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Diethylstilbestrol Is a Potent Inhibitor of Store-Operated Channels and Capacitative Ca^{2+} Influx

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ABSTRACT

We have recently found that diethylstilbestrol (DES), a synthetic estrogen agonist, inhibits thrombin-induced Ca^{2+} influx in human platelets, but it remains unclear to what extend this effect might be related to the store-operated Ca^{2+} influx pathway. To study the effect of DES on store-operated channels and capacitative Ca^{2+} influx, we used rat basophilic leukemia (RBL) cells, vascular smooth muscle cells (SMC), and human platelets, and recorded whole-cell Ca^{2+} release-activated Ca^{2+} (CRAC) currents and thapsigargin (TG)-induced capacitative Ca^{2+} influx. In this study, we demonstrate that extracellular DES produces a dose-dependent and reversible inhibition of CRAC currents in RBL cells (IC_{50}, ~0.5 μM), whereas intracellular DES (25 μM) has no effect. Extracellular DES (up to 30 μM) inhibited only CRAC but did not affect a whole-cell monovalent cation current mediated by TRPM7 channels. DES effectively inhibited TG-induced capacitative Ca^{2+} influx in a dose-dependent manner with an IC_{50} values of ~0.1 μM in RBL cells, <0.1 μM in SMC, and ~1 μM in human platelets. It is noteworthy that *trans*-stilbene, a close structural analog of DES that lacks hydroxyl and ethyl groups, had no effect on CRAC current and on store-operated Ca^{2+} influx. Thus, we found DES to be a very effective inhibitor of store-operated channels and Ca^{2+} influx in a variety of cell types.

Ca^{2+} influx is one of the major events triggered by agonists in a wide variety of cell types, but not all of the Ca^{2+} influx pathways are well understood. One of the most intriguing and controversial is the capacitative (or store-operated) Ca^{2+} influx activated by agonist-induced depletion of intracellular Ca^{2+} stores (for review, see Berridge et al., 2000; Lewis, 2001; Putney et al., 2002; Venkatachalam et al., 2002; Parekh, 2003; Prakriya and Lewis, 2003). So far, two specific store-operated Ca^{2+}-conducting (SOC) channels have been found that mediate this influx: the so called Ca^{2+} release-activated Ca^{2+}-selective (CRAC) channels, which could be only resolved on the level of the whole-cell currents in a variety of nonexcitable cells (Lewis, 2001; Parekh and Penner, 1997; Hoth and Penner, 1992), and store-operated nonselective cation channels of small (3 pS) conductance that were found in vascular smooth muscle cells (SMC) and human platelets (Trepakova et al., 2001; Albert and Large, 2002; Trepakova and Bolotina, 2002). Despite the great physiological importance of these channels, only a few pharmacological tools are available for their direct inhibition. Many different inhibitors have been proposed and tested, but none of them proved to be a truly specific and effective inhibitor of store-operated channels and capacitative Ca^{2+} influx. 2-Aminoethoxydiphenyl borate (2-APB), the most recent inhibitor to trigger a lot of hopes, turned out to be quite nonspecific. It was originally introduced as a specific membrane permeable modulator of inositol-1,4,5-trisphosphate–induced Ca^{2+} release (Maruyama et al., 1997); later, however, it was shown to also inhibit SOC channel-mediated Ca^{2+} influx (Diver et al., 2001; Dobrydneva and Blackmore, 2001; Gregory et al., 2001; Iwasaki et al., 2001; Bootman et al., 2002). The effect of 2-APB on CRAC currents seemed to be quite complicated, showing potentiation at low concentrations (< 5 μM) and inhibition at high concentrations (> 10 μM) (Prakriya and Lewis, 2001), as well as use-dependence (Peppiatt et al., 2003). 2-APB was shown to inhibit not only CRAC channels but also store-independent TRPM7 channels that exist and could be activated in many different cell types under the

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S.I.Z. and T.S. contributed equally to this work

ABBREVIATIONS: SOC, store-operated Ca^{2+}-conducting; CRAC, Ca^{2+} release-activated Ca^{2+}-selective; SMC, smooth muscle cells; 2-APB, 2-aminoethoxydiphenyl borate; DCF, divalent cation-free; DES, diethylstilbestrol; *trans*-S, *trans*-stilbene; TG, thapsigargin; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; DCF, divalent cation free.

same divalent cation-free (DCF) experimental conditions (Bakowski and Parekh, 2002b; Prakriya and Lewis, 2002; Kozak and Cahalan, 2003; Zakharov et al., 2003). The most recent studies show that 2-APB inhibits some other ion channels as well (Wang et al., 2002; Braun et al., 2003; Chinopoulos et al., 2003; Harks et al., 2003; Sydorenko et al., 2003). Thus, finding some new and potent inhibitors of SOC channels (and corresponding capacitative Ca^{2+} influx) that will be more specific to the store-operated channels remained an important and urgent matter.

We demonstrated recently that some agents that possess a stilbene structural moiety inhibit thrombin-induced Ca^{2+} influx in human platelets (Dobrydneva et al., 1999, 2002), but to what extent this effect could be related to a direct effect on the store-operated channels remained unclear. Among the stilbene analogs, we found diethylstilbestrol (DES) to be the most potent blocker (100% inhibition at 10 μM) (Dobrydneva et al., 2003). *trans*-Resveratrol, genistein, and tetrahydrochrysenes (rigid analogs of DES) inhibited thrombin-induced Ca^{2+} responses, but their effectiveness was considerably lower (50% inhibition at 10 μM) (Dobrydneva et al., 1999, 2002, 2003).

In this study, we tested and compared the effects of DES and its close derivative, *trans*-stilbene (*trans*-S) directly on the CRAC and TRPM7 currents in RBL cells and on the capacitative Ca^{2+} influx triggered by thapsigargin (TG, an inhibitor of sarco(endo)plasmic reticulum Ca^{2+} ATPase) in RBL cells, SMC, and human platelets. As a result of these studies, we found that DES effectively inhibits CRAC but not TRPM7 channels and inhibits capacitative Ca^{2+} influx with IC_{50} values below 100 nM in a variety of cell types, making DES the most effective inhibitor of CRAC current and capacitative Ca^{2+} influx yet known.

Materials and Methods

Cells. Primary culture SMC from rabbit aortas, RBL cells, and human platelets were used in this study as described previously (Dobrydneva and Blackmore, 2001; Trepakova et al., 2001; Smani et al., 2003).

Electrophysiology. Whole-cell currents were recorded in RBL cells using standard whole-cell (dialysis) patch-clamp technique. An Axopatch 200B amplifier (Axon Instruments, Union City, CA) was used; data were digitized at 5 kHz and filtered at 1 kHz. Pipettes were used with tip resistance of 2 to 4 MΩ. After breaking into the cell, holding potential was 0 mV and ramp depolarizations (from -100 to $+100$mV, 200 ms) were applied every 5 s. The time course of CRAC current development was analyzed at -80 mV in each cell (amplitude was expressed in picoAmperes per picoFarad). The capacitance of RBL cells was 19.6 $\pm$ 0.7 pF (n = 19). The maximum current density (in picoAmperes per picoFarad) at -80 mV was determined after 10 min of cell dialysis and summarized for all the cells tested (S.E. is shown in the figures). Representative current-voltage relationships are shown during ramp depolarization after 10 min of cell dialysis. Passive leakage current (0.1–0.5 pA/pF at -80 mV) with zero reversal potential at the moment of breaking into the cell was subtracted. To distinguish CRAC current from the current through TRPM7 channels, 3 mM $MgCl_2$ was added to the pipette, and 20 mM Ca^{2+} was used from outside when CRAC was recorded. For recording of CRAC current intracellular (pipette) solution contained 145 mM cesium glutamate, 3 mM $MgCl_2$, 10 mM BAPTA, and 10 mM HEPES, pH 7.2. For registration of TRPM7, $MgCl_2$ was omitted from the pipette solution. Extracellular solutions were 20 mM $CaCl_2$, 1 mM $MgCl_2$, 130 mM NaCl, 3 mM CsCl, and 5 mM HEPES, pH 7.4, for CRAC currents, and 0 Ca^{2+}/0 Mg^{2+} with 140 mM NaCl, 3 mM CsCl, 0.1 mM EGTA, 5 mM tetraethylammonium, and 10 mM HEPES, pH 7.4, for TRPM7 currents. For extracellular application, DES (different concentrations) was added to the bath after CRAC or TRPM7 currents reached their maximum. For intracellular application, DES (25 μM) was added to the pipette solution. Experiments were done at 20 to 22°C.

Intracellular Ca^{2+} Measurement. RBL and SMC cells were loaded with fura-2AM, and quantitative changes in intracellular Ca^{2+} (Fura-2, F_{340}/F_{380}) were monitored as described previously (Trepakova et al., 2001; Smani et al., 2003). For summary data, Δ Ratio was calculated as the difference between the peak ratio after extracellular Ca^{2+} was added, and its level right before Ca^{2+} addition. Summary data are shown after subtraction of the basal Ca^{2+} influx. Dual-excitation fluorescence imaging system (IonOptics) was used for studies of individual SMC and RBL cells. Data were summarized from the large number of individual SMC or RBL cells (from three to five different preparations). The basal Ca^{2+} influx was 0.3 $\pm$ 0.1 (n = 44) in RBL cells and 0.7 $\pm$ 0.1 (n = 22) in SMC and was subtracted from summary data but not from the original traces.

In human platelets, Ca^{2+} was measured using Fura-2 technique as described previously (Dobrydneva and Blackmore, 2001). In brief, platelets were isolated from citrated blood by centrifugation and incubated in modified Tyrode's buffer containing 1 mM EGTA. Platelets were incubated for 1 h with 2 μM Fura-2 acetoxymethyl ester, washed, and resuspended in Tyrode's buffer without calcium. Calcium measurements were performed on platelets in suspension using a SPEX ARCM spectrofluorometer.

Drugs. All the salts and drugs were from Sigma (St. Louis, MO).

Statistical Analysis. Summary data are presented as mean $\pm$ S.E. A logistic function was used to fit the dose-dependence. Student's t test was used to determine the statistical significance of the obtained data. The significance between multiple groups was evaluated using analysis of variance. Data were considered significant at $P < 0.01$ (*).

Results and Discussion

Dose-Dependent Inhibition of CRAC Currents by DES. Fig. 1A shows a typical example of the time course of development of the whole-cell CRAC current (at -80 mV) in RBL cells in the presence of 20 mM extracellular Ca^{2+} when the cells are dialyzed with 10 mM BAPTA (which is commonly used to deplete their Ca^{2+} stores). Application of the increasing doses of DES (1 and 10 μM) to RBL cells caused fast inhibition of CRAC current. Fig.1B presents current voltage relationships of CRAC currents in the presence of DES (1 and 10 μM). The dose dependence of DES-induced inhibition of CRAC current (Fig. 1C) shows that DES started to suppress CRAC at concentrations as low as 100 nM and totally inhibited CRAC when 10 μM was applied. It is noteworthy that the effect of DES was monophasic, and (contrary to 2-APB) it did not cause any activation of CRAC currents or Ca^{2+} influx at low concentrations. IC_{50} for DES-induced inhibition of CRAC currents was around 0.6 μM, making DES the most potent inhibitor of CRAC current known so far. We found the effect of DES to be reversible even when used at high concentrations: after application of 3 μM of DES, the amplitude of CRAC current was restored to 92 $\pm$ 2% of its original value after 12 to 15 min of washing (n = 4). Even after 30 μM DES was applied, 15 min of washing was enough to restore CRAC current to 82 $\pm$ 2% of its original value. To get a better understanding of the mechanism of DES action, we also tested its effects from inside the cell. Fig.2 shows that the dialysis of RBL cells with DES (even at a very high

concentration of 25 μM) did not affect the development, or the amplitude of CRAC current. Thus, we found that DES is inhibiting CRAC channels only when applied from outside the cell, which suggests that it is most probably a direct inhibitor of store-operated channels working from the outside that does not involve intracellular signaling targets or pathways.

Monovalent Cation Currents That Are Mediated by TRPM7 Channels Are Not Affected by DES. It is known that in the absence of extracellular divalent cations, CRAC channels start to conduct monovalent cations, and monovalent cation current through CRAC channels could be observed in RBL cells in divalent cation-free (DCF) conditions. However, it is now well accepted that not only CRAC but also TRPM7 channels [called also MagNuM (Nadler et al., 2001), MIC (Prakriya and Lewis, 2002), or MCC (Mubagwa et al., 1997; Zakharov et al., 2003)] are activated in RBL and other cells under DCF conditions (Bakowski and Parekh, 2002a; Hermosura et al., 2002; Prakriya and Lewis, 2002), and discrimination of these two currents poses a certain difficulty. 2-APB, which was originally thought and used as a specific inhibitor of CRAC channels, was found to also inhibit TRPM7 channels in almost the same range of concentrations (Bakowski and Parekh, 2002b; Prakriya and Lewis, 2002; Zakharov et al., 2003; Kozak and Cahalan, 2003). To determine whether the inhibitory effects of DES are specific to CRAC channels, we tested the effects of DES (up to 30 μM) on the monovalent cation currents that are mediated by TRPM7 channels. Fig. 3 shows a typical example of the development of the monovalent cation current (in the absence of extracellular divalents) through TRPM7 channels. Corresponding current voltage relationships are shown in Fig. 3B. Application of 20 μM DES (which totally inhibited CRAC current) produced no effect on TRPM7 current ($n = 6$), which could be subsequently inhibited by spermine (100 μM). We have recently shown that spermine specifically inhibits inward component of the monovalent current mediated by TRPM7 but not CRAC channels (Zakharov et al., 2003). Thus, DES seemed to be a potent inhibitor of CRAC but not TRPM7 channels and could be used as a new tool to effectively separate these two channels.

The Effects of DES and Its Structural Analog on Capacitative Ca^{2+} Influx in RBL Cells, SMC, and Platelets. Fig. 4A shows that 1 μM DES inhibited 93 $\pm$ 3% of the

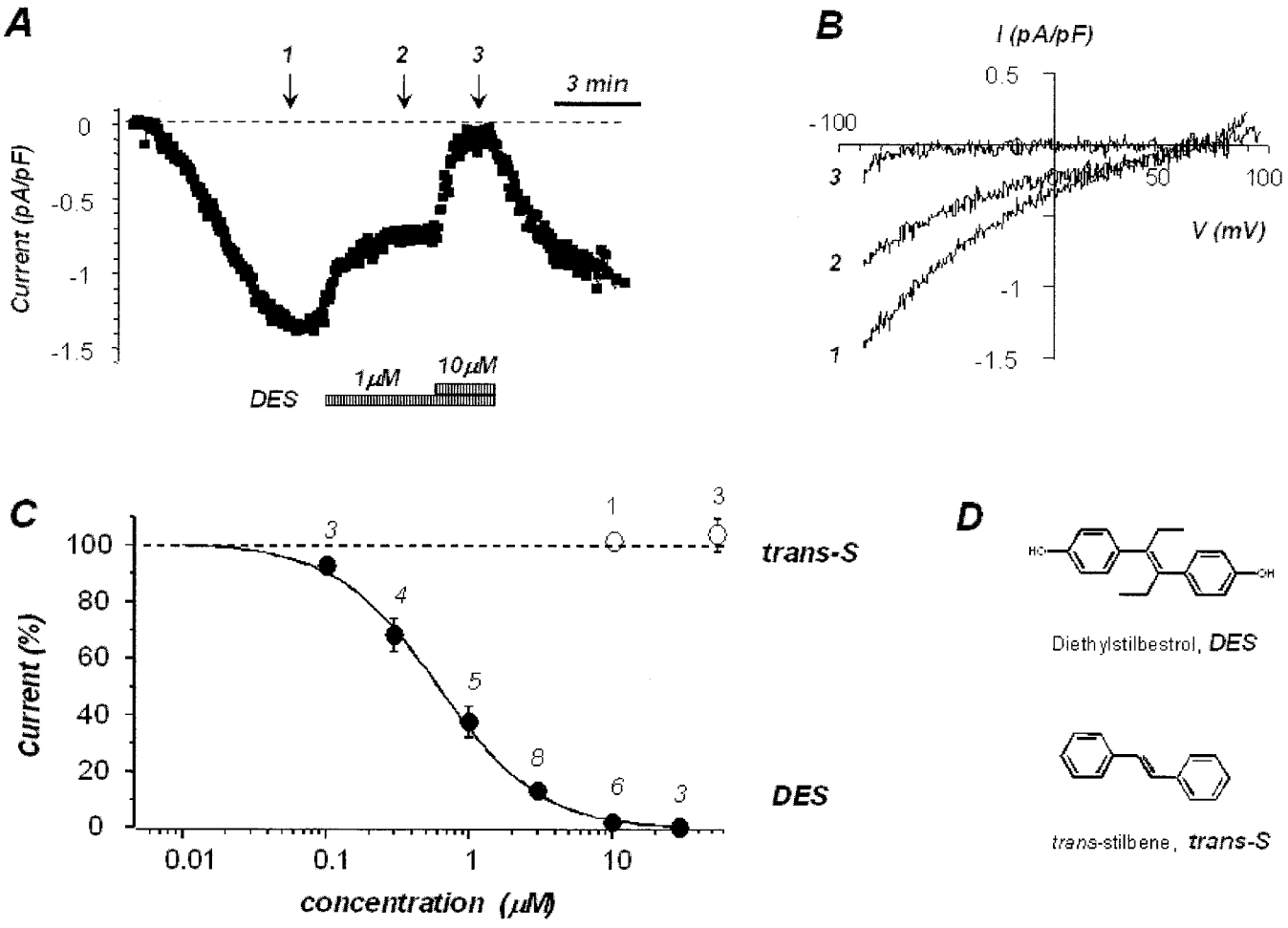

Fig. 1. Whole-cell CRAC current is inhibited by extracellular DES in RBL cells. A, representative time course of the whole-cell inward CRAC current that develops during cell dialysis with BAPTA-containing solution in control extracellular solution that contains 20 mM Ca^{2+}. DES (1 and 10 μM) was applied to the external solution at the times specified by the bars. The current density (in picoAmperes per picoFarad) is shown at -80 mV. Zero time corresponds to the beginning of cell dialysis. Passive leakage current (at the moment of breaking into the cell) was subtracted. B, typical example of the current-voltage relationships of CRAC current before (1) and after 1 μM (2) and 10 μM (3) DES was applied to the cell in the experiment shown in A. C, dose-dependence of the effects of DES and *trans*-S on CRAC current. Summary data (with S.E.), with each concentration tested in the number of cells (as specified by the numbers). D, the chemical structures of *trans*-S and DES are shown on the right.

capacitative Ca^{2+} influx triggered by TG-induced depletion of Ca^{2+} stores in RBL cells. The inhibitory effect of DES was dose-dependent with $IC_{50} \sim 0.1$ μM (Fig. 4B), which was even lower than for the CRAC currents. Thus, DES indeed seemed to be very potent in inhibiting both CRAC currents and capacitative Ca^{2+} influx in RBL cells.

Next, we tested whether DES effects were specific to Ca^{2+} selective CRAC channels, or if it could also inhibit store-operated nonselective cation channels, which are known to mediate capacitative Ca^{2+} influx in SMC and platelets. Figure 4, C and D, shows that indeed, capacitative Ca^{2+} influx in SMC is inhibited by DES in a dose-dependent manner with $IC_{50} < 0.1$ μM. In platelets, the same effect was observed (Fig. 4, E and F). Thus, DES was effective in inhibiting capacitative Ca^{2+} influx independently on whether it is mediated by CRAC (in RBL cells, Fig. 4, A and B), or by SOC (in SMC, Fig. 4, C and D; in platelets, Fig. 4, E and F) channels.

It is noteworthy that only DES, and not its very close structural analog *trans*-stilbene, was able to inhibit capacitative Ca^{2+} influx in RBL cells, SMC, and human platelets (Fig. 4). *trans*-Stilbene also had no effect on CRAC currents in RBL cells (Fig. 1C).

Summarizing our findings, we would like to propose DES as a new and very potent inhibitor of store-operated channels and capacitative Ca^{2+} influx in a variety of cell types. It could be a prototype drug to develop new potent and truly specific

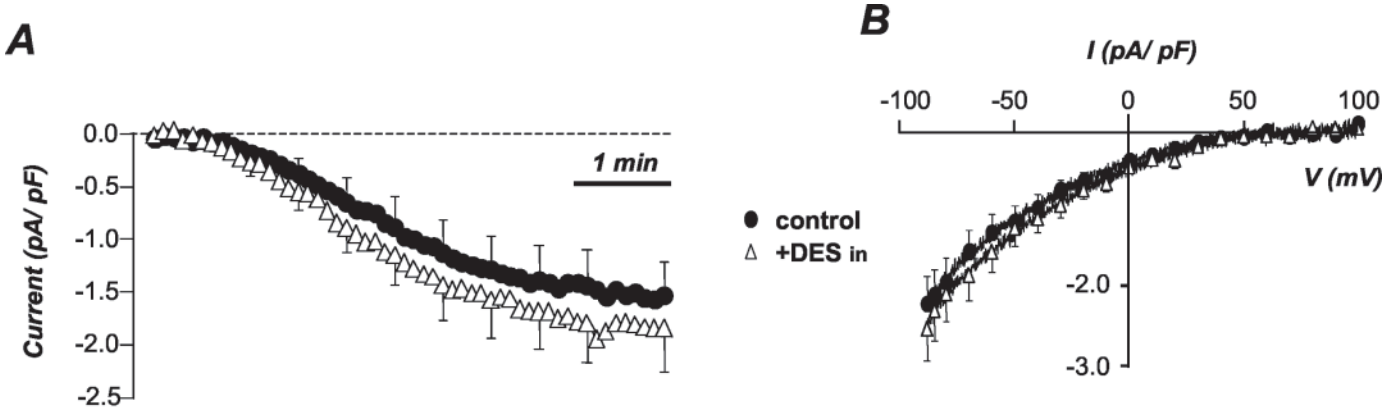

Fig. 2. CRAC current is not affected by intracellular application of DES. A, summary data showing the development of the whole-cell inward CRAC current in RBL cell dialysis with intracellular solution containing BAPTA (10 mM) with or without DES (25 μM). ●, the average (± S.E.) current density (at −80 mV) in control cells (n = 11); △, current density (at −80 mV) in the cells dialyzed with DES (n = 11). Zero time corresponds to the beginning of cell dialysis. B, average current-voltage relationships (± S.E.) of maximum CRAC current in control RBL cells (●) and in cells dialyzed with DES (25 μM) in the experiments shown in A.

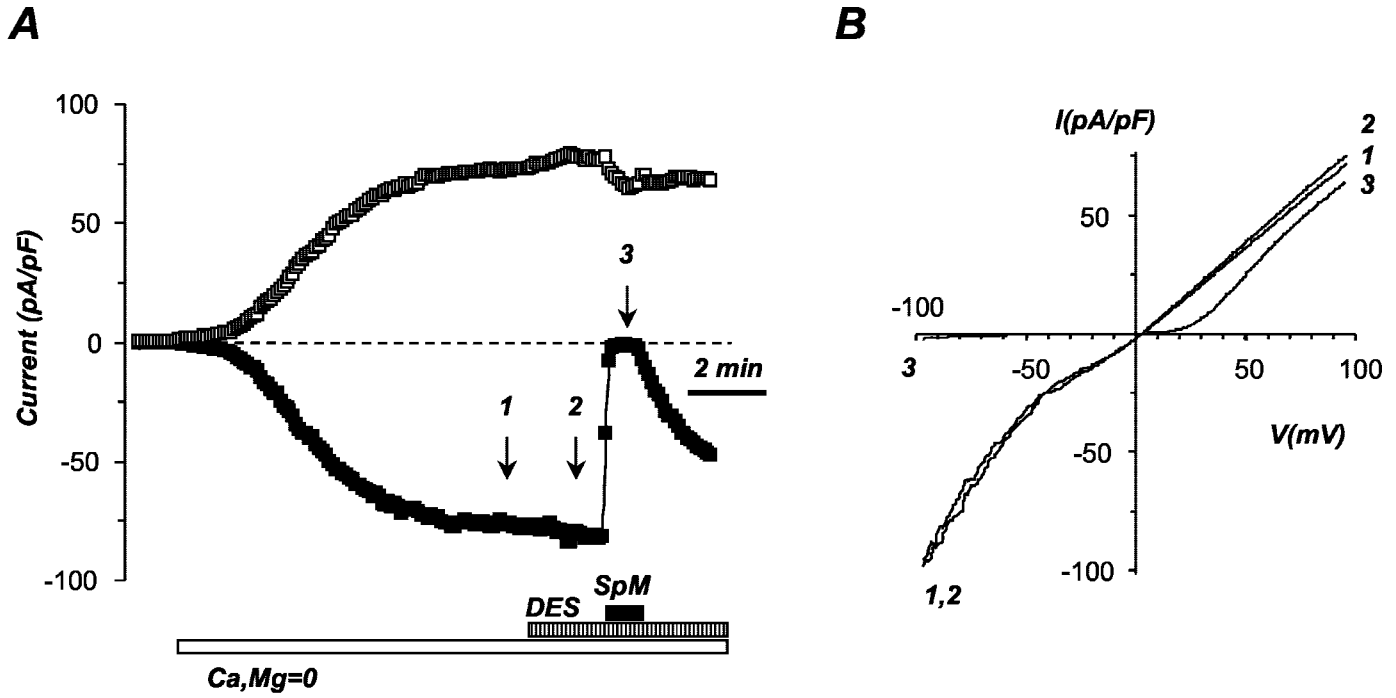

Fig. 3. DES does not affect whole-cell current through TRPM7 channels. A, representative time course of the whole-cell current that develops in divalent cation-free conditions (intracellular dialysis with 10 mM BAPTA and 0 Mg^{2+}, and extracellular 0 Ca^{2+}/0 Mg^{2+}). DES (20 μM) applied to the cell did not affect the current, whereas spermine (SpM; 10 μM) inhibited it. The current density (in picoAmperes per picoFarad) is shown at −80 mV. Zero time corresponds to the beginning of cell dialysis. Passive leakage current (at the moment of breaking into the cell) was not subtracted. B, typical example of the current-voltage relationships of TRPM7 current in the absence (1) and in the presence of 20 μM DES (2) and 100 μM spermine (3) DES was applied to the cell in the experiment shown in A.

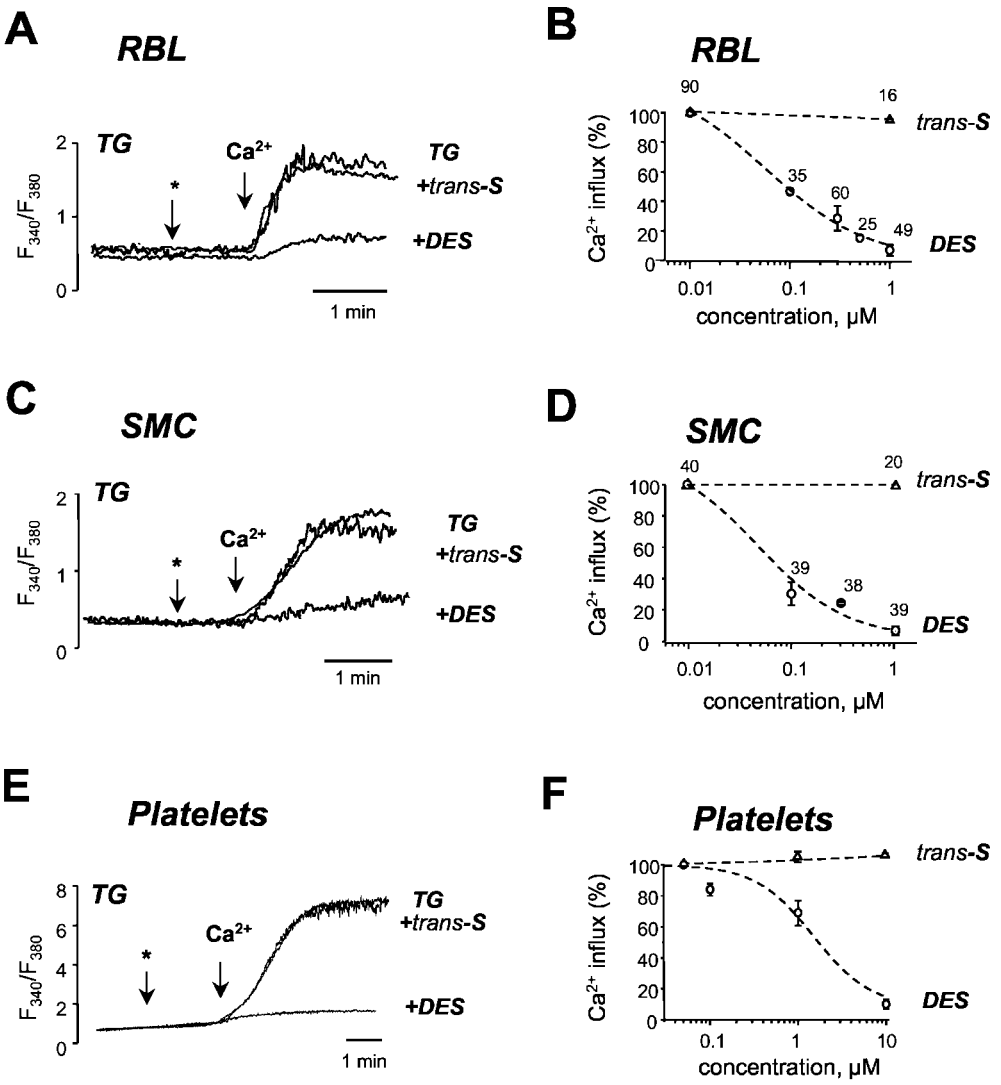

Fig. 4. Comparative analysis of the effects of DES and trans-S on capacitative Ca^{2+} influx in RBL cells, SMC, and human platelets. Intracellular Ca^{2+} (fura-2 ratio) was monitored in RBL cells, SMC, and human platelets after TG was added in the absence of extracellular Ca^{2+} to deplete their stores (2 μM for RBL and SMC; 0.1 μM for platelets). A, C, and E, representative traces showing the results of addition of extracellular Ca^{2+} (2 mM, 5 min after addition of TG) in control cells (TG) and when DES (1 μM) or *trans*-S (10 μM) was added 1 min before Ca^{2+} (at the time shown by the asterisk). In platelets, 10 μM DES was used. B, D, and F, dose dependence of the effects of DES and *trans*-S on TG-induced Ca^{2+} influx in RBL cells, SMC, and platelets. Summary data are shown for the number of SMC and RBL cells specified by the numbers above each data point (from three different cultures). For platelets, the data are summarized from five experiments. Basal Ca^{2+} influx is subtracted.

inhibitors of store-operated channels. It could be also used as a helpful tool in discriminating CRAC and TRPM7 channels. The molecular target of DES action on CRAC is unknown, but because DES inhibits store-operated channels only when applied from the outside, the effects are rapid and easily reversible, they most likely do not involve any intracellular targets or transcription/translation processes, and they may be localized to the channel itself or to some extracellularly located and closely related regulatory proteins. We may speculate that because DES is known to bind to the estrogen receptor, it is possible that the binding site for DES on the channel (or regulatory proteins) may share some homology with the binding site on the estrogen receptor.

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