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Dietary Phytoestrogens and Their Synthetic Structural Analogues as Calcium Channel Blockers in Human Platelets

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Summary: Phytoestrogens have been shown to inhibit platelet activation by blocking platelet calcium channels. This study examined the effect of several synthetic derivatives of *trans*-resveratrol, genistein, and daidzein on platelet free intracellular calcium ($[Ca^{2+}]_i$) elevation in thrombin-activated platelets and the possible mechanisms of this inhibitory effect. Studies were conducted on fresh human platelets from healthy volunteers. The fluorescent dye fura-2 was used to monitor $[Ca^{2+}]_i$ in platelets. At 10 μ*M trans*-resveratrol, triacetyl-*trans*-resveratrol, and trimethoxy-*trans*-resveratrol produced, respectively, 57 ± 4%, 40 ± 4%, and 21 ± 1% inhibition; genistein, acetylgenistein, and dihydrogenistein produced 51 ± 10%, 26 ± 7%, and 16 ± 2% inhibition, respectively; daidzein and diacetyldaidzein produced 56 ± 5% and 45 ± 10% inhibition of thrombin-induced $[Ca^{2+}]_i$ elevation. The inhibitory effect was immediate and appeared to directly affect the calcium influx channels. Phytoestrogen action on $[Ca^{2+}]_i$ did not cause alteration in nitric oxide signaling. Tyrosine phosphorylation was not involved in the inhibition of $[Ca^{2+}]_i$ elevation by phytoestrogens, because the percent inhibition produced by the tyrosine kinase inhibitor genistein and its inactive analogue daidzein on thrombin-induced and thapsigargin-induced $[Ca^{2+}]_i$ elevation was not significantly different for either compound at any concentration tested. Structure–activity relationship studies on this limited set of compounds reveal the requirements for the stilbene pharmacophore for the calcium-blocking activity. **Key Words:** Calcium—Genistein—Phytoestrogens—Platelets—Stilbene—Thrombin.

Consumption of phytoestrogen-rich foods has been reported to cause decreased platelet aggregation in vivo (1–5). Furthermore, phytoestrogens can inhibit platelet aggregation in vitro (6–7). Therefore, dietary intake of phytoestrogens with wine and certain phytoestrogen-rich foods, such as soy, may have beneficial effects on cardiovascular health. Excessive platelet aggregation plays a role in the pathogenesis of the diseases such as stroke

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and myocardial infarction. Platelet aggregation is initiated by an increase in the intracellular free calcium concentration ($[Ca^{2+}]_i$). Calcium enters platelets through specific and tightly regulated calcium channels, which open in response to a variety of stimulatory agonists (8). These agonists are produced at the site of vascular injury, including the most potent platelet agonist, thrombin (9).

Thrombin is generated in vivo as a result of the sequential activation of the enzymes of the coagulation cascade. The thrombin receptor (10) is a single polypeptide chain with seven hydrophobic transmembrane-spanning domains. An extracellular *N*-terminus has a proteolytic cleavage site at which thrombin cleaves its receptor after Arg^{41}, creating a new *N*-terminus, which in turn serves as the ligand for the receptor. It has been shown that this receptor-derived cleaved peptide can evoke all of the thrombin responses observed in platelets, fibroblasts, and endothelial cells (11). Interaction of the thrombin receptor with thrombin leads to the G_q-protein mediated, pertussis toxin–insensitive activation of PLCβ. This in turn causes generation of IP_3, which binds to the IP_3 receptor localized on the dense tubular system (endoplasmic reticulum), the primary storage site of the intracellular calcium. The IP_3 receptor serves as a ligand-gated channel and its activation causes rapid release of stored Ca^{2+} into the cytoplasm (12). This pathway has been extensively studied in many cell types (13). The so-called store-operated calcium channels (SOCC) are then activated in response to this intracellular calcium store depletion, promoting calcium influx from the external media. However, the molecular identity of these SOCC channels is still largely unknown. Evidence is accumulating that the members of the transient receptor potential-like family of proteins form the SOCC (14). Although the molecular identity of SOCC has been a subject of a number of publications (15,16), there is no agreement on which particular protein from this family is SOCC. There is evidence that in platelets, naturally expressed transient receptor potential-1 functions as SOCC (17). The nature of the signal that couples emptying of the internal stores to the opening of SOCC remains elusive despite numerous studies. The most recent evidence supports a conformational coupling hypothesis. This hypothesis proposes direct physical interaction between IP_3 receptors on the endoplasmic reticulum and SOCC in the plasma membrane (16–18). Thrombin, the most potent platelet agonist, causes calcium influx into activated platelets following the depletion of the internal calcium stores (11). Thus calcium influx into platelets in response to thrombin stimulation is attributed to SOCC.

We have previously shown that a possible mechanism by which phytoestrogens inhibit platelet aggregation may be associated with their ability to block calcium influx into thrombin-stimulated platelets and, therefore, prevent activation of platelets. We attributed this inhibition to the inhibition of SOCC. Thus, the dietary phytoestrogens *trans*-resveratrol, genistein, daidzein, and apigenin, at 10 μ*M*, produced, respectively, 57 ± 4%, 50 ± 5%, 56 ± 2%, and 50 ± 8% inhibition of thrombin-induced $[Ca^{2+}]_i$ elevation in platelets (19). We have now investigated how several synthetic derivatives of *trans*-resveratrol, genistein, and daidzein affect calcium channels in platelets and the possible mechanism of this effect.

MATERIALS AND METHODS

Reagents and material sources

The following were from Sigma Chemical: *trans*-resveratrol, apigenin (4′,5,7-trihydroxyflavone), genistein (4′,5,7-trihydroxyisoflavone), daidzein (4′,7-dihydroxyisoflavone), thrombin, Me_2SO (dimethylsulfoxide), and egtazic acid [ethylene glycol-bis(β-aminoethyl ether)N,*N*,*N*′,N′-tetra-acetic acid]. The following were from Calbiochem: ODQ (1H-[1,2,4]oxadiazolo[4,3-a]-quinoxalin-1-one), thapsigargin, and ionomycin. Fura-2/AM was from Molecular Probes. Other chemicals were from Fisher Scientific and Sigma Chemical.

Analytic methods

The structures of each of the compounds described in the "Discussion" section were confirmed by infrared, nuclear magnetic resonance spectroscopy (NMR), and elemental analysis (percent C and H). Infrared analysis spectra were obtained in the solid phase with KBr pellets using a Nicolet infrared spectrometer. NMR spectra were obtained in solution in either deuterated dimethyl sulfoxide, deuterated acetone, or deuterated chloroform using tetramethyl silane as the internal standard. Elemental analyses for percent C and H were obtained from Desert Analytical (Tucson, AZ, U.S.A.).

Syntheses of analogues of *trans*- resveratrol

3,4′,5-Triacetoxy-trans-*stilbene (triacetyl*-trans-*resveratrol). Trans*-resveratrol, 0.2 g, was refluxed in 15 ml of acetic anhydride for 6 h. The solution was diluted with methanol and then concentrated in vacuo to give a pale yellow oil. The oil was crystallized from methanol/water to give white needles of the triacetyl-*trans*-resveratrol with a 78% yield, mp 115°C. Infrared (KBr) yields a peak at 1,680 cm^{-1} (ester carbonyl).

3,4′,5′-Trimethoxy-trans-*stilbene (trimethoxy*-trans-*resveratrol). Trans*-resveratrol, 0.2 g, was dissolved in

25 ml of acetone and treated with excess methyl iodide (5 ml) and 3.6 m*M* of potassium carbonate. The solution was stirred for 24 h and then the acetone and excess methyl iodide were removed in vacuo. The resulting solid was suspended in ether and any potassium carbonate was removed by filtration to give an oily residue. The oil was resuspended in methanol and triturated with water to give pale beige crystals of the trimethoxy derivative of *trans*-resveratrol, 58% yield, mp 50–51°C. Infrared (KBr) yields peak at 1,150 cm^{-1} (ether C-O-C absorption). NMR (dimethylsulfoxide) yields chemicals shifts at 3.78 (singlet/9H), 6.83 (triplet/1H), 6.7 (doublet/2H), 6.94 (doublet/2H), 7.02 (doublet/1H), 7.22 (doublet/1H), and 7.53 (doublet/2H) ppm.

Synthesis of acetyl analogues of daidzein and genistein

4',7-Diacetoxyisoflavanone (diacetyldaidzein). Daidzein, 0.2 g, was dissolved in 10 ml of acetic anhydride and refluxed for 6 h. The solution was then concentrated in vacuo to yield a pale yellow oil. The addition of methanol/water initiated crystallization to give a 65% yield of the diacetyl derivative of daidzein, mp 111°C. Infrared (KBr) yields a peak at 1,725 cm^{-1} (ester carbonyl absorption). NMR (deuterated acetone) yields chemical shifts at 2.8 (two singlets/6H), 7.2 (doublet/1H), 7.3 (doublet/1H), 7.46 (doublet/1H), 7.65 (doublet/1H), 8.25 (doublet/1H0, and 8.4 (singlet/1H) ppm.

4',5,7-Triacetoxyisoflavanone (triacetylgenistein). Genistein, 0.2 g, was dissolved in 10 ml of acetic anhydride and refluxed for 6 h. The solution was concentrated in vacuo. The oily product was then treated with methanol/water, which caused the product to crystallize from solution in 53% yield, mp 203–204°C. Infrared (KBr) yields a peak at 1,725 cm^{-1} for ester carbonyl absorption.

2,3-Dihydroisoflavanone (dihydrogenistein). Synthesis was performed as described by Wahala and Hase (20). Genistein (0.05 g) was dissolved in 15 ml of methanol and treated with 0.05 g of 10% palladium on carbon and 0.2 g of freshly sublimed ammonium formate. This heterogeneous catalytic transfer hydrogenation resulted in a 59% yield of the corresponding 2,3-dihydrogenistein, mp 176–180°C, whose structure was confirmed by NMR. NMR (dimethyl sulfoxide) yields chemical shifts at 4.0 (triplet/1H), 4.5 (doublet/2H), 5.9 (quartet/2H, aromatic), 6.75 (doublet/2H), and 7.05 (doublet/2H, aromatic) ppm.

Blood donors and platelet preparation

All donors were healthy nonsmoking volunteers (aged 20–60 years) who had not consumed any medication known to affect platelet function (e.g., calcium channel blockers or aspirin) for at least 10 days prior to the study. Venous blood was collected into 1/10 volume of ACD (74.8 m*M* sodium citrate, 38.1 m*M* citric acid, and 123 m*M* dextrose pH 6.4) (Baxter Healthcare Corp.). The blood was centrifuged at 250 × *g* for 10 min at room temperature to obtain platelet-rich plasma. The platelet-rich plasma was centrifuged at 550 × *g* for 12 min to sediment the platelets. The platelets were then resuspended in a modified Tyrode physiologic salt solution (NaCl, 145 m*M*; KCl, 4 m*M*; $MgSO_4$, 1 m*M*; Na_2HPO_4, 0.5 m*M*; Na/4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 10 m*M*; glucose, 6 m*M*; pH 7.4) containing 1.0 m*M* egtazic acid, which acted to prevent spontaneous aggregation during the various experimental manipulations by binding extracellular Ca^{2+} (21). The platelets were loaded with fura-2 as described here.

Phytoestrogen solution preparations

Stock solutions of the phytoestrogens and their derivatives in Me_2SO (1 m*M*) were prepared and stored at −20°C. Just before each experiment, aliquots were thawed and diluted with Me_2SO up to a desirable concentration (see specific figure legends for concentrations used). Stock solutions of phytoestrogens and their derivatives were diluted 200-fold into the spectrofluorometric cuvette containing platelets to give the desired final concentration.

Platelet loading with fura-2 and measurement of $[Ca^{2+}]_i$

Intracellular calcium measurements $[Ca^{2+}]_i$ used the fluorescent dye fura-2, which involved incubating the platelets with the cell permeant acetoxymethyl ester (fura-2/AM). Suspensions of human platelets (isolated as described here) were incubated with 2 μ*M* of fura-2/AM for 1 h at room temperature (on a rocking platform). Excess fura-2/AM was removed by centrifugation (500 × *g* for 10 min) and the platelets were suspended in modified Tyrode buffer, without added egtazic acid. Aliquots of platelet suspension (0.5 ml) were added to a 1.0-ml cuvette containing a Teflon-coated stirrer bar (Chrono-Log, Havertown, PA, U.S.A.). Approximately 30 seconds before $[Ca^{2+}]_i$ measurements were performed, Ca^{2+} was added back to the buffer to a final concentration of 2 m*M*; then phytoestrogens (various concentrations in 2.5 μl) and thrombin 0.01 units ml^{-1} were added (see individual figure legends for specific details). The measurements of $[Ca^{2+}]_i$ were performed at room temperature in a SPEX ARCM spectrofluorometer using excitation wavelengths of 340 nm and 380 nm and an emission wavelength of 505 nm. Calibration was performed as

previously described for human sperm (22). $[Ca^{2+}]_i$ was calculated by using the SPEX dM3000 software package.

Thapsigargin-induced $[Ca^{2+}]_i$ entry

After Ca^{2+} was added back to the platelet suspensions, thapsigargin (dissolved in Me_2SO) was added to the platelet suspension to a final concentration of 10^{-7} *M* and the fluorescence was monitored as described here.

Measurement of Sr^{2+} entry

Basal Ca^{2+} channel activity can be measured without agonist stimulation in Ca^{2+}-free medium monitoring Sr^{2+} entry (23). Sr^{2+} at 20 m*M* was added to the platelets and fluorescent ratio was measured.

Statistical analysis

Data are reported as mean ± SEM for the number of individual experiments specified in the figure legends. Comparisons were made using Student *t* test, with a p value < 0.05 considered significant. Different platelet donors were used for each experiment.

RESULTS

Certain *trans*-resveratrol derivatives possess lower inhibitory activity compared with *trans*-resveratrol

Synthetic *trans*-resveratrol derivatives, such as triacetyl-*trans*-resveratrol and trimethoxy-*trans*-resveratrol (Fig. 1), produced a lower inhibition of the thrombin-induced $[Ca^{2+}]_i$ increase at 10 μ*M* compared with the same dose of *trans*-resveratrol (Fig. 2). Thus, 10 μ*M* of *trans*-resveratrol, triacetyl-*trans*-resveratrol, and trimethoxy-*trans*-resveratrol produced 54 ± 7%, 40 ± 4%, and 24 ± 1% inhibition, respectively, which are significantly different from the control values ($p < 0.001$) (Fig. 2). The trimethoxy derivative decreased the calcium-blocking ability of *trans*-resveratrol more than the corresponding triacetyl derivative.

Genistein derivatives possess lower inhibitory activity compared with genistein

To evaluate the importance of the free phenolic hydroxyls and the rigidity of the A/B ring system in the isoflavonoids, several synthetic analogues were tested. Genistein, triacetylgenistein, and dihydrogenistein (Fig. 1) were compared for their ability to block thrombin-induced $[Ca^{2+}]_i$ elevation in platelets (Fig. 3). Genistein at 10 μ*M* displayed the highest degree of inhibition (51 ± 10%), triacetylgenistein at 10 μ*M* displayed less inhibition (26 ± 7%), and dihydrogenistein displayed the lowest level of inhibition (16 ± 2%). These values are significantly different from the control ($p < 0.001$). Diacetyldaidzein at 10 μ*M* produced a slightly lower inhibition of thrombin-induced elevation of $[Ca^{2+}]_i$ in platelets (Fig. 3) than daidzein (45 ± 10% versus 56 ± 5%); these values are not significantly different from each other and are significantly different from the control ($p < 0.001$).

Genistein and *trans*-resveratrol rapidly inhibit Sr^{2+} entry into platelets

Previously obtained data suggest that *trans*-resveratrol has a direct inhibitory effect on platelet calcium channels. Sr^{2+} and Ba^{2+} spontaneously enter platelets in the absence of agonist stimulation. These cations form fluorescent complex in the cytoplasm with fura-2 and therefore can be used as calcium surrogates in calcium-free medium. Basal calcium channel activity can be measured by monitoring the entry of these divalent cations (19,23). The addition of 20 m*M* of Sr^{2+} to nonstimulated platelets causes a rapid increase in fluorescence due to the formation of this fluorescent complex in the cytoplasm. When *trans*-resveratrol or genistein (10 μ*M*) was added (at approximately 75 seconds) to the platelets that were taking up Sr^{2+}, it caused an immediate drop in fluorescence and cessation of further Sr^{2+} entry (Fig. 4). Because Sr^{2+} entry was associated with basal calcium channel activity, addition of phytoestrogens appears to block spontaneous Sr^{2+} entry through nonstimulated channels and therefore suggests that phytoestrogens may have a site of action on the cell surface directly modifying these calcium channels.

Tyrosine phosphorylation does not seem to be involved in the inhibition of $[Ca^{2+}]_i$ elevation by phytoestrogens

It has been shown that genistein inhibits tyrosine phosphorylation, and genistein is widely used as a tyrosine kinase inhibitor (24–26). Daidzein is devoid of one hydroxyl group on the aromatic ring, which renders it inactive as a tyrosine kinase inhibitor (26). Therefore, if tyrosine phosphorylation plays a role in phytoestrogen blocking $[Ca^{2+}]_i$ elevations in platelets, genistein would inhibit thrombin-mediated elevation of $[Ca^{2+}]_i$ and daidzein would be inactive as a calcium channel blocker. Our previous findings (19) showed, however, that both genistein and daidzein at 10 μ*M* block $[Ca^{2+}]_i$ elevation in thrombin-stimulated platelets with equal effectiveness.

Thapsigargin, a cell-permeable inhibitor of the SERCA pump, is a specific tool to study SOCC (27). When the SERCA pump is inhibited, internally stored calcium leaks into cytoplasm, thus promoting calcium influx through SOCC without a concomitant rise in IP_3 (28,29). Genistein and daidzein demonstrated a similar

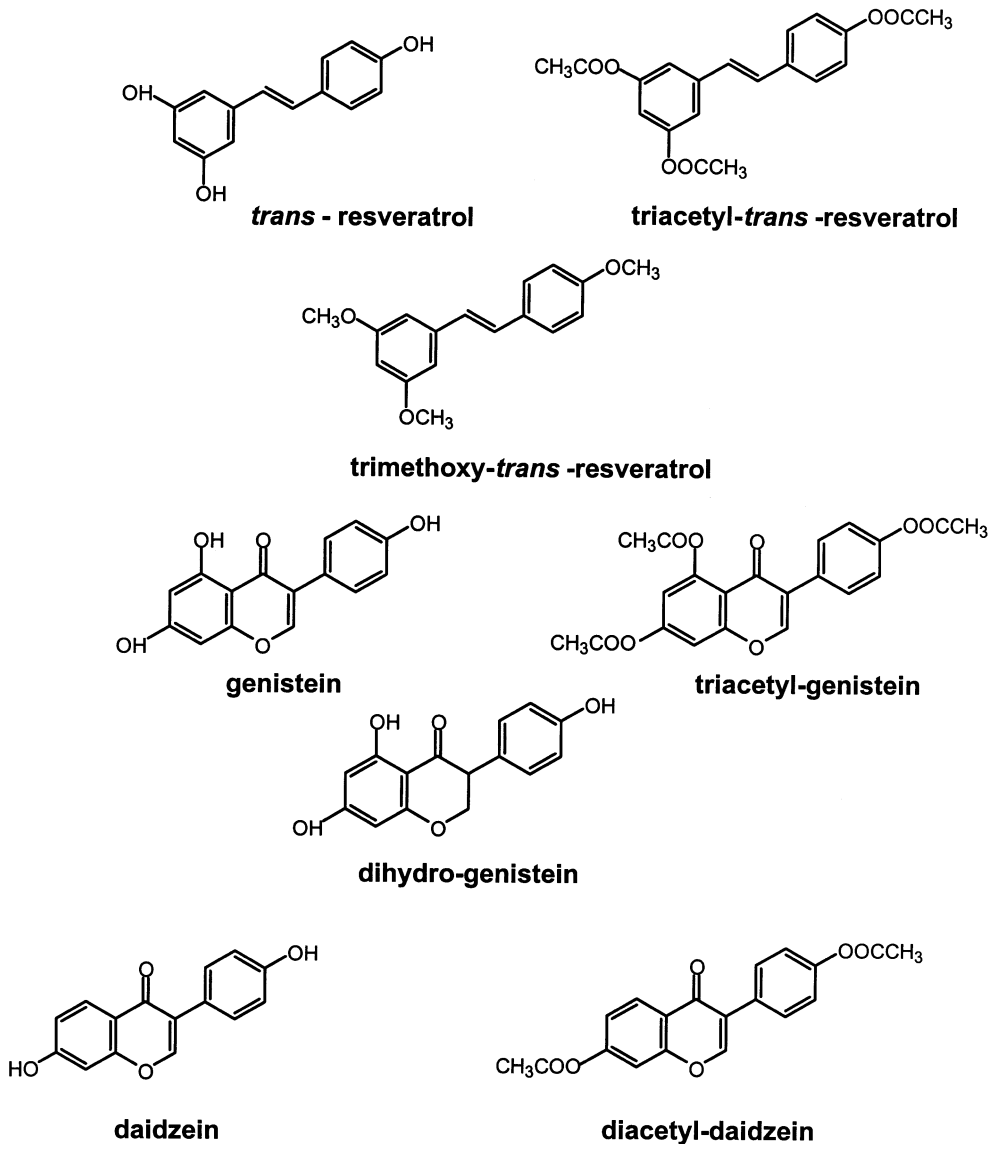

FIG. 1. Structures of *trans*-resveratrol, genistein, and daidzein analogues.

mode of inhibition of thrombin-induced $[Ca^{2+}]_i$ elevation (Fig. 5) and thapsigargin-induced calcium influx (Fig. 5). Thus, genistein at 0.1, 1, and 10 μM produced, respectively, 19 ± 5%, 29 ± 3%, and 50 ± 5% inhibition, whereas daidzein at 0.1, 1, and 10 μM produced, respectively, 17 ± 5%, 24 ± 4%, and 56 ± 2% inhibition of thrombin. These values for daidzein and genistein were not significantly different from each other at the corresponding concentrations. Both genistein and daidzein were able to inhibit thapsigargin-induced $[Ca^{2+}]_i$ elevation in platelets. Genistein at 0.1, 1, and 10 μM produced, respectively, 2 ± 4%, 5 ± 4%, and 76 ± 7% inhibition of thapsigargin-induced $[Ca^{2+}]_i$ elevation. Daidzein at 0.1, 1, and 10 μM produced, respectively, 0 ± 1%, 1 ± 2%, and 51 ± 4% inhibition of thapsigargin-induced $[Ca^{2+}]_i$ elevation, which suggests that both of these agents were blocking SOCC.

Inhibition of thrombin-evoked $[Ca^{2+}]_i$ elevation by phytoestrogens does not involve nitric oxide signal transduction ascade

It has been shown (30) that nitric oxide plays a role in the regulation of capacitative calcium entry in platelets. In some cells, lower levels of nitric oxide stimulate SOCC, possibly through the mechanism of *S*-nitrosylation, and higher doses inhibit SOCC through

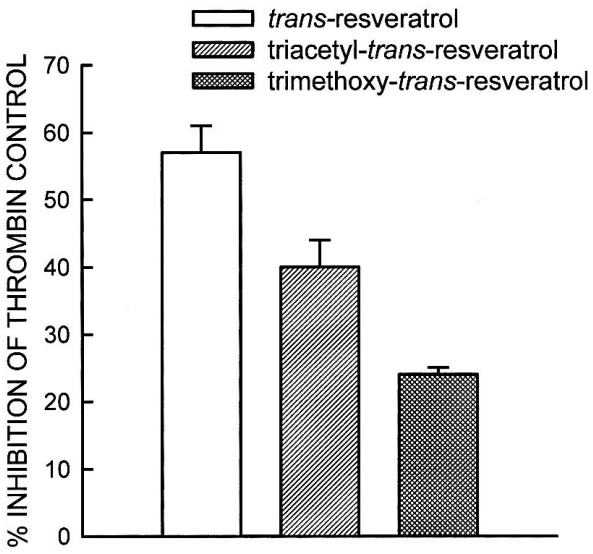

FIG. 2. *Trans*-resveratrol derivatives at 10 μ*M* have lower inhibitory activity to block thrombin-induced $[Ca^{2+}]_i$ elevation compared with 10 μ*M* of *trans*-resveratrol. Platelet suspensions were challenged with thrombin 0.01 U/ml in the presence of 2 m*M* extracellular Ca^{2+} so as to initiate calcium influx. Elevation of $[Ca^{2+}]_i$ above the baseline (nonstimulated level) served as a control (0% inhibition). Each *trans*-resveratrol derivative solution in dimethyl sulfoxide (DMSO) was added to the platelet suspension to yield final concentration of 10 μ*M*. Thrombin was added immediately, within 5 seconds, after each derivative. The percent inhibition was obtained by comparing the peak effect on $[Ca^{2+}]_i$, with the compound present, to the control effect on $[Ca^{2+}]_i$ observed with thrombin alone plus DMSO (solvent control). Data were obtained from the experiments with 4 different platelet donors. Numerical values of the percent inhibition ± SEM and p values are given in the text.

generation of cGMP (31,32). Pharmacologic agents that increase nitric oxide production or cGMP generation inhibit $[Ca^{2+}]_i$ elevation and inhibit platelet activation (30). To rule out the chance that phytoestrogens might inhibit $[Ca^{2+}]_i$ elevation through the activation of nitric oxide signaling cascade, we have investigated whether phytoestrogens affect nitric oxide generation or have an effect on guanylyl cyclase.

Sodium nitroprusside, 100 n*M*, was first added to the platelets to increase the level of nitric oxide and thrombin was added 30 seconds later (Fig. 6). Addition of sodium nitroprusside produced inhibition of thrombin-evoked $[Ca^{2+}]_i$ elevation compared with the thrombin control (without sodium nitroprusside). Thus, the addition of nitric oxide donor inhibits thrombin-induced $[Ca^{2+}]_i$ elevation.

ODQ, which selectively inhibits nitric oxide–sensitive guanylyl cyclase, thus preventing cGMP-mediated signal transduction, had a very slight potentiating effect on thrombin-induced elevation of $[Ca^{2+}]_i$ (data not shown) (Fig. 7). These data suggest that nitric oxide–mediated responses do not make a large contribution to a thrombin-induced $[Ca^{2+}]_i$ elevation. When ODQ at 10 μ*M* was added to the platelets 30 seconds before the addition of sodium nitroprusside, it restored the thrombin-induced $[Ca^{2+}]_i$ increase to near the control level (Fig. 6). This was to be expected, because no cellular response through the cGMP cascade would be possible. The effect of a submaximally effective concentration of sodium nitroprusside (100 n*M*) to inhibit thrombin-induced $[Ca^{2+}]_i$ increase was not inhibited by a submaximally effective concentration of genistein (1 μ*M*); (Fig. 8) this result suggests that genistein was not acting as a nitric oxide scavenger. The effects of submaximal concentrations of genistein and sodium nitroprusside were additive; this result implies that each agent was inhibiting thrombin-induced $[Ca^{2+}]_i$ increase via separate mechanisms. Similar results were obtained with *trans*-resveratrol (data not shown).

The inhibition of thrombin-induced $[Ca^{2+}]_i$ increase

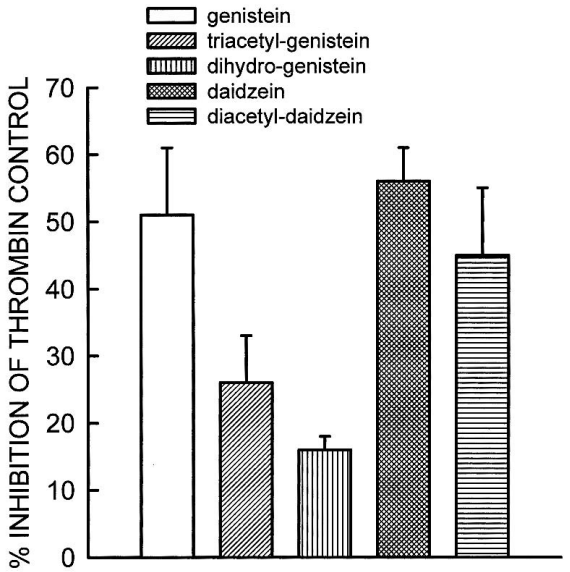

FIG. 3. Inhibition of 10 μ*M* genistein and daidzein derivatives to block thrombin-induced $[Ca^{2+}]_i$ elevation in platelets compared with underivatized genistein and daidzein. Platelet suspension was challenged with thrombin 0.01 U/ml in presence of 2 m*M* extracellular Ca^{2+} to initiate calcium influx. Elevation of $[Ca^{2+}]_i$ above the baseline (nonstimulated level) served as a control (0% inhibition). Thrombin was added immediately, within 5 seconds, after the phytoestrogenic compounds. The percent inhibition was obtained by comparing the peak effect on $[Ca^{2+}]_i$, with the compound present, to the control effect on $[Ca^{2+}]_i$ observed with thrombin alone plus dimethyl sulfoxide (solvent control). Data were obtained from experiments with 4 different platelet donors. Numerical values of the percent inhibition ± SEM and p values are given in the text.

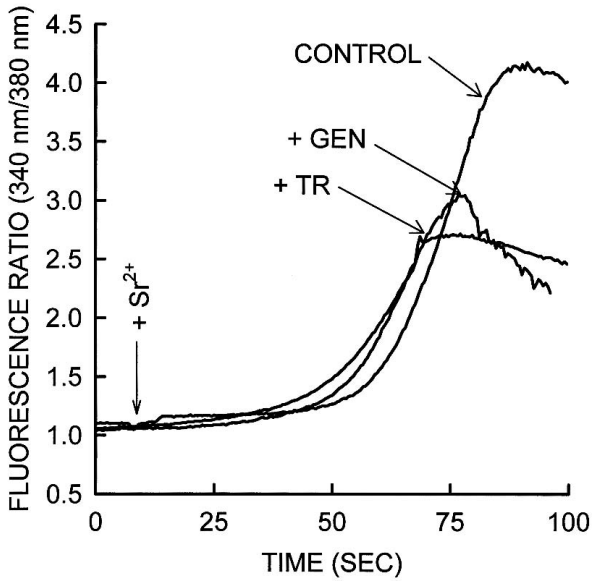

FIG. 4. *Trans*-resveratrol (TR) and genistein (GEN) rapidly inhibit Sr^{2+} entry into platelets. Increase in the 340 nm/380 nm fluorescence ratio was due to Sr^{2+} spontaneously entering the platelet through Ca^{2+} channels and binding to fura-2. Control trace represents Sr^{2+}, 20 m*M*, added alone at 20 seconds. *Trans*-resveratrol at 10 μ*M* or genistein at 10 μ*M* were added when Sr^{2+} was entering the platelets at its maximal rate, as indicated by the rapidly increasing fluorescence ratio. Addition of these compounds immediately blocked Sr^{2+} entry into platelets as seen by the decrease in the fluorescent ratio. One representative experiment of 3 is shown.

by *trans*-resveratrol in platelets was not attenuated by ODQ at 10 μ*M* (Fig. 7). ODQ had a very small potentiating effect on thrombin-induced $[Ca^{2+}]_i$ elevation, therefore demonstrating that soluble guanylyl cyclase–mediated effects do not play a significant role in thrombin-evoked calcium response. The concomitant addition of 10 μ*M* of ODQ and 10 μ*M* of *trans*-resveratrol did not attenuate the inhibition of $[Ca^{2+}]_i$ elevation that was exhibited by *trans*-resveratrol alone. Inhibition of thrombin-induced $[Ca^{2+}]_i$ elevation by genistein was also not attenuated by ODQ (data not shown).

If *trans*-resveratrol and genistein inhibit the thrombin effect to elevate $[Ca^{2+}]_i$ through the activation of soluble guanylyl cyclase, then ODQ would have counteracted this inhibition. However, this was not the case; thus *trans*-resveratrol and genistein do not affect soluble guanylyl cyclase. Therefore, the experimental data suggest that the inhibition of thrombin-evoked $[Ca^{2+}]_i$ increase by *trans*-resveratrol and genistein is unrelated to the stimulation of nitric oxide production or stimulation of soluble guanylyl cyclase by these compounds (Figs. 7 and 8).

DISCUSSION

Rapid effect of phytoestrogens and their derivatives to block $[Ca^{2+}]_i$ elevation

All the compounds tested in the study displayed rapid inhibition of thrombin-induced $[Ca^{2+}]_i$ increase. To reach an inhibitory effect, no preincubation was required, and the effect was immediate on contact with the cells. *Trans*-resveratrol and genistein immediately blocked Sr^{2+} entry into the platelets, and no latency was observed (Fig. 4). Preincubation would be a likely requirement if this effect were mediated by a second messenger system. Thus, in order for genistein to act as a tyrosine kinase inhibitor in platelets, preincubation of 30 min is necessary (33,34). Therefore, the rapid inhibitory effect of phytoestrogens on $[Ca^{2+}]_i$ in platelets suggests that these compounds are not acting through a phosphorylation cascade.

Trans-resveratrol and genistein are moderately hydrophobic compounds. Both compounds have three hydroxyl groups, and genistein has a polar 1,2-benzopyrone moiety. It is likely that the uptake of these compounds into cells would not be instantaneous but would probably

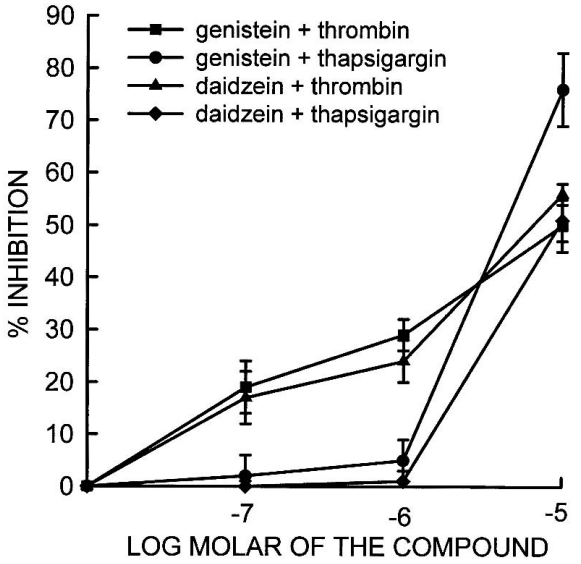

FIG. 5. Dose responses of genistein and daidzein to inhibit thrombin-induced and thapsigargin-induced $[Ca^{2+}]_i$ elevation in platelets. Platelet suspensions were challenged with thrombin 0.01 U/ml or thapsigargin 100 n*M* in presence of 2 m*M* extracellular Ca^{2+} to initiate calcium influx. Elevation of $[Ca^{2+}]_i$ above the baseline (nonstimulated level) served as a control (0% inhibition). The percent inhibition was obtained by comparing the peak effect with the compound present to the control effect observed with thrombin alone and dimethyl sulfoxide blank. Data were obtained from the experiments with 6 different platelet donors. Numerical values of the percent inhibition ± SEM and p values are given in the text.

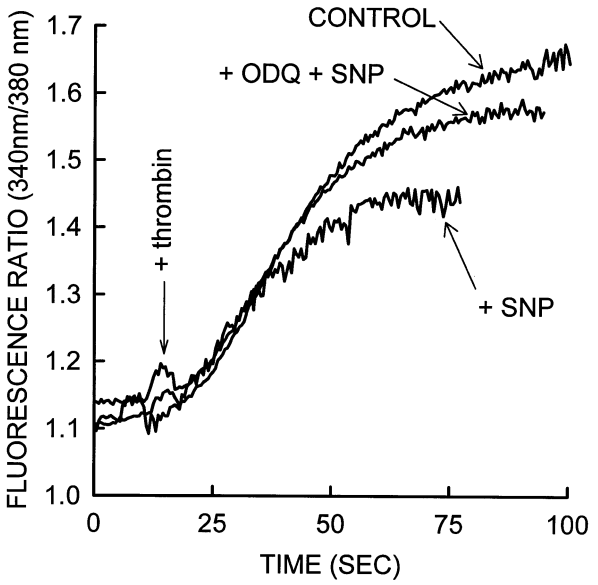

FIG. 6. ODQ, inhibitor of nitric oxide (NO)–sensitive guanylyl cyclase, reverses sodium nitroprusside (SNP) inhibition of thrombin-induced $[Ca^{2+}]_i$ elevation. SNP at 100 n*M* was added to the platelets to increase the level of NO (SNP was added 10 seconds before data collection). At 20 seconds thrombin 0.01 U/ml was added. Addition of SNP inhibited thrombin-evoked $[Ca^{2+}]_i$ elevation compared with the thrombin control (without SNP), as expected. When ODQ 10 μ*M* was added to the platelets 30 seconds before SNP (not shown on the trace). ODQ restored the thrombin-induced $[Ca^{2+}]_i$ increase to control levels. One representative experiment is shown.

require up to 60 min (35). Because the observed inhibitory effects were immediate, the most likely mode of action for the phytoestrogens would be on the cell surface, probably on the channel itself.

Phytoestrogens and structurally related compounds have direct effect on calcium channels

Ba^{2+} and Sr^{2+} enter platelets through calcium channels spontaneously, and genistein and *trans*-resveratrol have a similar mode of action in inhibiting Sr^{2+} entry into the platelets. Genistein and *trans*-resveratrol block Sr^{2+} entry into the platelets immediately, which suggests that the common feature of these compounds is to modify some property of the calcium channel directly and independently from second messenger generation. This hypothesis can be supported by the experiments in which genistein and daidzein demonstrate similar degrees of inhibition of thrombin-induced $[Ca^{2+}]_i$ elevation. Genistein is known to be a tyrosine kinase inhibitor and daidzein is inactive. Therefore, similarities in the dose response of these compounds to inhibit thrombin and thapsigargin-induced $[Ca^{2+}]_i$ elevation strongly suggest that tyrosine phosphorylation is not responsible for the inhibition. The role of genistein as a tyrosine kinase inhibitor is well established (26) and genistein is often used as a broad-range tyrosine kinase inhibitor. However, this effect requires preincubation of 20–30 min (36) and a large concentration of genistein (10–50 μ*M*) (37). Thus, to inhibit tyrosine phosphorylation in ADP-stimulated platelets, genistein was used at 100 μ*M* for 30 min (38). In addition, it has been shown (25) that when platelets were treated with tyrosine kinase inhibitors (including genistein) at concentrations up to 100 μ*M* for 30 min, thapsigargin promoted calcium influx into platelets independently of tyrosine phosphorylation. These authors concluded that genistein inhibited calcium influx into BAPTA-loaded platelets independently from tyrosine phosphorylation. Also, when platelets were stimulated with phorbol esters, genistein was reported to activate mitogen-activated protein kinase activity (39) rather than inhibit this protein tyrosine kinase. The authors concluded that this activation is independent from cAMP and protein kinase C pathways. It also has been reported

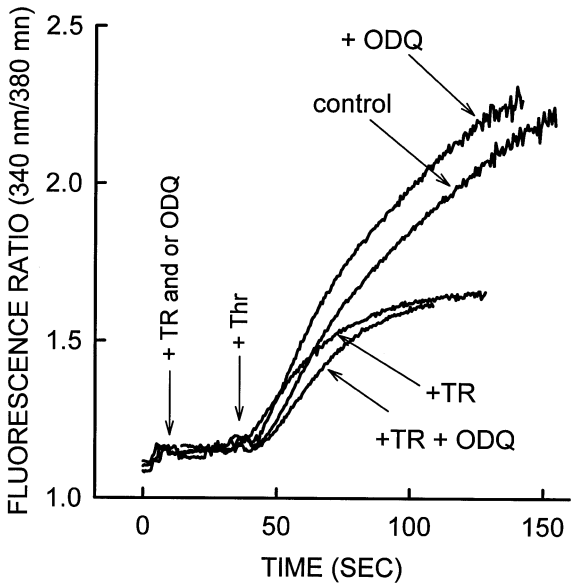

FIG. 7. ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) does not relieve inhibition of *trans*-resveratrol on thrombin-evoked $[Ca^{2+}]_i$ elevation. Control trace represents $[Ca^{2+}]_i$ elevation caused by thrombin in presence of 5 μl dimethyl sulfoxide (control solvent for ODQ). Addition of ODQ at 10 μ*M* caused small potentiation of the thrombin effect to elevate $[Ca^{2+}]_i$. *Trans*-resveratrol at 10 μ*M* inhibited thrombin-induced $[Ca^{2+}]_i$ elevation as previously shown. When 10 μ*M* *trans*-resveratrol and 10 μ*M* ODQ were added to the platelets together, the inhibition of thrombin by *trans*-resveratrol was unchanged by ODQ. These data suggest that *trans*-resveratrol does not activate soluble guanylyl cyclase in platelets. One representative experiment is shown. SNP, sodium nitroprusside.

(24) that *trans*-resveratrol inhibits tyrosine kinases in the standard assay, but it was also described as a weak inhibitor. Tyrosine phosphorylation therefore does not seem to be involved in the inhibition of $[Ca^{2+}]_i$ elevation by *trans*-resveratrol and genistein, and most likely these phytoestrogens have a direct effect on platelet calcium channels.

Phytoestrogen action to block thrombin-induced $[Ca^{2+}]_i$ increase does not include alteration in nitric oxide production

A growing number of studies imply the role of nitric oxide production in regulation of intracellular calcium homeostasis and, in particular, regulation of SOCC. These reports (32,40) provide evidence that SOCCs are activated by *S*-nitrosylation and that this process is stimulated by emptying of internal calcium stores. Therefore, nitric oxide donors such as sodium nitroprusside can bypass the normal coupling process between mobilization of the internal stores and channels, thus modifying the channels directly. This pathway has been shown to take place in a smooth muscle cell line and in a fibroblast cell line (40). There is no evidence indicating that this pathway exists in platelets. In platelets, nitric oxide donors have been shown to inhibit the activity of SOCC through activation of soluble guanylyl cyclase and the generation of cGMP, with concomitant protein phosphorylation (41,42). There is also a study suggesting that nitric oxide exposure inhibits SOCC indirectly by promoting the refilling of the intracellular stores (30). Physiologic activation of endothelial nitric oxide synthase occurs in response to an increase in $[Ca^{2+}]_i$. It is possible that the nitric oxide cascade serves as a feedback loop regulating the degree of calcium influx into platelets. Possibly lower doses of nitric oxide initially produced in response to agonist-induced calcium increase stimulate further calcium entry via *S*-nitrosylation, and the subsequent rise in nitric oxide production will eventually shut down further calcium entry. To date, however, there is no comprehensive study that would confirm this pathway in platelets.

Therefore, agents that raise the level of nitric oxide, either directly or by activation either of the endothelial nitric oxide synthase or guanylyl cyclase or by any other mechanism, should be capable of inhibiting SOCC. We have investigated the hypothesis that phytoestrogens were able to induce cGMP production or interfere with nitric oxide production. Data shown in the Fig. 6, when the nitric oxide donor sodium nitroprusside at 100 n*M* inhibited $[Ca^{2+}]_i$ elevation in thrombin-activated platelets, are consistent with other studies (43,44). ODQ, a specific inhibitor of guanylyl cyclase, reverses this inhibition caused by sodium nitroprusside. At this concentration, ODQ has a small effect to enhance thrombin-induced $[Ca^{2+}]_i$ elevation (Fig. 7), suggesting that nitric oxide/cGMP–dependent responses do not play a major role in thrombin-induced $[Ca^{2+}]_i$ increase in platelets. If phytoestrogens were able to inhibit $[Ca^{2+}]_i$ elevation by increasing the level of cGMP, then ODQ should abolish or alleviate this inhibition. Results represented in Fig. 7 show that ODQ does not interfere with inhibition of thrombin-induced calcium influx by 10 μ*M* of *trans*-resveratrol (similar results were obtained for 10 μ*M* genistein, not shown). Therefore it appears that genistein and *trans*-resveratrol are not inducing nitric oxide/cGMP generation.

Because *trans*-resveratrol and isoflavonoids are reported to inhibit free radical formation (45,46) a more likely site of action for these phytoestrogens would be scavenging nitric oxide. This possibility was also investigated, though nitric oxide scavenging would eventually lead to a potentiation rather than blocking the thrombin-induced calcium increase. The experimental data (Fig. 8) show that 1 μ*M* genistein does not prevent 100 n*M* so-

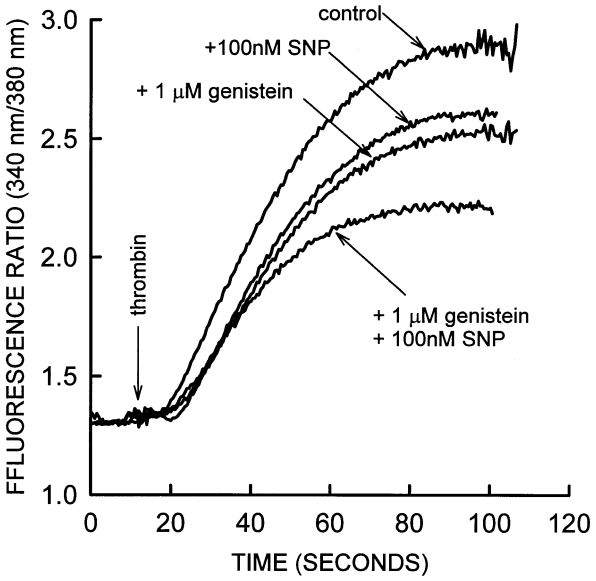

FIG. 8. Submaximally effective concentrations of sodium nitroprusside (SNP)- and genistein-mediated inhibition of thrombin-induced $[Ca^{2+}]_i$ increase are additive. Control trace represents $[Ca^{2+}]_i$ elevation caused by thrombin 0.01 U/ml in presence of 5 μl dimethyl sulfoxide blank. SNP 100 n*M* was added to the platelets 10 seconds before data collection was started. Thrombin was added at 15 seconds. SNP inhibited of thrombin-evoked $[Ca^{2+}]_i$ elevation. Genistein (1 μ*M* added 10 seconds before data collection) inhibited thrombin-evoked $[Ca^{2+}]_i$ elevation as previously shown. When 1 μ*M* genistein and 100 n*M* SNP were added together, the inhibition of thrombin-induced elevation of $[Ca^{2+}]_i$ was additive. One representative experiment is shown.

dium nitroprusside inhibition of thrombin-evoked $[Ca^{2+}]_i$ increase. In fact, the actions of sodium nitroprusside and genistein to inhibit thrombin-induced $[Ca^{2+}]_i$ increase were additive. Because genistein does not increase nitric oxide, ODQ does not inhibit the effect of genistein (data not shown), and it does not inhibit sodium nitroprusside–induced nitric oxide production (Fig. 8), genistein is acting independently of nitric oxide generation. Similar results were observed with 1 μ*M* of *trans*-resveratrol and 100 n*M* of sodium nitroprusside (Fig. 7). Therefore phytoestrogens do not block calcium responses through the modification of nitric oxide signal transduction cascades, but rather through another mechanism most likely involving direct calcium channel blockade.

Synthetic derivatives of *trans*-resveratrol and genistein possess lower inhibitory activity than *trans*-resveratrol and genistein

Certain synthetic derivatives of *trans*-resveratrol and genistein were shown to have significantly lower activity to inhibit thrombin-induced $[Ca^{2+}]_i$ elevation. In these derivatives, the free hydroxyl groups were converted into either an ether or an ester group. It appears that the presence of the phenolic hydroxyl groups is playing a role in a hydrogen bond formation at the target site. Thus, triacetylresveratrol is more active than the trimethoxy derivative, whereas daidzein and diacetyldaidzein have similar activities. In this instance possible hydrogen bond formation is facilitated in the presence of the ester oxygen compared with the ether oxygen.

The importance of the double bond, which restricts flexibility of the isoflavonoid ring system, was demonstrated in experiments with dihydrogenistein, which is more flexible. Isoflavonoids and flavonoids do not have a central ethylene bond, like *trans*-resveratrol, but rather a 1,2-benzopyrone moiety containing a double bond. This double bond places phenolic rings of isoflavonoids at a particular angle relative to each other (47). Dihydrogenistein had activity lower than that of genistein and the triacetylgenistein derivative. However, the activity was not completely lost, indicating that the ligand-binding pocket can still accommodate this more flexible pharmacophore.

It also seems that the binding pocket tolerates the presence of two oxygen atoms of 1,2-benzopyrone moiety of isoflavonoids quite well, because the activity of genistein and daidzein to block SOCC were similar. Phenolic rings of genistein are tilted relative one to another when cocrystallized with ERβ (48). Apigenin, which is a flavonoid and a positional isomer of genistein in regard to the 1,2-benzopyrone moiety, also inhibited $[Ca^{2+}]_i$ elevation in a similar manner (50 ± 5% for 10 μ*M* of genistein, 50 ± 8% for 10 μ*M* of apigenin) (19). Recently a new calcium inhibitor 2APB (2-aminoethoxydiphenyl borate) was described which effectively inhibited agonist-induced increases in $[Ca^{2+}]_i$ in several different cell lines. Its ability to inhibit agonist-induced increases in $[Ca^{2+}]_i$ in platelets has been attributed to inhibition of the internal release and also direct inhibition of SOCC channels (49). This widely used synthetic inhibitor has potency close to that of naturally occurring phytoestrogenic compounds. Thus, 10 μ*M* 2APB inhibited thrombin-induced $[Ca^{2+}]_i$ elevation by 47 ± 8%, which was close to the inhibition observed with 10 μ*M* phytoestrogenic compounds used in the present study.

Therefore, this study demonstrates that certain synthetic analogues of genistein, daidzein, and *trans*-resveratrol possess lower inhibitory activity to block thrombin-induced $[Ca^{2+}]_i$ elevation in human platelets than corresponding phytoestrogens. Action of genistein and *trans*-resveratrol to block thrombin-induced $[Ca^{2+}]_i$ elevation or Sr^{2+} influx was immediate. Genistein and daidzein demonstrated similar dose responses to inhibit thrombin-induced $[Ca^{2+}]_i$ elevation and thapsigargin-induced free elevation, which rules out the involvement of the tyrosine phosphorylation into the inhibitory mechanism. Inhibition of thrombin-induced $[Ca^{2+}]_i$ elevation by genistein and *trans*-resveratrol does not involve alterations in nitric oxide production. As was established earlier for *trans*-resveratrol (19), in the experiments with thapsigargin, genistein and daidzein inhibit thrombin-mediated Ca^{2+} influx through inhibition of SOCC. These findings may be helpful in elucidating how dietary phytoestrogens may modulate platelet functions and may help to explain how phytoestrogen-rich foods are claimed to have a beneficial effect on the cardiovascular system and identify a novel class of potential anti-platelet compounds.

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