-induced Ca²⁺ release in permeabilized A7r5 cells. If KN-93 and Ca²⁺/CaM act through different sites, it can be expected that the effect of the two inhibitory factors together should be additive. However, whereas the inclusion of either KN-93 (25 μM) or CaM (3 μM) each caused a significant reduction in IP₃-induced Ca²⁺ release, the combination of both antagonists did not cause a larger inhibition than that caused by KN-93 alone (Fig. 6). Similar results were obtained when using other concentrations of KN-93 or of CaM (data not shown).

CaM is also a partial inhibitor of [³H]IP₃ binding to IP₃R-1 (8, 12). Although it is not yet known how the effects of CaM on IP₃ binding relate to its effect on channel function, it was important to verify whether KN-93 could interfere with the ability of CaM to inhibit IP₃ binding. The need to use neutral pH for observing the inhibitory effect of CaM on IP₃ binding (8), combined with the quite low density of IP₃-binding sites in A7r5 cells, forced us to shift for this type of analysis to Sf9 cells heterologously expressing IP₃R-1 (12, 28). KN-93 (50 μM) by itself did not affect IP₃ binding to Sf9 microsomes, but when added together with a supramaximal concentration of CaM (20 μM), it completely reversed the inhibitory effect of the latter (Table II).

Finally, we investigated whether KN-93 could affect IP₃-induced Ca²⁺ release in permeabilized 16HBE14o- cells, which predominantly express IP₃R-3 (49). The significance of these experiments lies in the fact that the IP₃R-3 lacks the high affinity Ca²⁺/CaM-binding site present in the modulatory region of IP₃R-1 (10). KN-93 was, however, markedly less effective at inhibiting IP₃-induced Ca²⁺ release in 16HBE14o- cells compared with A7r5 cells (only 22% inhibition at 50 μM KN-93, compared with 67% inhibition in A7r5 cells under the same conditions), whereas KN-92 and KN-62 were virtually ineffective (data not shown).

**DISCUSSION**

Although KN-93 reportedly acts as a specific inhibitor of CaMII activity (17), several other effects of the compound, unrelated to CaMII inhibition, have been reported, including blockade of voltage-dependent K⁺ channels in vascular myocytes (50) and interference with Ca²⁺ influx in pancreatic β-cells (51). The results of our current study provide further evidence of additional effects of KN-93 when the compound is used to probe CaMII-related cellular functions; however, our results also give rise to the exciting possibility that KN-93 may be a valuable tool for the study of IP₃-related cellular functions. Through a series of experiments that probed critical regulatory mechanisms of IP₃-mediated Ca²⁺ signaling, we have demonstrated that KN-93 antagonizes IP₃-induced Ca²⁺ release in both mouse MII eggs and permeabilized A7r5 smooth muscle cells. This inhibition involves direct modulation of IP₃R-1 function via a mechanism that is not related to a putative regulation of the IP₃R-1 by CaMII activity. Furthermore, we directly evaluated several functional properties of the IP₃R-1 and found that KN-93 does not prevent IP₃ binding through competitive or allosteric inhibition and that the capac-
ity of KN-93 to modulate the IP₃-R-1 may depend on the conformational status of the receptor, as dependent on [IP₃] and [Ca²⁺], and on the presence of a critical CaM-binding site.

CaMKII has been shown to phosphorylate the IP₃-R both in vitro and in vivo (52, 53); however, the functional significance of this phosphorylation is not clear. Zhu et al. (16) and Matifat et al. (53) have reported that CaMKII activity is inhibitory with respect to IP₃-induced Ca²⁺ release in HeLa cells and Xenopus oocytes, respectively, and may represent one of the mechanisms by which Ca²⁺ release is terminated at elevated [Ca²⁺]. In contrast, the results obtained by Zhang et al. (15) in permeabilized 3T6 fibroblasts imply that CaMKII activity may increase the sensitivity of the IP₃-R to IP₃, thus potentiating Ca²⁺ release during the initiation of a [Ca²⁺] rise, although the
kinase in question could not be directly identified as CaMKII. A stimulatory function for CaMKII could explain the severe abrogation of Ca\(^{2+}\) release observed in MII eggs and A7r5 cells in response to KN-93 treatment; however, this is unlikely, due to the inability of other CaMKII inhibitors to mimic the results obtained with KN-93. We can further discount a role of CaMKII to the inability of other CaMKII inhibitors to mimic the results in response to KN-93 treatment; however, this is unlikely, due to an unknown side effect of KN-93. Due to the small size of mammalian eggs and an inability to simultaneously stimulate a large number of eggs, measurements of phosphoinositide turnover cannot currently be performed in mouse MII eggs. However, since it is known that Ca\(^{2+}\) can stimulate PLC activity (54), it is possible that KN-93 may have an indirect, negative effect on PLC due to its ability to abrogate IP\(_3\)-induced Ca\(^{2+}\) release.

The activity of the IP\(_3\)-R can be modulated either by compounds acting on the IP\(_3\)-binding site or by compounds acting on the opening of the Ca\(^{2+}\) release channel. Analysis of the kinetics of KN-93 inhibition indicate a noncompetitive mechanism, since KN-93 does not alter the \(K_{i}\) for IP\(_3\). Direct measurement of \([^{3}H]IP_3\) binding to the IP\(_3\)-R-1 and analysis of IP\(_3\)-R-1 down-regulation in eggs indicated that KN-93 did not abrogate IP\(_3\) binding to the IP\(_3\)-R-1. Therefore, it is likely that the inhibition imposed by KN-93 occurs downstream of IP\(_3\) binding. Since CaM is an important modulator of both IP\(_3\) binding (8, 12) and IP\(_3\)-R function (9, 13, 14) and since KN-93 is known to interact with the CaM-binding site of at least CaMKII (17), we hypothesized that KN-93 might inhibit the IP\(_3\)-R by interacting with one or more of its CaM-binding sites. Up to now, three different CaM-binding sites have been described for the IP\(_3\)-R-1 (9–11), but the functional significance of each is still unclear. The Ca\(^{2+}\)-independent CaM-binding site present in the N terminus of the receptor (9) might be responsible for the inhibitory effects on IP\(_3\) binding (8, 12, 55), but it is unclear whether the high affinity Ca\(^{2+}\)/CaM-binding site located in the modulatory region is involved in the regulation of the channel function of IP\(_3\)-R-1 (56, 57) as originally thought. Moreover, IP\(_3\)-induced Ca\(^{2+}\) release in cells expressing predominantly IP\(_3\)-R-3 is also inhibited by CaM (9, 58), although a characteristic structural feature of IP\(_3\)-R-3 is the absence of the high affinity Ca\(^{2+}\)/CaM-binding site conserved in IP\(_3\)-R-1 and IP\(_3\)-R-2 (10). Significantly, we found that KN-93 is far less effective at inhibiting IP\(_3\)-induced Ca\(^{2+}\) release in permeabilized 16HBE14o cells, which predominantly, but not exclusively, express the IP\(_3\)-R-3 (49). Independently of the physiological site of action of Ca\(^{2+}\)/CaM, a mechanism whereby KN-93 inhibits IP\(_3\)-R-1 function by occupying the high affinity Ca\(^{2+}\)/CaM binding site is therefore consistent with the lack of inhibition in 16HBE14o cells and with our finding that inhibition of IP\(_3\)-R-1 occurs downstream of IP\(_3\) binding. Moreover, we evaluated the effect of exogenous Ca\(^{2+}\)/CaM on the ability of KN-93 to inhibit IP\(_3\)-induced Ca\(^{2+}\) release to determine whether the two antagonists act synergistically. Since no addi-

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**Table II**

KN-93 does not affect \([^{3}H]IP_3\) binding to microsomes from Sf9 cells heterologously expressing IP\(_3\)-R-1

| Treatment                  | \([^{3}H]IP_3\) binding (percentage of control) |
|----------------------------|-----------------------------------------------|
| KN-93 (50 \(\mu\)M)       | 136 ± 28                                      |
| CaM (20 \(\mu\)M)         | 73 ± 4*                                       |
| KN-93 (50 \(\mu\)M) + CaM (20 \(\mu\)M) | 158 ± 48                                     |

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**Fig. 6.** Inhibition by KN-93 may depend on one of the Ca\(^{2+}\)/CaM-binding sites on IP\(_3\)-R-1. Fractional Ca\(^{2+}\) loss from nonmitochondrial Ca\(^{2+}\) stores in permeabilized A7r5 cells in the absence (open symbols) or the presence of 1 \(\mu\)M IP\(_3\) (closed symbols). The basal Ca\(^{2+}\) leak (open squares) was measured in the absence of KN-93, CaM, and Ca\(^{2+}\). The effect of 2 \(\mu\)M Ca\(^{2+}\) on the leak pathway in the absence of KN-93 and CaM is also shown (open circles). Ca\(^{2+}\) was added for 2 min, as indicated by the upper bar. IP\(_3\) was added together with Ca\(^{2+}\) in the absence of CaM and KN-93 (closed squares) or in the presence of 25 \(\mu\)M KN-93 alone (inserted triangles), 3 \(\mu\)M CaM alone (triangles), or of both 25 \(\mu\)M KN-93 and 3 \(\mu\)M CaM (diamonds). CaM and KN-93 were present in the medium from the start of the efflux up to 12 and 16 min, as indicated by the middle and lower bars, respectively. Results are representative of three experiments, each performed in duplicate. S.D. is not indicated when smaller than the symbol.
tive effect of both inhibitors was observed, our results are at least consistent with the notion that both antagonists act through the same binding site. Alternatively, the possibility exists that the interaction of either CaM or KN-93 induces a large conformational change of the receptor, precluding the binding of the other. Additionally, the fact that KN-93 can abrogate the inhibitory effect of CaM on IP₃ binding suggests that it can interact in a direct or indirect way with the CaM-binding site located in the N-terminal region of the receptor. The relation between this CaM-binding site, which is essentially Ca²⁺-independent, and the inhibitory effect on IP₃R function, which is essentially Ca²⁺-dependent, is however not yet known.

In conclusion, our results indicate that the widely used CaMKII inhibitor KN-93 potently inhibits IP₃-mediated Ca²⁺ signals by interacting directly with the IP₃R. This effect may be mediated by its interaction with one or several of the CaM-binding sites on the receptor. This is relevant, since, to date, there is no known highly specific and reversible inhibitor of the IP₃R available. KN-93 can therefore be a helpful tool in the structure-function analysis of the IP₃R. Moreover, it may be possible that related compounds could be developed with higher specificity for the regulatory CaM-binding site on IP₃R.

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