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Suramin and Disulfonated Stilbene Derivatives Stimulate the Ca^{2+}-Induced Ca^{2+}-Release Mechanism in A7r5 Cells

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ABSTRACT

We have described previously a novel Ca^{2+}-induced Ca^{2+}-release (CICR) mechanism in permeabilized A7r5 cells (embryonic rat aorta) and 16HBE14o- cells (human bronchial mucosa) cells (*J Biol Chem* **278:**27548–27555, 2003). This CICR mechanism was activated upon the elevation of the free cytosolic calcium concentration $[Ca^{2+}]_c$ and was not inhibited by pharmacological inhibitors of the inositol-1,4,5-trisphosphate (IP_3) receptor nor of the ryanodine receptor. This CICR mechanism was inhibited by calmodulin $(CaM)_{1234}$, a Ca^{2+}-insensitive CaM mutant, and by different members of the superfamily of CaM-like Ca^{2+}-binding proteins. Here, we present evidence that the CICR mechanism that is expressed in A7r5 and 16HBE14o- cells is strongly activated by suramin and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS). We found several indications that both activation mechanisms are indeed two different modes of the same release system. Suramin/DIDS-induced Ca^{2+} release was only detected in cells that displayed the CICR mechanism, and cell types that do not express this type of CICR mechanism did not exhibit suramin/DIDS-induced Ca^{2+} release. Furthermore, we show that the suramin-stimulated Ca^{2+} release is regulated by Ca^{2+} and CaM in a similar way as the previously described CICR mechanism. The pharmacological characterization of the suramin/DIDS-induced Ca^{2+} release further confirms its properties as a novel CaM-regulated Ca^{2+}-release mechanism. We also investigated the effects of disulfonated stilbene derivatives on IP_3-induced Ca^{2+} release and found, in contrast to the effect on CICR, a strong inhibition by DIDS and 4′-acetoamido-4′-isothiocyanostilbene-2′,2′-disulfonic acid.

Changes in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) mediate a variety of cellular processes, ranging from fertilization to cell death. The endoplasmic reticulum (ER) and sarcoplasmic reticulum (SR) serve as the main sources of releasable Ca^{2+} for cytosolic cellular signaling. Calcium pumps of the sarco(endo)-plasmic-reticulum Ca^{2+}-ATPase family import Ca^{2+} into the organelle lumen. Two families of intracellular Ca^{2+}-release channels are primarily responsible for the release, the inositol-1,4,5-trisphosphate receptor (IP_3R) and the ryanodine receptor (RyR) (Berridge et al., 2003).

Recent studies have emphasized the role of novel types of intracellular Ca^{2+}-release channels possibly playing an important role in intracellular Ca^{2+} signaling. Wissing et al. (2002) identified a novel Ca^{2+}-induced Ca^{2+}-release (CICR) mechanism in permeabilized hepatocytes that responded to modest increases in $[Ca^{2+}]_c$. A CICR atypical of the SR type was found in mouse pancreatic β-cells (Beauvois et al., 2004). Polycystin-2, the product of the gene mutated in type-2 autosomal dominant polycystic kidney disease and a prototypical member of a subfamily of the transient receptor potential channel superfamily (TRP), is expressed abundantly in the ER. It was shown recently that polycystin-2 expressed in the ER of epithelial cells is a Ca^{2+}-activated channel that is permeable for divalent cations. Increased levels of intracellular Ca^{2+} activated polycystin-2-mediated release of Ca^{2+} from intracellular stores (Koulen et al., 2002). Moreover,

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ABBREVIATIONS: $[Ca^{2+}]_c$, cytosolic free calcium concentration; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; IP_3R, inositol-1,4,5-trisphosphate receptor; RyR, ryanodine receptor; CICR, calcium-induced calcium release; TRP, transient receptor potential; CaM, calmodulin; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; SITS, 4′-acetoamido-4′-isothiocyanostilbene-2′,2′-disulfonic acid; IICR, inositol-1,4,5-trisphosphate-induced Ca^{2+} release; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; TG, thapsigargin; IP_3, inositol-1,4,5-trisphosphate; XeC, xestospongin C; RuRed, ruthenium red; S1P, sphingosine-1-phosphate.

there are indications that some bona fide plasmalemmal Ca^{2+}-permeable TRP channels (e.g., TRPV1 and TRPM8) also reside in intracellular membranes where they may function as Ca^{2+}-release channels (Turner et al., 2003; Zhang and Barritt, 2004). In a previous study, we described a novel CICR mechanism in permeabilized A7r5 cells, a permanent cell line derived from embryonic rat aorta (Nadif Kasri et al., 2003). This CICR mechanism was activated upon the elevation of the $[Ca^{2+}]_c$ and was not inhibited by pharmacological inhibition of the IP_3R or of the RyR. Moreover, we found that this CICR mechanism could be inhibited by CaM_{1234}, a Ca^{2+}-insensitive CaM mutant, and by different members of the superfamily of CaM-like Ca^{2+}-binding proteins. Our data suggested that the CICR mechanism described here may represent a novel type of Ca^{2+}-release channel, which is silent at low $[Ca^{2+}]_c$ because of inhibition by bound apocalmodulin and which becomes activated by the Ca^{2+}-dependent interaction with CaM.

Suramin and disulfonic stilbene derivatives have been extensively used as pharmacological probes to study the transport kinetics and molecular structures of a wide range of membrane transporters. An excellent example of this includes the structure of the ATP-binding site of the sarco(endo)plasmic-reticulum Ca^{2+}-ATPase (Hua and Inesi, 1997). 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) has been widely used to understand the role and mechanism of various ion transport processes in the muscle sarcolemma and the SR (Cabantchik and Greger, 1992). 4′-acetoamido-4′-isothiocyanostilbene-2′,2′-disulfonic acid (SITS) is commonly used for the study of anion transporters, and like DIDS it possesses structural similarities to suramin. Suramin (1,3,5-naphthylenetrisulfonic acid) is a trypanoside that acts as an ATP antagonist for P2-purinoceptors (Hoyle et al., 1990). On the RyR it acts as a strong activator, and regulates the RyR via a binding site that is distinct from its adenine nucleotide binding site (Emmick et al., 1994). Suramin has also been postulated to act on the RyR via binding to the CaM-binding site (Klinger et al., 1999). Suramin was found to bind directly to CaM-binding sites on the RyR and the IP_3R (Klinger et al., 1999; Nadif Kasri et al., 2004). Although suramin and disulfonic stilbene derivatives have been extensively used to study the RyR, data on the effects on IP_3Rs or IP_3-induced Ca^{2+} release (IICR) are scarce. Previous results, however, have shown that endogenous sulfonate derivatives can regulate IP_3R function (Watras et al., 2000). In this study, therefore, we have used suramin and stilbene derivatives to further characterize the previously detected CICR mechanism, and we also investigated the effects of these compounds on IICR.

In a first part, we present evidence that the CICR mechanism that is expressed in A7r5 and 16HBE14o- cells is strongly activated by suramin and DIDS. Other cell types that do not express this type of CICR mechanism did not exhibit any suramin/DIDS-induced Ca^{2+} release. Furthermore, we show that this suramin-stimulated Ca^{2+} release is regulated in a similar way as the previously described CICR mechanism (Nadif Kasri et al., 2003). The pharmacological characterization further confirms the properties of this CICR mechanism as a novel CaM-regulated Ca^{2+}-release mechanism. We also investigated the effects on IICR and found, in contrast to the effect on CICR, a strong inhibition by DIDS and SITS.

Materials and Methods

Materials. Suramin was purchased from Sigma-Aldrich (Bornem, Belgium). DIDS, SITS, and 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) were purchased from Molecular Probes (Leiden, The Netherlands).

$^{45}Ca^{2+}$ Fluxes. A7r5 cells, which are derived from embryonic rat aorta smooth muscle cells, were obtained from the American Type Culture Collection (Manassas, VA) (CRL 1444). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 3.8 mM L-glutamine, 0.9% (v/v) nonessential amino acids, 85 IU/ml penicillin, 85 μg/ml streptomycin, and 20 mM HEPES, pH 7.4. For 16HBE14o- (human bronchial mucosa) cells, a mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium was used, and for LLC-PK_1 cells minimal essential medium-α was used. L15 cells were obtained by stable exogenous expression of IP_3R1 in Lvec cells, whereas Lvec cells represent the control cells expressing the empty vector (Miyawaki et al., 1990; Mackrill et al., 1996). $^{45}Ca^{2+}$ fluxes were performed on saponin-permeabilized cells. The cells were seeded in 12-well clusters (Costar, Cambridge, MA) at a density of approximately 4×10^4 cm^{-2}. Experiments were carried out on confluent monolayers of cells (3×10^5 cells/well) between the seventh and ninth day after plating. Cells were permeabilized by incubating them for 10 min with a solution containing 120 mM KCl, 30 mM imidazole-HCl, pH 6.8, 2 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA, and 20 μg/ml saponin at 25°C. The nonmitochondrial Ca^{2+} stores were loaded for 45 min at 25°C in 120 mM KCl, 30 mM imidazole-HCl, pH 6.8, 5 mM $MgCl_2$, 5 mM ATP, 0.44 mM EGTA, 10 mM NaN_3, and 150 nM free $^{45}Ca^{2+}$ (28 μCi/ml). The cells were then washed twice with 1 ml of efflux medium containing 120 mM KCl, 30 mM imidazole-HCl, pH 6.8, 1 mM EGTA, and 10 μM thapsigargin (TG). TG was added to block the ER Ca^{2+} pumps during subsequent additions of Ca^{2+}. The efflux medium was replaced every 2 min, and the efflux was performed at 25°C. The additions of $^{40}Ca^{2+}$, IP_3, suramin, and stilbene derivatives are indicated on the figures (arrow). Free $[Ca^{2+}]$ was calculated by the Cabuf program (ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/cabuf.zip) and based on the stability constants as published previously (Fabiato and Fabiato, 1979). At the end of the experiment, the $^{45}Ca^{2+}$ remaining in the stores was released by incubation with 1 ml of a 2% sodium dodecyl sulfate solution for 30 min. Ca^{2+} release in some experiments is plotted as the fractional loss (i.e., the amount of Ca^{2+} released in 2 min divided by the total store Ca^{2+} content at that time). The latter value was calculated by summing in retrograde order the amount of tracer remaining in the cells at the end of the efflux and the amounts of tracer collected during the successive time intervals.

CaM-Sepharose Pull-Down Assay. CaM-Sepharose 4B (50 μl) (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) was incubated with 500 ng of recombinant IP_3R1 purified from Sf9 cells for 2 h at 4°C in incubation buffer [i.e., one part Tris-buffered saline (20 mM Tris-HCl, pH 7.2, and 150 mM NaCl) mixed with one part bacterial ProFound lysis buffer containing a Tris-buffered solution of 75 mM NaCl with 1% of a nonionic detergent (according to the manufacturer's protocol; Pierce Chemical, Rockford, IL), and supplemented with 1 mM β-mercaptoethanol]. Unbound protein was removed by washing the Sepharose beads four times with 500 μl of the incubation buffer. Bound IP_3R1 protein was eluted by incubating the beads with LDS (Invitrogen, Carlsbad, CA) for 10 min at 70°C, and the beads were removed by centrifugation at 20,000*g* for 1 min. All samples were separated on NuPAGE 3 to 8% Tris-acetate SDS-polyacrylamide gel electrophoresis gels and analyzed by Western blotting, using a rabbit polyclonal antibody against IP_3R1 (Rbt03) as the primary antibody (Parys et al., 1995)

[^{3}H]IP_3-Binding Experiments. Binding studies were performed as described previously. [^{3}H]IP_3 binding was performed at 0°C in 100 μl of binding buffer containing 50 mM Tris-HCl, pH 7.0, 1 mM

EGTA, 10 mM β-mercaptoethanol, and 10 nM [^{3}H]IP_3. Nonspecific binding was determined in the presence of 12.5 μM unlabeled IP_3. After 30 min of incubation, the samples were rapidly filtered through glass-fiber filters. The amount of Sf9 microsomes expressing IP_3R1 ranged between 100 and 150 μg. Statistical analysis was performed using the paired Student's t test. Values were considered significantly different when $P < 0.05$.

Expression and Purification of Recombinant Proteins. Recombinant CaM and CaM_{1234} were expressed and purified as described previously (Sienaert et al., 2002).

Expression and Purification of IP_3R1 from Sf9 Cells. Production of recombinant viruses, expression and purification of recombinant IP_3R1 proteins in Sf9 insect cells, and preparation of the microsomes were as described previously (Sipma et al., 1999; Vermassen et al., 2004).

Results

Suramin Stimulates Ca^{2+} Release from Intracellular Stores. In A7r5 cells, Ca^{2+} release from intracellular stores, mainly from the ER, occurs to a large extent via production of the second messenger IP_3. In the permeabilized cell system, a maximal effective dose of IP_3 (100 μM) can release up to 95% of the intracellular Ca^{2+} content (Missiaen et al., 1990). We previously identified a CICR mechanism that contributed significantly to the Ca^{2+} release from the ER (40%) (Nadif Kasri et al., 2003). This CICR mechanism was found to be highly regulated by CaM. Because suramin has already been used extensively to study intracellular Ca^{2+}-release channels and has been reported to bind directly on CaM-binding sites (Klinger et al., 1999, 2001), we tested the effects of suramin on intracellular Ca^{2+} release in A7r5 cells. The nonmitochondrial stores of permeabilized A7r5 cells were loaded to steady state with $^{45}Ca^{2+}$ and then incubated in a nonlabeled efflux medium containing 10 μM thapsigargin. The loss of Ca^{2+} from the stores under these conditions is plotted as ER Ca^{2+} content as a function of time (Fig. 1A). After 10 min of efflux, the cells were challenged with 100 μM suramin during 2 min, as indicated by the arrow. Suramin (100 μM) strongly increased the rate of Ca^{2+} release (Fig. 1A). In these conditions, the amount of the stored Ca^{2+} that could be released by suramin was 33% (Fig. 1B). The total amount of releasable Ca^{2+} was measured by treating the cells with 5 μM ionophore A23187 (triangles). A dose-response curve is shown in Fig. 1B. Suramin stimulated Ca^{2+} release from the intracellular stores with an EC_{50} of 93 ± 9 μM, and the activation curve had a Hill coefficient of 1.0 ± 0.1. A saturating concentration of suramin was found to release up to 70% of the stored Ca^{2+} in A7r5 cells.

Suramin-Induced Ca^{2+} Release Is Expressed in the Same Cell Types as the CICR Mechanism. Next, we investigated whether this suramin-induced Ca^{2+} release could be observed in other cell types that express the CICR mechanism described previously in A7r5 cells. Therefore, we performed the same experiments in 16HBE14o- cells, in which a similar CICR mechanism is expressed, and in COS-1, LLC-PK_1, L15, and Lvec cells, in which we could not detect this mechanism (Nadif Kasri et al., 2003). Suramin-induced Ca^{2+} release was only observed in 16HBE14o- cells and not in the other cell types (Fig. 2). Suramin maximally released 40% of the stored Ca^{2+} with an EC_{50} of 104 ± 8 μM in 16HBE14o- cells, which is identical to that observed in A7r5 cells. These data indicate that the Ca^{2+} release induced by suramin is not a property of all cells and is therefore not caused by a nonspecific leak. Hence, these data suggest that suramin-induced Ca^{2+} release is a property of cells that express the previously described CICR mechanism.

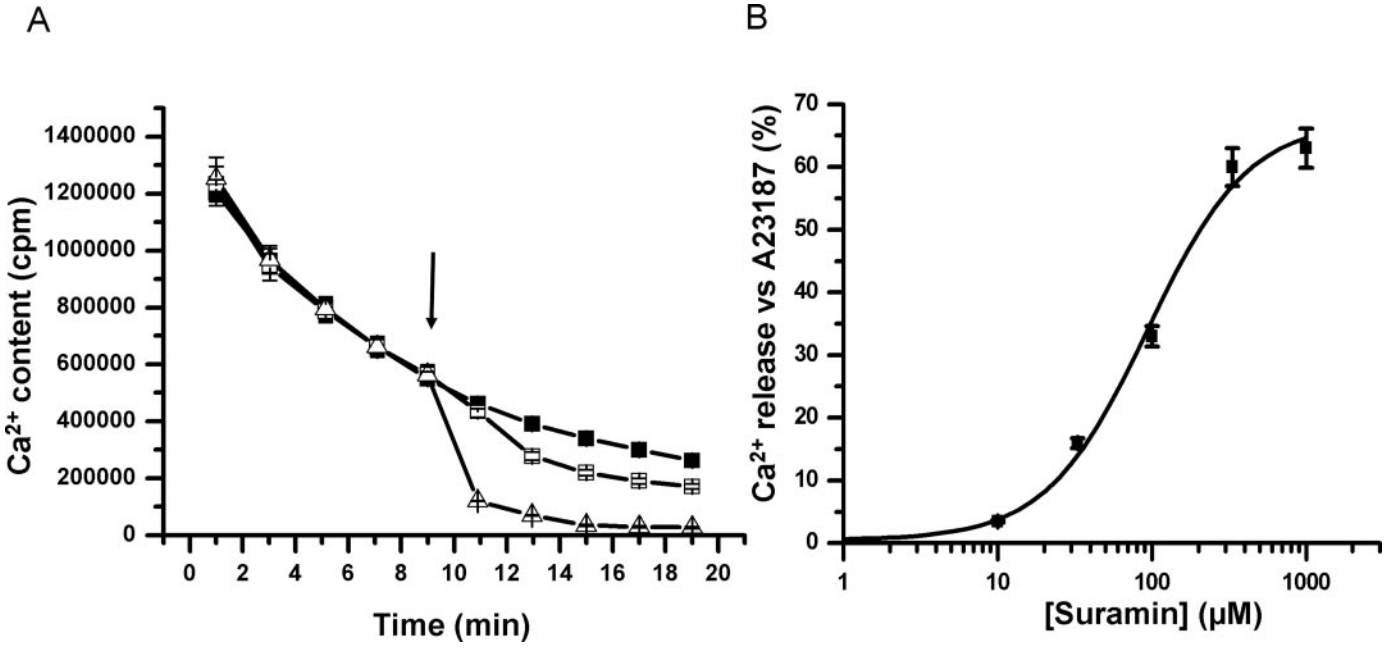

Fig. 1. Suramin-induced Ca^{2+} release. A, permeabilized A7r5 cells were loaded during 45 min in 150 nM free $^{45}Ca^{2+}$. From time 0 onward, cells were incubated in efflux medium. The traces illustrate how the $^{45}Ca^{2+}$ content of the stores decreased during the efflux (■) and how this Ca^{2+} content was affected by a 2-min application (arrow) of 100 μM suramin (□). A23187 (5 μM) was applied to measure the total releasable Ca^{2+} (△). Results represent the means ± S.E.M. for three wells. B, after loading of permeabilized A7r5 cells during 45 min in 150 nM free $^{45}Ca^{2+}$, efflux was started. After 10 min, suramin was added to the cells for a time period of 2 min. The Ca^{2+} release was plotted as a function of the applied [suramin]. The Ca^{2+} release was normalized to the total releasable fraction by 5 μM A23187, which was taken as 100%. Results represent the means ± S.E.M. of three independent experiments each performed in 2-fold.

Suramin-Induced Ca^{2+} Release Is Neither IP_3R- nor RyR-Mediated. The two major classes of intracellular Ca^{2+}-release channels are the IP_3Rs and the RyRs. In A7r5 cells, only IP_3R1 (73%) and IP_3R3 (26%) are expressed (De Smedt et al., 1994). No evidence has been found for a functional activity of the RyR in A7r5 cells, as there was no measurable Ca^{2+} release by caffeine or cyclic ADP-ribose (Missiaen et al., 1990).

Because suramin was shown to potently activate several types of ion channels (Hill et al., 2004, and references therein), we wanted to investigate the possibility that suramin acted directly on the IP_3R. Heparin and xestospongin C (XeC) are often used as antagonists of the IP_3R. In Fig. 3A, we show that these compounds did not affect the fractional loss induced by 100 μM suramin, strongly suggesting that the IP_3R was not involved in this mechanism. Although there is no evidence for a functional RyR in A7r5 cells, we also used an antagonist of the RyR to exclude any role of the RyR in this suramin-induced Ca^{2+} release. Figure 3B illustrates that 100 μM ruthenium red (RuRed) had no effect on the fractional loss induced by 100 μM suramin.

Ca^{2+} release stimulated by sphingosine-1-phosphate (S1P) (Pyne and Pyne, 2000) and NAADP (Genazzani and Galione, 1996) has been observed in a number of cell types. However, it is unlikely that one of these mechanisms is activated by suramin in A7r5 cells because no S1P- or NAADP-stimulated Ca^{2+} release was observed in A7r5 cells under our assay conditions (data not shown).

Characteristics of the Observed Suramin-Induced Ca^{2+} Release. Fig. 4A illustrates that the suramin-induced Ca^{2+} release was controlled by the level of store loading. Ca^{2+} stores from permeabilized A7r5 cells loaded to steady state with $^{45}Ca^{2+}$ were incubated in Ca^{2+}-free efflux medium and their Ca^{2+} content plotted as a function of time. Suramin (100 μM) was added either after 2 min (○, full stores) or after 20 min (△, less filled stores). The relative amount of Ca^{2+}

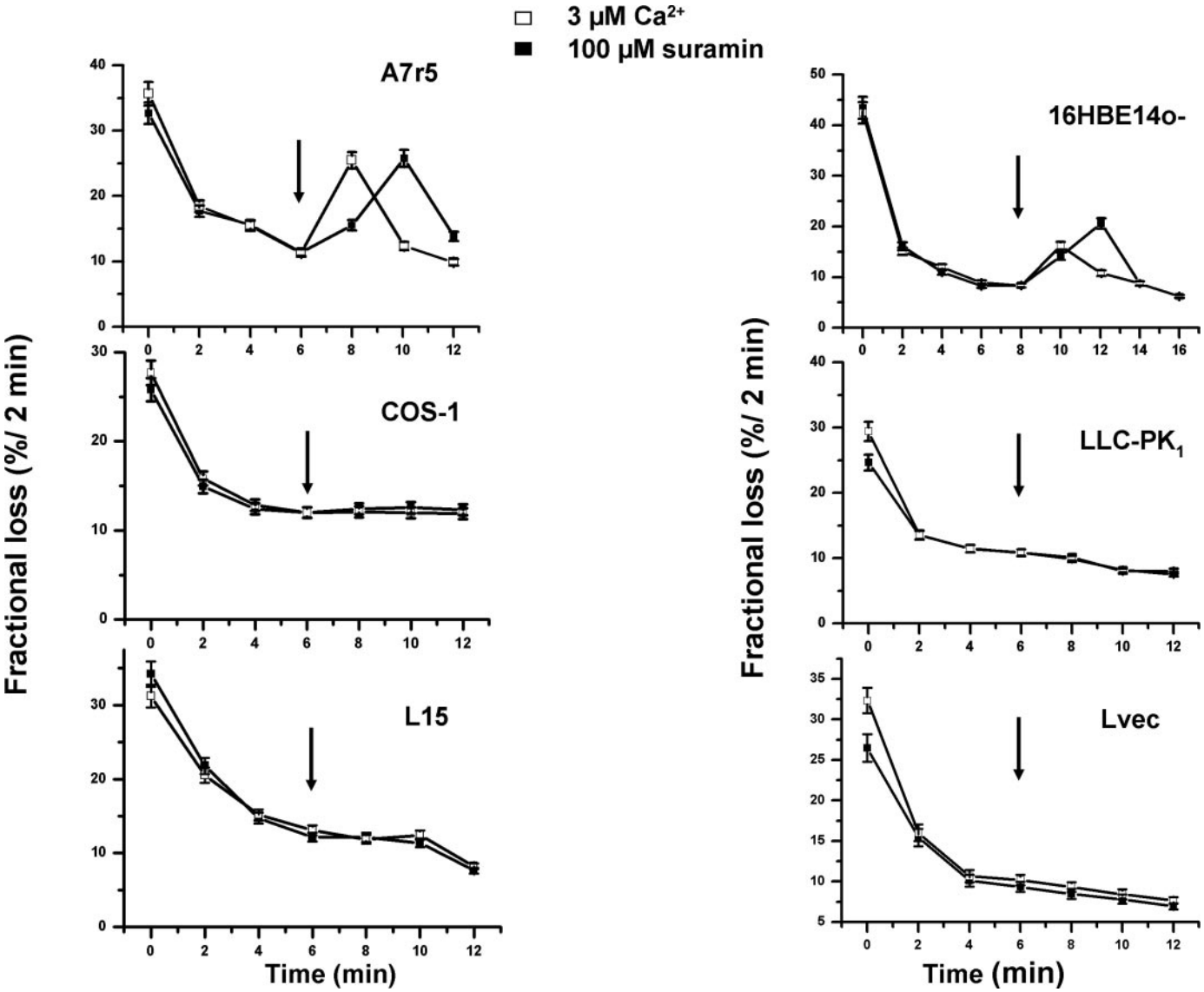

Fig. 2. Suramin-induced Ca^{2+} release and the CICR mechanism are expressed in the same cell types. Suramin-induced Ca^{2+} release and CICR were measured in different cell lines. Permeabilized A7r5, 16HBE14o-, COS-1, LLC-PK_1, L15, and Lvec cells were loaded with $^{45}Ca^{2+}$ during 45 min. After stabilization of the efflux cells were challenged with 3 μM free $^{40}Ca^{2+}$ (□) or with 100 μM suramin (■) (arrow). Fractional loss was measured as a function of time and is defined as the amount of $^{45}Ca^{2+}$ released in 2 min, divided by the total amount of $^{45}Ca^{2+}$ stored at that moment. Each curve represents the means ± S.E.M. for three wells.

released by suramin from fully loaded stores was higher than the release from less filled stores. The Ca^{2+} content of the cells induced by suramin after 2 min was depleted more extensively than the Ca^{2+} content of cells that were induced after 20 min. These results indicate that the suramin-induced Ca^{2+} release was controlled by the luminal $[Ca^{2+}]$. In this respect, the suramin-induced Ca^{2+} release shows the same dependence on the luminal Ca^{2+} content as described for both the CICR (Nadif Kasri et al., 2003) or for the IICR mechanism in those cells (Missiaen et al., 1992).

We have shown previously that the CICR mechanism in A7r5 cells originated from the IP_3-sensitive and TG-sensitive stores. We investigated whether the suramin-induced Ca^{2+} release described here was originating from the same IP_3- and TG-sensitive stores. Permeabilized cells were loaded with $^{45}Ca^{2+}$ in the presence or absence of a saturating dose of IP_3 (300 μM) or TG (10 μM). Efflux was then performed in Ca^{2+}-free medium (EGTA). After 10 min, cells were incubated for 2 min with 100 μM suramin. No Ca^{2+} release was observed in cells that were loaded in the presence of IP_3 or TG (data not shown). This finding suggests that suramin can only release Ca^{2+} from the IP_3-sensitive and TG-sensitive stores.

To confirm that CICR and suramin-induced Ca^{2+} release originate from the same stores, we first challenged cells with 300 μM suramin and subsequently activated CICR by addition of 3 μM free $^{40}Ca^{2+}$. Cells exposed with suramin did not show subsequent CICR, in contrast to nontreated cells (Fig. 4B). From these experiments we conclude that CICR and suramin-induced Ca^{2+} release originated from the same Ca^{2+} stores in A7r5 cells.

In the next step, we have measured the effect of the free $[Ca^{2+}]$ on the suramin-induced Ca^{2+} release. When the Ca^{2+} release induced by 100 μM suramin was plotted as a function of the free $[Ca^{2+}]_c$, we observed a Ca^{2+}-dependent component with an EC_{50} of 900 ± 46 nM free Ca^{2+} (Fig. 4C). This Ca^{2+} dependence had a similar EC_{50} as that previously measured for activation of the CICR mechanism (700 nM free Ca^{2+}) (Nadif Kasri et al., 2003). We also observed that suramin-induced Ca^{2+} release and CICR were only partially additive. This could suggest that suramin and Ca^{2+} activate the same mechanism and that Ca^{2+} is also a modulator of the suramin-induced Ca^{2+} release.

In contrast to the CICR mechanism, Ca^{2+} release induced by suramin was not potentiated by ATP (data not shown). Suramin was identified previously as an ATP antagonist for P2-purinoceptors and RyRs (Hoyle et al., 1990; Mallard et al., 1992; Emmick et al., 1994). Therefore, we can hypothesize that the lack of activation by ATP could be explained by binding of suramin to a ATP-binding site.

An important characteristic of CICR described previously was the role of CaM as a Ca^{2+} sensor (Nadif Kasri et al., 2003). We showed that when cells were incubated with CaM_{1234}, CICR was completely blocked. Further characterization revealed that CaM acted as a Ca^{2+} sensor for this CICR mechanism and that CaM_{1234} had dominant negative properties. Assuming that suramin activates the same CICR mechanism, CaM_{1234} should also block suramin-induced Ca^{2+} release. Indeed, when 10 μM CaM_{1234} was added to permeabilized cells, suramin-induced Ca^{2+} release was nearly completely inhibited (89 ± 4%). This was not the case when CaM itself was added (Fig. 5). Thus, suramin-induced Ca^{2+} release shows remarkable similarities with the CICR mechanism in A7r5 cells with respect to the Ca^{2+} and CaM dependence. Suramin-induced Ca^{2+} release, however, does also occur in the complete absence of Ca^{2+} (EGTA), which suggests that suramin is an independent activator and not merely a regulator of the CICR mechanism.

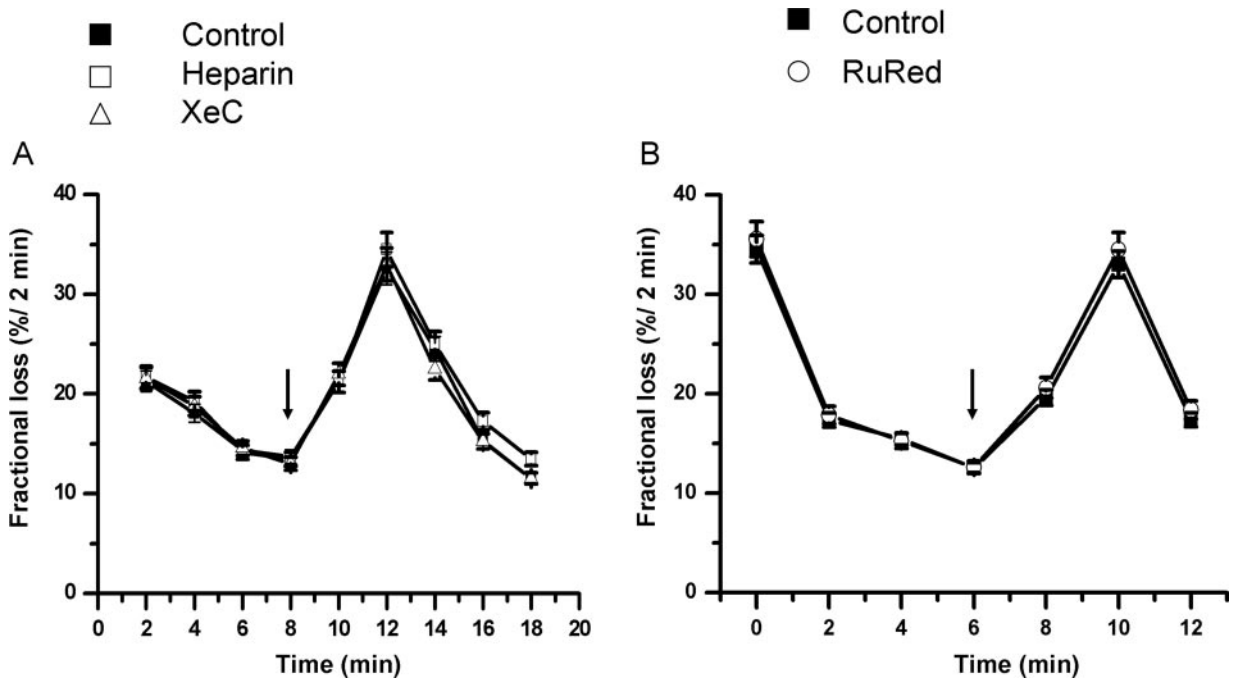

Fig. 3. Effects of IP_3R and RyR antagonists on suramin-induced Ca^{2+} release. Cells were incubated for a 2-min period (arrow) with 100 μM suramin alone (■) or in the presence of IP_3R (A) or RyR antagonists (B): 1 mg/ml heparin (□), 5 μM XeC (△), or 100 μM RuRed (○). Fractional loss was plotted and compared with fractional loss induced with 100 μM suramin alone. None of the antagonists significantly altered the fractional loss. Results represent the means ± S.E.M. of three independent experiments each performed in 2-fold.

Stilbene Derivatives Induce Ca^{2+} Release in A7r5 Cells. DIDS and SITS (Fig. 6A) are normally regarded as chemical probes for the study of anion transporters. However, because they possess structural similarities to suramin, they have also been used as a ligand for purinoceptors and RyRs. We have tested the effects of those derivatives on intracellular Ca^{2+} release in A7r5 cells. When applied to permeabilized cells, 100 μM DIDS induced a Ca^{2+} release (18 $\pm$ 3%) comparable with the activation of suramin, whereas SITS and DNDS were not effective (Fig. 6B). A dose-response curve of the activation by DIDS revealed that the EC_{50} for DIDS activation was higher than for suramin (Fig. 6C). Similar to the suramin-induced Ca^{2+} release, CaM_{1234} inhibited Ca^{2+} release induced by DIDS by 83 $\pm$ 3% (data not shown), suggesting that both suramin and DIDS activate the same Ca^{2+}-release mechanism.

Stilbene Derivatives Inhibit IICR. Although stilbene derivatives have been extensively used to study several types of ion channels, no data are available for IP_3Rs. We have therefore investigated the effects of DIDS, SITS, and DNDS on IICR. To prevent any interference with the previously described suramin/DIDS-induced Ca^{2+}-release mechanism, we used permeabilized L15 and Lvec fibroblast cells, which do not express CICR and in which suramin did not promote Ca^{2+} release (Fig. 2). Western blots indicated a 3:1 ratio for IP_3R1/IP_3R3 for L15 and the reverse ratio for Lvec cells (data not shown). This comparison was made because differences between both IP_3R isoforms may yield different results, as was shown for RyR isoforms (Sitsapesan, 1999; O'Neill et al., 2003; Hill et al., 2004).

In permeabilized L15 or Lvec cells, the addition of 200 nM IP_3 to the efflux medium induced Ca^{2+} release from the nonmitochondrial internal stores. IICR was inhibited in a concentration-dependent way by both DIDS and SITS, whereas DNDS did not have any effect. Because the inhibition occurred to the same extent in both cell lines, it can be concluded that these effects are not isoform specific. Dose-response curves for DIDS and SITS inhibition in L15 cells are presented in Fig. 7A. With 200 nM IP_3 in L15 cells, DIDS half-maximally inhibited IICR at a concentration of 0.7 $\pm$ 0.02 μM and SITS at a concentration of 9.3 $\pm$ 0.47 μM.

As indicated in Fig. 7B, the blocking effect of DIDS or SITS was essentially irreversible. Permeabilized L15 cells were preincubated with 100 μM DIDS or SITS for 6 min followed

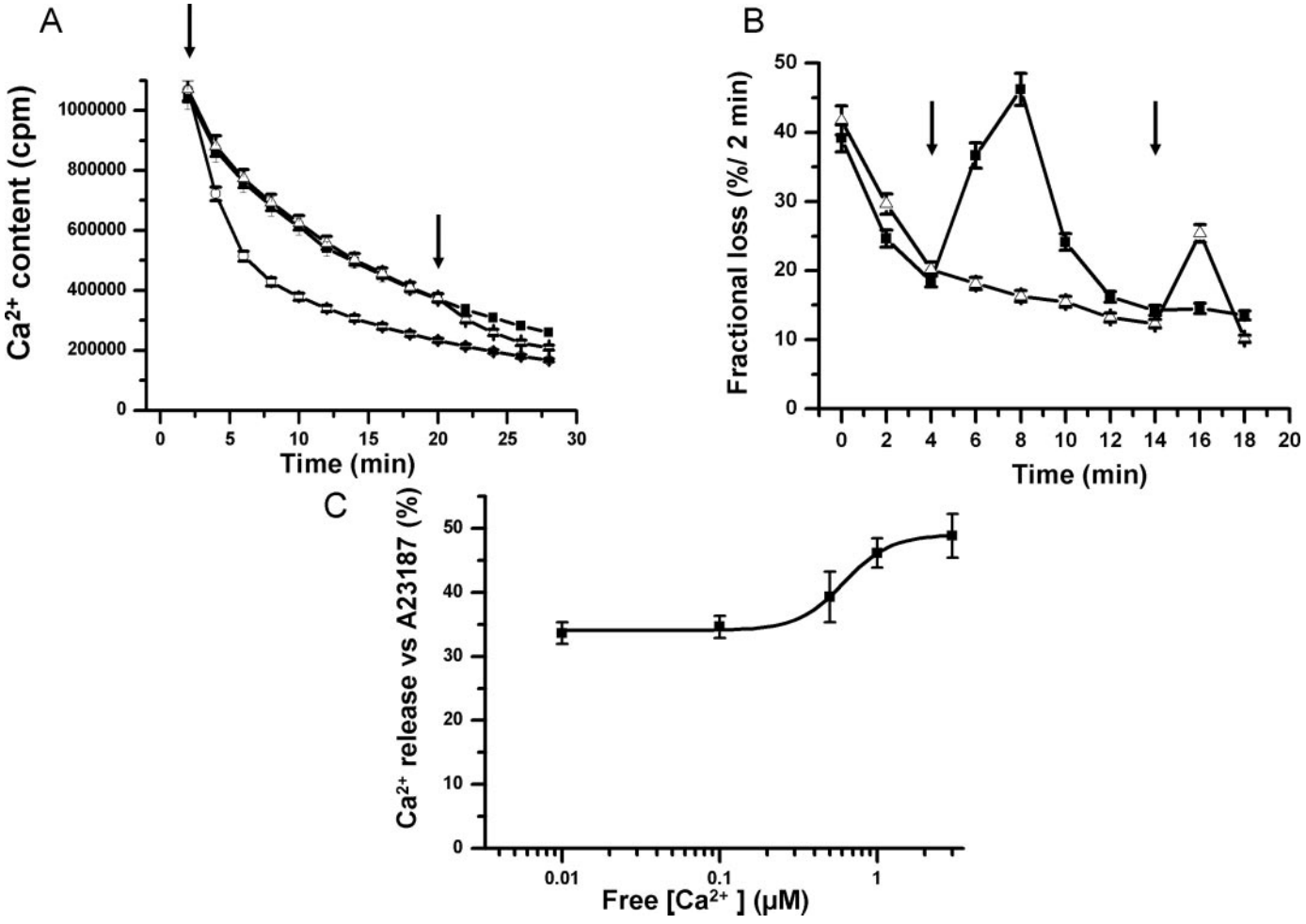

Fig. 4. The CICR store overlaps with suramin-induced Ca^{2+}-release store. A, loading dependence of the suramin-induced Ca^{2+} release. The stores were loaded for 45 min with 150 nM free $^{45}Ca^{2+}$ and from time 0 onward incubated in efflux medium. The traces illustrate how the $^{45}Ca^{2+}$ content of the stores decreased during the efflux (■) and how this Ca^{2+} content was affected by a 2-min application of 100 μM suramin after 2 min (○) or after 20 min (△). Results represent the means $\pm$ S.E.M. for three wells. B, fractional loss as a function of time. Cells were first challenged with 300 μM suramin and subsequently with 3 μM $^{40}Ca^{2+}$ (■) or only with 3 μM $^{40}Ca^{2+}$ (△). Results represent the means $\pm$ S.E.M. for three wells. C, Ca^{2+} release by 100 μM suramin was plotted as a function of the free [Ca^{2+}]. The Ca^{2+} release was normalized to the total releasable fraction by 5 μM A23187, which was taken as 100%. Results represent the means $\pm$ S.E.M. of three independent experiments each performed in 2-fold.

by a washout period of 4 min before activation with 200 nM IP_3. The decrease of IICR was compared with cells that were incubated with 100 μM DIDS or SITS for 12 min with no washout period before activation with 200 nM IP_3. Cells that were treated for 6 min showed a similar decrease in IICR compared with cells that were incubated for 12 min. DIDS completely inhibited IICR, whereas SITS inhibited IICR by 74 ± 4% (Fig. 7B). We conclude that washing away the stilbene derivatives did not reverse the inhibition of IICR. Irreversible binding of stilbene derivatives was also observed for the interaction with the RyR (O'Neill et al., 2003) and may involve isothiocyanate groups on DIDS and SITS.

For suramin, we have previously shown that it decreased the apparent sensitivity of the IP_3R for IP_3, presumably by binding to the N-terminal CaM-binding sites of the receptor (Nadif Kasri et al., 2004). Therefore, we performed IP_3-binding experiments to see whether DIDS, SITS, or DNDS interfered in a similar way with the IP_3R. IP_3-binding measurements were performed in the presence of 50 μM stilbene derivative on microsomes from Sf9 cells expressing IP_3R1. In contrast with suramin, DIDS, SITS, and DNDS had no effect on IP_3 binding (Fig. 7C).

Another property of the suramin interaction was the interference with the CaM-binding sites of the IP_3R. We previously showed that suramin interacted with the two different CaM-binding sites on the IP_3R1 (Nadif Kasri et al., 2004). Pull-down experiments with CaM-Sepharose 4B were performed in the presence of 1 μM suramin, DIDS, SITS, or DNDS. Suramin inhibited the binding of IP_3R1 to the CaM-Sepharose 4B by 58 ± 5% (Fig. 7D). In contrast, none of the other tested stilbene reagents showed a significant interference. Higher concentrations of DIDS, SITS, and DNDS (100 μM) also did not result in an inhibition of the binding of IP_3R1 to the beads (data not shown). From this part, we conclude that DIDS and SITS are both potent inhibitors of IICR. Although both compounds are structurally related to suramin, the inhibition of IICR occurs via a different mechanism.

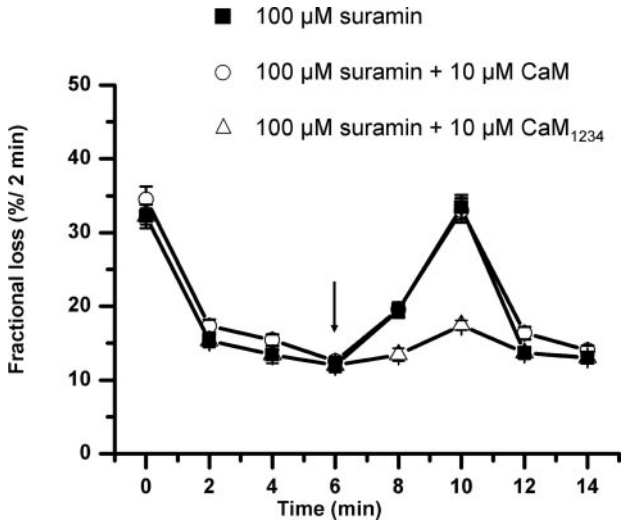

Fig. 5. Effects of CaM and CaM_{1234} on suramin-induced Ca^{2+} release. Ca^{2+} release from permeabilized cells incubated during 45 min in loading buffer expressed as fractional loss. After incubation in efflux buffer, cells were challenged with 100 μM suramin alone (■) or together with 10 μM CaM (○) or 10 μM CaM_{1234} (△) during 2 min. Results represent the means ± S.E.M. for three wells.

Discussion

The present data clearly show that suramin and disulfonic stilbene derivatives potently induce Ca^{2+} release from the ER in A7r5 and 16HBE14o- cells (Fig. 1). The properties of this suramin-induced Ca^{2+} release closely resemble those of the recently identified novel CICR mechanism (Nadif Kasri et al., 2003).

Disulfonic stilbene derivatives are known to affect ion channels and transporters by both reversible and nonreversible mechanisms. Kawasaki and Kasai (1989) were the first to report that DIDS and SITS activated RyR1 from rabbit skeletal muscle in SR vesicles and lipid bilayers. They found that these compounds locked RyRs in an open state. Irreversible effects are related to reactive isothiocyanate groups that form covalent bonds with a variety of amino acid residues by reacting with NH_2 groups on lysine residues, OH-groups on serine residues, and aromatic groups on tyrosine and cysteine residues. DIDS has two isothiocyanate groups, whereas SITS has only one and DNDS lacks reactive isothiocyanate groups (Fig. 6A). It is therefore not surprising that DIDS, but not SITS or DNDS, induced Ca^{2+} release in A7r5 cells similarly to suramin. An increase in intracellular Ca^{2+} release by DIDS has also been shown for rat pulmonary artery smooth muscle cells (Cruickshank et al., 2003). This suggests that such an increase in Ca^{2+} release by disulfonic stilbene derivatives not only occurs in cultured cells but also in some primary cells.

There are two main families of intracellular Ca^{2+}-release channels, RyRs and IP_3Rs. However, there is growing evidence that other, not yet identified, intracellular Ca^{2+}-release pathways could also play important roles. This was already proposed for the release induced by S1P (Pyne and Pyne, 2000) and NAADP (Genazzani and Galione, 1996), but the molecular identity of both release channels is largely unknown. Besides the "classic" Ca^{2+}-release pathways, a poorly understood Ca^{2+} leak from the ER has also been postulated, but its molecular nature is also still unknown (Camello et al., 2002), although it has recently been proposed to be a property of a hyperphosphorylated IP_3R1 (Oakes et al., 2005) or a ribosome-translocon complex (Lomax et al., 2002). Our data lend support to the idea that additional intracellular Ca^{2+}-release pathways may be present in some cell types. We showed evidence that suramin activates a release pathway very similar to a CICR mechanism described previously in A7r5 and 16HBE14o- cells (Nadif Kasri et al., 2003). Such a CICR mechanism was already found in hepatocytes (Wissing et al., 2002) and pancreatic β-cells (Beauvois et al., 2004).

The exact mechanism by which suramin and DIDS activate Ca^{2+} release remains largely unknown. We found that CaM plays an important role in the regulation of suramin-induced Ca^{2+} release. We previously proposed that the CICR mechanism in A7r5 cells was activated upon binding of Ca^{2+} to CaM. Dissociation or relocalization of CaM would lead to activation of CICR. Because suramin has been shown to interact directly with the CaM-binding sites on the IP_3R and RyR (Klinger et al., 2001; Papineni et al., 2002; Nadif Kasri et al., 2004), suramin might induce Ca^{2+} release by replacing endogenous CaM. However, DIDS, which also stimulates Ca^{2+} release, does not interact in the same way with CaM-binding sites. Suramin was also shown to interact with ATP-

binding sites on purinoceptors (Hoyle et al., 1990) and RyRs (Emmick et al., 1994). Therefore, it is possible that suramin activates Ca^{2+} release through direct binding to a regulatory ATP-binding site. This could explain why ATP did not further enhance suramin-induced Ca^{2+} release. However, when comparing with the RyR, we can expect binding of suramin and DIDS to be more complex. It was demonstrated that suramin exerts a triphasic effect on the open probability of the RyR, indicating the presence of high-, intermediate- and low-affinity suramin-binding sites (Hill et al., 2004). The most probable mechanism for the activation of Ca^{2+} release in A7r5 cells could therefore also involve multiple binding sites for suramin and DIDS on an as yet unidentified protein. Some of these interaction sites are supposedly irreversible. Within the time resolution of our experiments, we could observe that CICR was faster than the suramin-induced Ca^{2+} release (Fig. 2). This could indicate that the suramin interaction might be more complex compared with the Ca^{2+} effects. Ca^{2+} could act directly on a binding site on the protein, whereas the suramin interaction could involve a more complex interference with other modulators such as CaM or ATP. Concerning the Ca^{2+} dependence of the suramin-induced Ca^{2+} release, we concluded that suramin can release an important part of the stored Ca^{2+} in the absence of free Ca^{2+}, although we also observed a modulation of the suramin-induced Ca^{2+} release by $[Ca^{2+}]_c$. Because to a large extent, the suramin- and Ca^{2+}-induced Ca^{2+} release was not additive, this would suggest that suramin and Ca^{2+} could act on the same release mechanism and that Ca^{2+} can also modulate the suramin-induced Ca^{2+} release.

We also found that DIDS and SITS are potent blockers of IICR (Fig. 7), similar to suramin (Nadif Kasri et al., 2004). However, we found that DIDS and SITS act differently on the IP_3R. Whereas suramin decreased the affinity for IP_3 by binding to CaM-binding sites, neither DIDS nor SITS affected IP_3 binding to IP_3R1 (Fig. 7C). Moreover, there was no

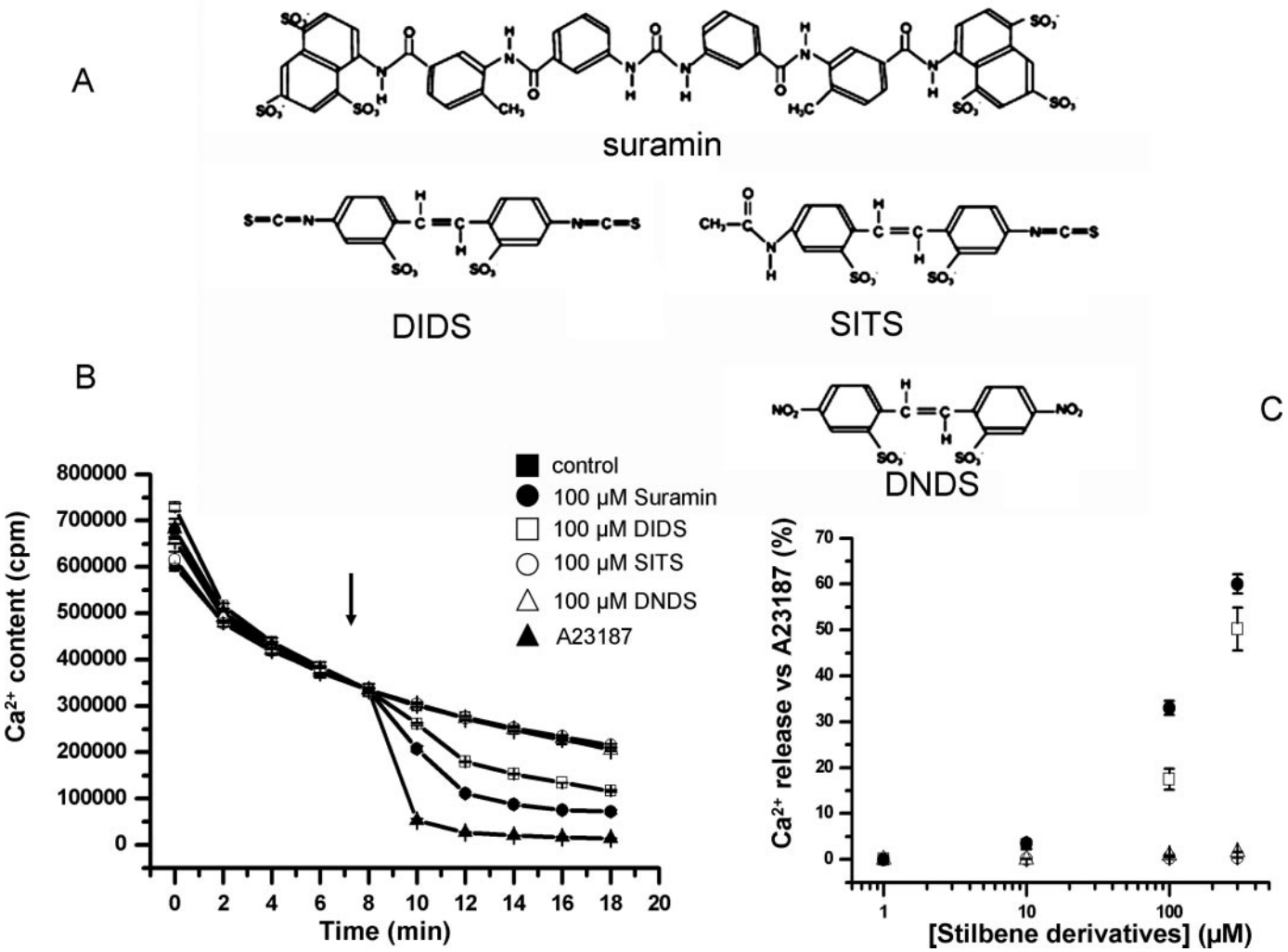

Fig. 6. Effects of the stilbene derivatives DIDS, SITS, and DNDS on intracellular Ca^{2+} release in A7r5 cells. A, chemical structure of suramin, DIDS, SITS, and DNDS. B, intracellular Ca^{2+} release induced by stilbene derivatives. Permeabilized A7r5 cells were loaded during 45 min in 150 nM $^{45}Ca^{2+}$. From time 0 onward, cells were incubated in efflux medium. The traces illustrate how the $^{45}Ca^{2+}$ content of the stores decreased during the efflux (■) and how this Ca^{2+} content was affected by a 2-min application (arrow) of 100 μM suramin (●), 100 μM DIDS (□), 100 μM SITS (○), or 100 μM DNDS (△). A23187 was applied to measure the total releasable Ca^{2+} (▲). Results represent the means ± S.E.M. for three wells. C, intracellular Ca^{2+} release by suramin (●) and the stilbene derivatives, DIDS (□), SITS (○), and DNDS (△) was plotted as a function of their concentration. Permeabilized A7r5 cells were loaded during 45 min in 150 nM $^{45}Ca^{2+}$. Cells were then incubated in efflux medium and after 10 min an increasing concentration of the stilbene derivative were added to the cells for 2 min. A23187 (5 μM) was applied to measure the total releasable Ca^{2+}. Results represent the means ± S.E.M. of three independent experiments each performed in 2-fold.

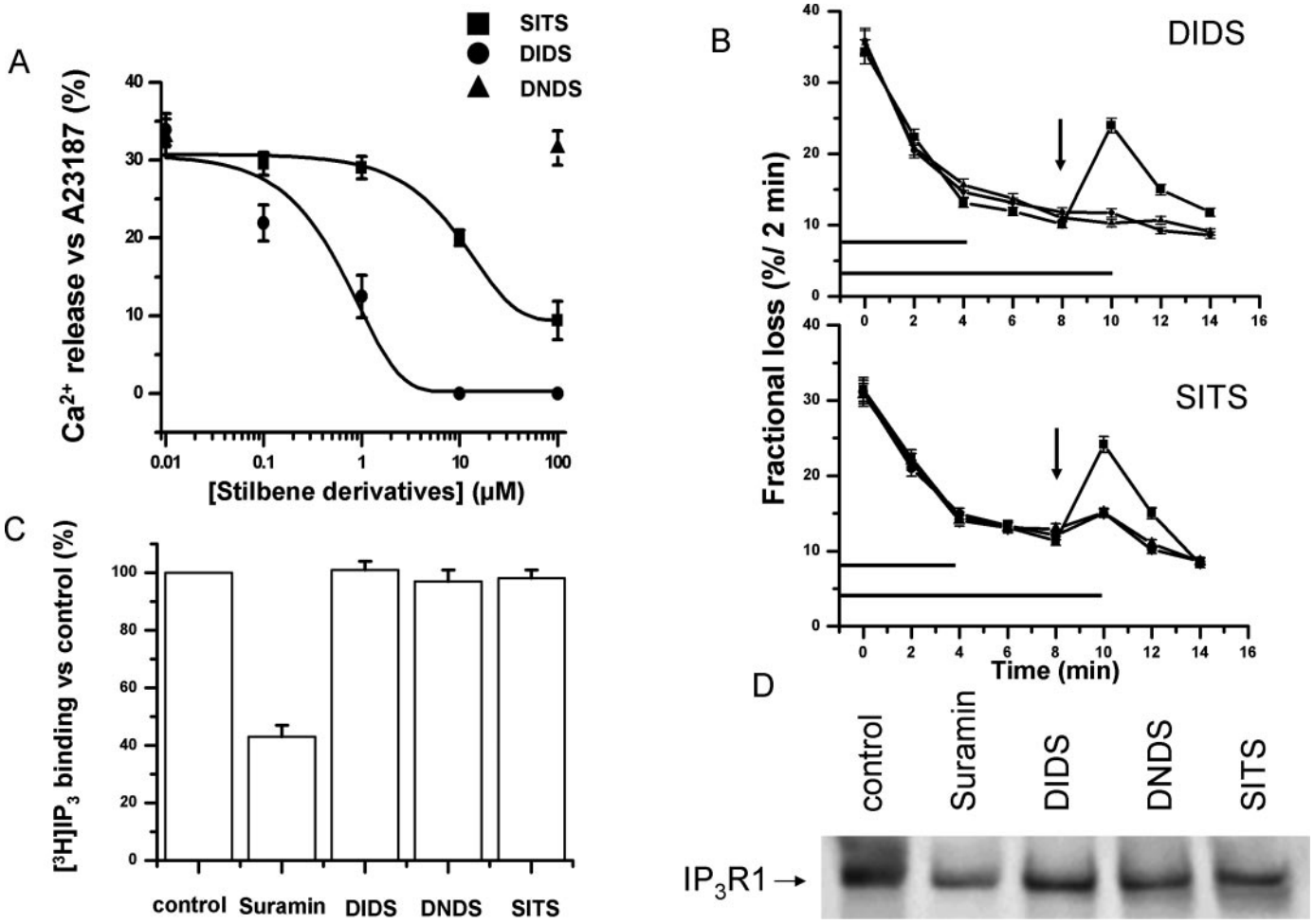

Fig. 7. Effects of stilbene derivatives on IP_3R activity. A, effect of various concentrations of DIDS (●), SITS (■), and DNDS (▲) on IICR in permeabilized L15 cells induced by 200 nM IP_3. Ca^{2+} release was expressed as the percentage of Ca^{2+} release induced by 5 μM A23187 (100%). B, irreversible inhibition of IICR by 100 μM DIDS and 100 μM SITS. IICR induced by 200 nM IP_3 (arrow) in permeabilized L15 cells was measured in the absence of stilbene derivatives (■) or in the presence of 100 μM DIDS (top) or SITS (bottom) added during 6 min (●) or 12 min (▲) as indicated by the bars. C, effect of 50 μM stilbene derivatives on [^{3}H]IP_3 binding to Sf9 microsomes expressing IP_3R1. Binding was measured at pH 7.0 in the presence of 1 mM EGTA and 10 nM [^{3}H]IP_3. D, immunoblot showing the effects of 1 μM suramin, 1 μM DIDS, 1 μM DNDS, or 1 μM SITS on the interaction of purified IP_3R1 from Sf9 cells with CaM-Sepharose 4B.

effect on the binding of CaM to the IP_3R1 (Fig. 7D). This suggests that DIDS and SITS may interact in a more complex way with IP_3Rs and that they could inactivate the IP_3R by irreversible binding. It has also been reported that endogenous sulfonates can play an important role in regulating IICR in cerebellum. An endogenous IP_3R inhibitor was identified and was found to be a sulfonated compound (Watras et al., 2000). The presence of an endogenous inhibitor in neuronal tissues may be important for extending the dynamic range of IICR. It is possible that suramin, DIDS, or SITS could work in a similar way on the IP_3R.

We showed that stilbene derivatives may have activatory or inhibitory effects, depending on which Ca^{2+}-release mechanism they work on. Therefore, caution must be taken in using these pharmacological reagents to study the properties of the intracellular Ca^{2+}-release mechanisms.

In summary, in this study, we confirmed the presence in A7r5 and 16HBE14o- cells of an as-yet-unidentified intracellular Ca^{2+}-release pathway that is clearly independent from the IP_3R and RyR. We have shown here that this pathway can be activated in both cell lines by the pharmacological agents suramin and DIDS. In contrast, the frequently used stilbene derivatives DIDS and SITS were also found to be potent inhibitors of the IP_3R in the cells. This clearly shows the promiscuous effect of these compounds on different types of Ca^{2+} channels.

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