

# Action of the natural compound esculetin on Ca<sup>2+</sup> movement and survival in prostate cancer cells

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## Research Article

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# Abstract

Esculetin is derived from coumarin and is shown to be the main constituent of the Chinese herb *Cortex Fraxini*. The molecular paths underlying the action of esculetin are intensively studied. The outcome of esculetin on  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) in prostate cells is unexplored. Fura-2 was used to detect  $\text{Ca}^{2+}$  changes. Death was assessed by using WST-1. At doses of 25-100  $\mu\text{M}$ , esculetin evoked  $[\text{Ca}^{2+}]_i$  raises. This signal was lessened by 15% by exclusion of  $\text{Ca}^{2+}$ . Esculetin (100  $\mu\text{M}$ ) induced  $\text{Mn}^{2+}$  entry that implied  $\text{Ca}^{2+}$  influx. Esculetin-evoked  $\text{Ca}^{2+}$  influx was curbed by 50% by nifedipine (1  $\text{mM}$ ), econazole (0.5  $\text{mM}$ ) and SKF96365 (5  $\text{mM}$ ); phorbol 12-myristate 13 acetate (PMA; 1  $\text{nM}$ ; a protein kinase C [PKC] activator); and GF109203X (2  $\text{mM}$ ; a PKC inhibitor. In the absence of  $\text{Ca}^{2+}$ , pretreatment with the endoplasmic reticulum (ER)  $\text{Ca}^{2+}$  pump inhibitor thapsigargin (1  $\text{mM}$ ) eradicated esculetin-induced  $[\text{Ca}^{2+}]_i$  raises. U73122, a phospholipase C (PLC) suppressor got rid of esculetin-caused  $[\text{Ca}^{2+}]_i$  rises. Esculetin (20-70  $\text{mM}$ ) evoked death which was not restrained by treatment with the  $\text{Ca}^{2+}$  binder BAPTA/AM. In summary, in PC3 cells, esculetin stimulated  $[\text{Ca}^{2+}]_i$  raises by  $\text{Ca}^{2+}$  influx through PKC-sensitive store-operated  $\text{Ca}^{2+}$  entry and PLC-associated ER  $\text{Ca}^{2+}$  discharging. Esculetin provoked  $\text{Ca}^{2+}$ -independent cell death.

## Background

Esculetin is derived from coumarin, and is the active constituent of the Chinese herb *Cortex Fraxini*. The study of paths underlying the action of esculetin has become intense<sup>1</sup>. Esculetin was shown to induce many cellular processes in many cell types<sup>2</sup>. Esculetin evoked apoptosis of hepatoma cells<sup>3</sup>, suppressed lung cancer<sup>4</sup>, and modulated viability in leukemia cells<sup>5</sup>.

Evidence shows that esculetin inhibited human gastric cancer<sup>6</sup>, restrained<sup>7</sup>, induced apoptosis in pancreatic cancer cells<sup>8</sup>, and evoked death of colon cancer cell<sup>9</sup>.

Thus far, only one study explored the action of esculetin on  $[\text{Ca}^{2+}]_i$  and viability in breast cancer cells<sup>10</sup>. Hence to study the action of esculetin on  $\text{Ca}^{2+}$  movement and the pertinent responses in other cells appears to be necessary.

Physiologically,  $\text{Ca}^{2+}$  is a unique ion. It is a key second envoy involved in triggering cellular events like enzyme activation, muscle contraction, fluid secretion, apoptosis, fertilization, etc.<sup>11</sup>. In cells, the cytosolic  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) is  $\sim 50$   $\text{nM}$  against external  $\text{Ca}^{2+}$  concentration of 2  $\text{mM}$ <sup>12</sup>. A  $\text{Ca}^{2+}$  stimulation is evoked by  $\text{Ca}^{2+}$  influx from extracellular solution or  $\text{Ca}^{2+}$  discharging from internal depots<sup>13</sup>. In most cells that lack voltage-gated  $\text{Ca}^{2+}$  channels, the store-operated  $\text{Ca}^{2+}$  influx has a major part in  $\text{Ca}^{2+}$  entry<sup>14</sup>. ER can discharge  $\text{Ca}^{2+}$  through phospholipase C (PLC)-coupled responses or repression of ER  $\text{Ca}^{2+}$  ATP pumps<sup>15</sup>.  $\text{Ca}^{2+}$  signaling intermingles with other cytosolic proteins like protein kinases<sup>16</sup>. The action of

esculetin on  $[Ca^{2+}]_i$  is unknown in prostate cells. Due to the important role of a  $Ca^{2+}$  signal, it is pivotal to study the paths of esculetin-provoked  $[Ca^{2+}]_i$  rises to understand the action of esculetin on prostate cells.

## Results

### The chemical structural formation of esculetin (Fig. 1).

#### Esculetin increased $[Ca^{2+}]_i$ .

The influence of esculetin on basal  $[Ca^{2+}]_i$  was explored. The basal  $[Ca^{2+}]_i$  was  $52 \pm 3$  nM. In the presence of  $Ca^{2+}$ , esculetin provoked  $[Ca^{2+}]_i$  raises in a dose-dependent fashion at 25-100 mM. Esculetin evoked  $[Ca^{2+}]_i$  raises that reached  $51 \pm 2$  nM at 100 mM. At 150 mM, esculetin provoked a response akin to that stimulated by 100 mM esculetin (not shown), therefore, the  $Ca^{2+}$  response attained saturation at 100 mM esculetin (Fig. 2(a)). Esculetin (25-100 mM) provoked dose-associated raises in  $[Ca^{2+}]_i$  in  $Ca^{2+}$ -free medium (Fig. 2(b)). Fig. 2(c) depicted the dose-signal relationships of esculetin-evoked  $[Ca^{2+}]_i$  elevations. The  $EC_{50}$  was  $63 \pm 2$  mM in  $Ca^{2+}$ -containing medium or  $42 \pm 1$  mM in  $Ca^{2+}$ -free medium. The numbers were acquired by using Hill equations. Esculetin-provoked  $[Ca^{2+}]_i$  rises were decreased by 15% in  $Ca^{2+}$ -free medium.

#### Esculetin induced $Mn^{2+}$ influx.

The following set of assays were performed to detect if  $Ca^{2+}$  entry participated in esculetin-caused  $[Ca^{2+}]_i$  raises.  $Mn^{2+}$  and  $Ca^{2+}$  are structurally similar ions, and can enter cells via similar pathways, but  $Mn^{2+}$  quenches fluorescence at all excitation wavelengths<sup>18</sup>. Therefore, quenching of fura-2 fluorescence excited at the  $Ca^{2+}$ -insensitive excitation wavelength of 360 nm by  $Mn^{2+}$  indirectly implicated  $Ca^{2+}$  influx. Since esculetin-elicited  $Ca^{2+}$  response attained saturation at 100  $\mu$ M, in the next analyses the  $Ca^{2+}$  response elicited by 100 mM esculetin was applied as control. Esculetin induced an instantaneous decrease in the 360 nm excitation signal that reached a maximal value of  $120 \pm 2$  arbitrary units at 75 s (Fig. 3). Data suggested  $Ca^{2+}$  influx participated in esculetin-elicited  $Ca^{2+}$  responses.

#### The pathways of esculetin-caused $Ca^{2+}$ influx.

Assays were performed to unveil the  $Ca^{2+}$  influx mechanisms of esculetin-evoked  $[Ca^{2+}]_i$  raises. Phorbol 12-myristate 13 acetate (PMA; 1 nM; a protein kinase C [PKC] activator) and GF109203X (2 mM; a PKC inhibitor); nifedipine (1 mM) and the store-operated  $Ca^{2+}$  influx suppressors: econazole (0.5 mM) and

SKF96365 (5 mM) were given 1 min before 100 mM esculetin. Results suggest PMA, GF109203X, nifedipine, econazole or SKF96365 decreased esculetin-elicited  $[Ca^{2+}]_i$  rises by 50% ( $p < 0.05$ ) (Fig. 4).

## Depots of esculetin-caused $Ca^{2+}$ discharging.

ER is established to be the main  $Ca^{2+}$  depository<sup>19</sup> in cells. Thus the role ER in esculetin-caused  $Ca^{2+}$  discharging in PC3 cells was investigated. Analyses were performed in the absence of  $Ca^{2+}$  to prevent interference of  $Ca^{2+}$  entry. Administration of 1 mM thapsigargin<sup>20</sup>, an ER  $Ca^{2+}$  pump suppressor, elicited  $[Ca^{2+}]_i$  raises of  $26 \pm 3$  nM (Fig. 5a). Administration of esculetin (100 mM) at 500 s did not induce  $[Ca^{2+}]_i$  elevations. On the contrary, administration of 1 mM thapsigargin at 500 s still evoked  $[Ca^{2+}]_i$  elevations with an extent comparable to control of 100 mM esculetin-induced  $[Ca^{2+}]_i$  rises (Fig. 5b).

## Participation of phospholipase C (PLC) in esculetin-caused $[Ca^{2+}]_i$ elevations.

PLC is known to be an important intracellular enzyme that regulates the discharging of  $Ca^{2+}$  from  $Ca^{2+}$  depository. Because esculetin discharged  $Ca^{2+}$  from ER, the part of PLC in this process was explored. The PLC suppressor U73122<sup>20</sup> was applied to investigate whether U73122 had a role in esculetin-evoked  $Ca^{2+}$  discharging<sup>21-23</sup>. ATP (10 mM) evoked  $[Ca^{2+}]_i$  elevations of  $47 \pm 3$  nM at 200 s (Fig. 6a). ATP is a PLC-dependent trigger of  $[Ca^{2+}]_i$  elevations<sup>24</sup>, and thus was applied to explore if U73122 inhibited PLC. Pretreatment with 2 mM U73122 fell short to influence basal  $[Ca^{2+}]_i$ , however extinguished ATP-elicited  $[Ca^{2+}]_i$  elevations (Fig. 6b). These data implied U73122 efficiently suppressed PLC. The data also suggested 2 mM U73122 did not affect basal  $[Ca^{2+}]_i$ , nevertheless extinguished 100 mM esculetin-caused  $[Ca^{2+}]_i$  rises ( $P < 0.05$ ). An inert structural analogue (U73343 (2 mM)) of U73122, did not decrease ATP-caused  $[Ca^{2+}]_i$  raises (not shown).

## Esculetin decreased viability dose-dependently.

In the company of different doses of esculetin, WST-1 analyses were applied to analyze cell death. Esculetin (0-70 mM) was added to cells overnight, results depicted viability was decreased concentration-dependently between 20-70 mM (Fig. 7). Subsequently, the question if esculetin-evoked death was due to  $[Ca^{2+}]_i$  raises was explored. The  $Ca^{2+}$  binder BAPTA/AM (5 mM)<sup>25</sup> was used to curb  $[Ca^{2+}]_i$  rises during treatment with esculetin. Esculetin (100 mM) failed to cause  $[Ca^{2+}]_i$  rises in BAPTA/AM-incubated cells (not shown). Fig. 7 shows 5 mM BAPTA/AM incubation failed to affect control cell viability. BAPTA/AM pretreatment failed to influence esculetin-induced death in the presence of 20-70 mM esculetin.

## Discussion

$\text{Ca}^{2+}$  homeostasis regulates numerous aspects of cellular physiology<sup>26</sup>. The action of esculetin on  $\text{Ca}^{2+}$  signaling in PC3 cells is unclear. Esculetin brought on  $[\text{Ca}^{2+}]_i$  elevations in a dose-dependent fashion at 25-100 mM. Results implied esculetin evoked  $[\text{Ca}^{2+}]_i$  increases by discharging  $\text{Ca}^{2+}$  from depots and provoking  $\text{Ca}^{2+}$  entry because removing  $\text{Ca}^{2+}$  decreased 100 mM esculetin-evoked  $[\text{Ca}^{2+}]_i$  raises by 15%. The data suggest  $\text{Ca}^{2+}$  entry and  $\text{Ca}^{2+}$  discharging occurred during 220 s of assays. The data also show  $\text{Ca}^{2+}$  entry included store-operated  $\text{Ca}^{2+}$  channels<sup>27-29</sup>, because esculetin-caused  $[\text{Ca}^{2+}]_i$  raises were eliminated by nifedipine, econazole and SKF96365. These compounds are used as suppressor of the  $\text{Ca}^{2+}$  entry<sup>27-29</sup>.

Our data show PKC regulators, nifedipine, SKF96365, and econazole suppressed half of the signal-caused  $\text{Ca}^{2+}$  response by 100 mM esculetin. This indicated the reagents eradicated esculetin-induced  $\text{Ca}^{2+}$  entry and also suppressed some portion of esculetin-induced  $\text{Ca}^{2+}$  discharging from ER. Kinases are known to interact with  $\text{Ca}^{2+}$  handling<sup>30</sup>. Consistently, in PC3 cells, previous data show stimuli-evoked  $\text{Ca}^{2+}$  entry included PKC-regulated store-operated  $\text{Ca}^{2+}$  entry<sup>31,32</sup>.

Concerning  $\text{Ca}^{2+}$  depots that were involved in esculetin-evoked  $\text{Ca}^{2+}$  discharging, the thapsigargin-sensitive ER stores appear to be dominant. As esculetin failed to influence thapsigargin-induced  $\text{Ca}^{2+}$  discharging, it seems that even though esculetin and thapsigargin discharged  $\text{Ca}^{2+}$  from ER, the thapsigargin-sensitive  $\text{Ca}^{2+}$  depot is greater than that sensitive to esculetin. Results depicted  $\text{Ca}^{2+}$  discharging was via a PLC-associated path, since the discharging was eradicated when PLC was suppressed. Therefore the findings implicated esculetin elicited  $\text{Ca}^{2+}$  discharging from ER in a PLC-associated fashion. Our findings suggest esculetin caused death in PC3 cells at doses similar to that induced  $[\text{Ca}^{2+}]_i$  rises.

In literature, it was reported that 75 mM esculetin induced death by 6.25% in PC3 cells pretreated with WST-1 for 48 h. In contrary, the present results suggest that treatment of 70 mM esculetin overnight killed 95% of cells<sup>7</sup>. Therefore it seems that the discrepancy was caused by different methodology. The harmful action of esculetin on PC3 cells is not consistent in different reports. Viability was reported to be unchanged by esculetin<sup>33</sup>.

Of note, in  $[\text{Ca}^{2+}]_i$  assays esculetin at 25-100 mM did not harm cells, but in cytotoxicity assays, 20-70 mM esculetin wiped out cells in a concentration-associated fashion. This may be because  $[\text{Ca}^{2+}]_i$  experiments were stopped within 20 min, and cytotoxicity assays were performed after overnight pretreatment for WST-1 to have an effect. The cytotoxic effect of esculetin seemed to be dissociated of  $[\text{Ca}^{2+}]_i$  elevations as BAPTA/AM incubation stalled 100 mM esculetin-caused  $[\text{Ca}^{2+}]_i$  rises without reversing cytotoxicity<sup>11</sup>. Cytotoxic action may be  $\text{Ca}^{2+}$ -associated or -dissociated<sup>34</sup>. In PC3 cells, Chang et al<sup>10</sup> reported the natural

product resveratrol induced cell proliferation  $\text{Ca}^{2+}$ -dependently. In the contrary, diindolylmethane was shown to induce cell death  $\text{Ca}^{2+}$ -dissociatedly<sup>32</sup>.

Together, esculetin evoked  $\text{Ca}^{2+}$  influx through PKC-modulated store-operated  $\text{Ca}^{2+}$  influx and  $\text{Ca}^{2+}$  discharging from ER PLC-dependently in PC3 cells. Esculetin elicited  $\text{Ca}^{2+}$ -independent death. The action of esculetin on  $\text{Ca}^{2+}$  homeostasis and viability in other cells deserves exploration.

## Methods

### Reagents.

The chemicals used for cell culture were acquired from Gibco<sup>®</sup> (Gaithersburg, MD, USA). Aminopolycarboxylic acid/acetoxymethyl (fura-2/AM) and 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid/acetoxymethyl (BAPTA/AM) were purchased from Molecular Probes<sup>®</sup> (Eugene, OR, USA). All other chemicals were purchased from Sigma-Aldrich<sup>®</sup> (St. Louis, MO, USA).

### Cell culture.

PC3 human prostate cancer cells were purchased from Bioresource Collection and Research Center (Taiwan). Cells were cultured in RPMI-1640 medium. The medium had penicillin (100 units/mL)-streptomycin (100  $\mu\text{g}/\text{mL}$ ) and fetal bovine serum (10%) kept at 37°C in a humidified 5%  $\text{CO}_2$  atmosphere.

### Solutions used in $[\text{Ca}^{2+}]_i$ measurements.

$\text{Ca}^{2+}$ -containing medium (pH 7.4) contained 140 mM NaCl, 5 mM KCl, 1 mM  $\text{MgCl}_2$ , 2 mM  $\text{CaCl}_2$ , 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 5 mM glucose.  $\text{Ca}^{2+}$ -free medium contained similar chemicals as  $\text{Ca}^{2+}$ -containing medium except that  $\text{CaCl}_2$  was replaced with 0.3 mM *ethylene glycol tetraacetic acid* (EGTA) and 2 mM  $\text{MgCl}_2$ . Esculetin was dissolved in ethanol as a 0.1 M stock solution. The other chemicals were dissolved in water, ethanol or dimethyl sulfoxide (DMSO). The concentration of solvents in the experimental solutions did not exceed 0.1%, and did not affect viability or basal  $[\text{Ca}^{2+}]_i$ .

### $[\text{Ca}^{2+}]_i$ analyses.

Confluent cells grown on 6 cm dishes were trypsinized and made into a suspension in culture medium at a concentration of  $10^6 \text{mL}^{-1}$ . Trypan blue exclusion was used to determine cell viability. After the treatment,

the viability was greater than 95%. Then cells were loaded with 2 mM fura-2/AM for 30 min at 25°C in the same medium. Afterwards, cells were washed with Ca<sup>2+</sup>-containing medium twice and were made into a suspension in Ca<sup>2+</sup>-containing medium at a concentration of 10<sup>7</sup>mL<sup>-1</sup>. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette had 1 mL of medium and 0.5 million cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer immediately after 0.1 mL cell suspension was added to 0.9 mL Ca<sup>2+</sup>-containing or Ca<sup>2+</sup>-free medium, by recording excitation signals at 340 nm and 380 nm and emission signal at 510 nm at 1-s intervals. During the recording, reagents (PMA (1 nM); GF109203X (2 mM); econazole (0.5 mM), nifedipine (1 mM), SKF96365 (5 mM), thapsigargin (1 mM), U73122 (2 mM), or ATP (4 mM)) were administered to the cuvette by pausing the recording for 2 s to open and close the cuvette. After completion of the experiments, the detergent Triton X-100 (0.1%) and CaCl<sub>2</sub> (5 mM) were added to the cuvette to obtain the maximal fura-2 fluorescence; then the Ca<sup>2+</sup> chelator EGTA (10 mM) was added to chelate Ca<sup>2+</sup> in the cuvette to obtain the minimal fura-2 fluorescence for calibration of [Ca<sup>2+</sup>]<sub>i</sub> after 20 min of fluorescence measurements. Control experiments showed that cells bathed in a cuvette had a viability of 95%. [Ca<sup>2+</sup>]<sub>i</sub> was calculated as described previously<sup>17</sup>. Mn<sup>2+</sup> smothering of fura-2 fluorescence was performed in Ca<sup>2+</sup>-containing medium containing 50 mM MnCl<sub>2</sub>. MnCl<sub>2</sub> was added to cell suspension in the cuvette 30 s before the fluorescence recording was started. Data were recorded at excitation signal at 360 nm (Ca<sup>2+</sup>-insensitive) and emission signal at 510 nm at 1-s intervals as described previously<sup>18</sup>.

## Cell viability assays.

Cell viability measurements were based on the ability of cells to cleave tetrazolium salts by dehydrogenases. Increases in the intensity of color correlated with the number of live cells. Assays were conducted based on manufacturer's instructions (Roche Molecular Biochemical, Indianapolis, IN, USA). Cells were seeded in 96-well plates at a concentration of 10<sup>4</sup> cells/well in culture medium for 24 h in the presence of 0-70 mM esculetin. The cell viability detecting tetrazolium reagent 4-[3-[4-Iodophenyl]-2-(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] (WST-1; 10 mL pure solution) was added to samples after esculetin treatment, and cells were incubated for 30 min in a humidified atmosphere. In experiments using BAPTA/AM to chelate cytosolic Ca<sup>2+</sup>, cells were treated with 5 mM BAPTA/AM for 1 h before esculetin (0-70 mM) incubation. The cells were washed once with Ca<sup>2+</sup>-containing medium and incubated with or without esculetin for 24 h. The absorbance of samples (A<sub>450</sub>) was analyzed using an enzyme-linked immunosorbent assay (ELISA) reader. Absolute optical density was normalized to the absorbance of unstimulated cells in each plate and expressed as a percentage of the control value.

## Statistics.

Data are reported as mean ± SEM (standard error of the mean) of three separate assays and were analyzed by one-way analysis of variances (ANOVA) using the Statistical Analysis System (SAS<sup>®</sup>, SAS

Institute Inc., Cary, NC, USA). Multiple comparisons between group means were performed by *post-hoc* analysis using the Tukey's HSD (honestly significantly difference) procedure. A p-value less than 0.05 were considered significant.

## Declarations

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## Author contributions

JLW, WCL designed the protocol of study and performed; RAL, SHC, CCK were response for collecting data; LJH and CTC wrote manuscript and conducted statistics analyses; CTC and CRJ reviewed the manuscript and responsible for submitting the manuscript.

## Competing interests

We declared no conflicts of interests.

## Data availability

All data are available upon request.

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## Figures

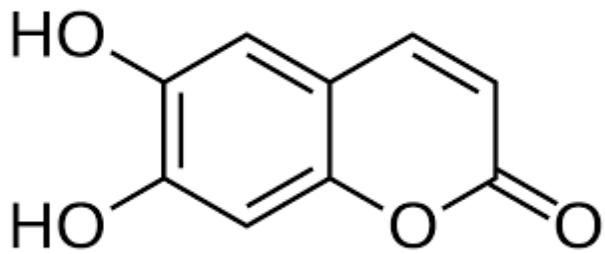


Figure 1

Chemical structure of esculetin.

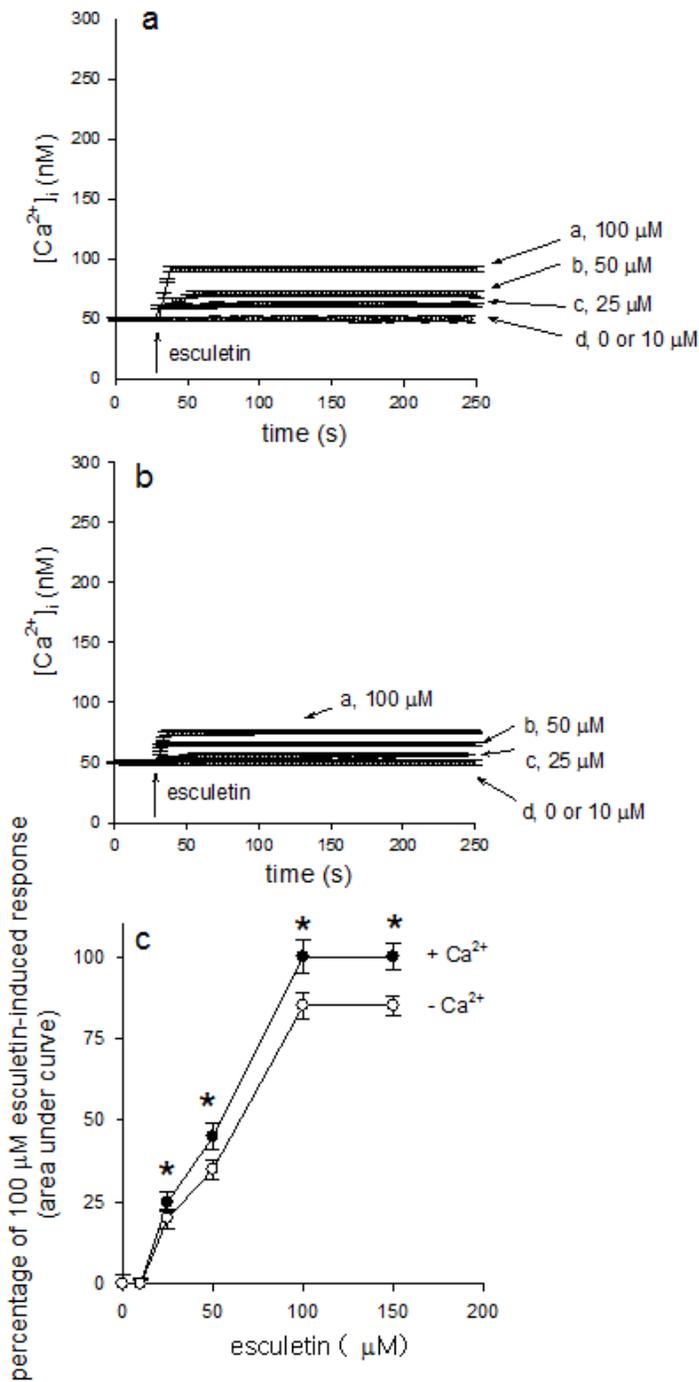


fig 2

## Figure 2

Action of esculetin on Ca<sup>2+</sup> signaling in cells treated with fura-2. (A) Esculetin- provoked [Ca<sup>2+</sup>]<sub>i</sub> raises . Esculetin was administrated at 25 s. The dose of esculetin was shown. The assays were performed Ca<sup>2+</sup>-containing medium. (B) Action of esculetin on [Ca<sup>2+</sup>]<sub>i</sub> in Ca<sup>2+</sup>-free medium. Esculetin was administrated at 25 s in the absence of Ca<sup>2+</sup>. (C) Dose-signal relationships of esculetin-provoked [Ca<sup>2+</sup>]<sub>i</sub> raises. Y axis is the percentage of the area under the recording (25-250 s) of the [Ca<sup>2+</sup>]<sub>i</sub> raises provoked by 100 μM

esculetin in the presence of  $\text{Ca}^{2+}$  (control). Results were mean  $\pm$  standard deviation from three unrelated assays. \* $P < 0.05$  in comparison to filled circles.

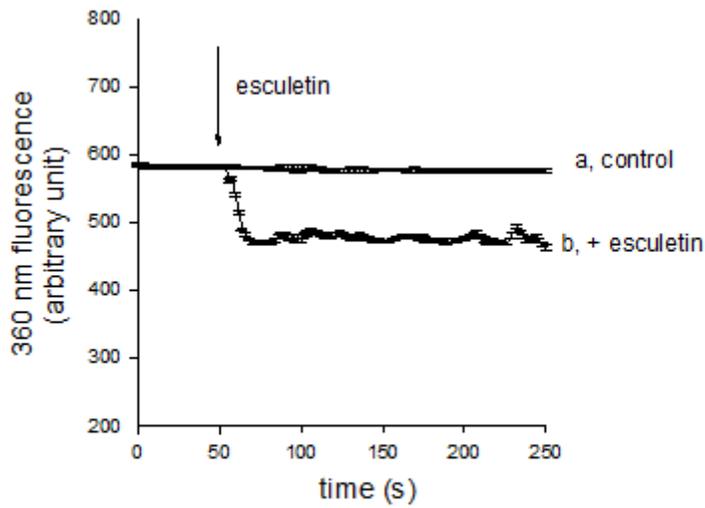


fig 3

### Figure 3

Action of esculetin on  $\text{Ca}^{2+}$  entry by detecting  $\text{Mn}^{2+}$  smothering of Fura-2 fluorescence. Assays were conducted in the presence of  $\text{Ca}^{2+}$ .  $\text{MnCl}_2$  ( $50 \mu\text{M}$ ) was administrated to cells 1 min prior to assays. The y axis is fluorescence intensity (in random units) recorded at the  $\text{Ca}^{2+}$ -insensitive excitation wavelength of 360 nm and the emission wavelength of 510 nm. Trace a: no esculetin was present. Trace b: esculetin ( $100 \mu\text{M}$ ) was present. Results were mean  $\pm$  standard deviation from three unrelated assays.

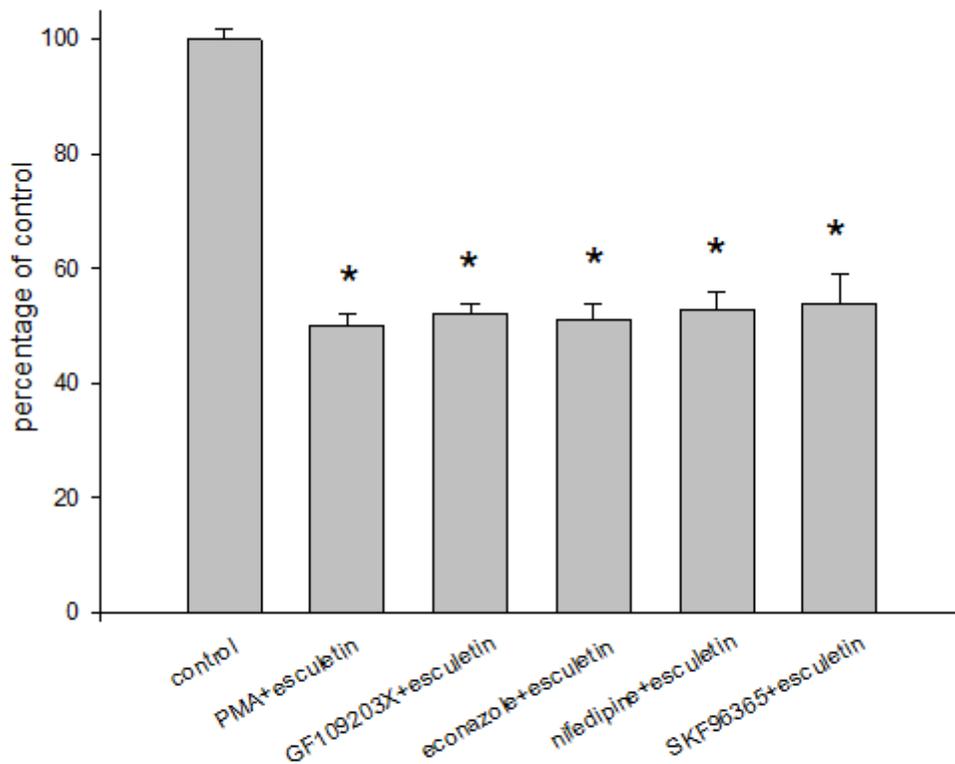


fig 4

## Figure 4

Action of  $Ca^{2+}$  entry controllers on esculetin-provoked  $[Ca^{2+}]_i$  raises. In regulators-pretreated groups, the compound was administrated 1 min prior to esculetin (100  $\mu$ M). The dose was 10 nM for phorbol 12-myristate 13-acetate (PMA), 2  $\mu$ M for GF109203X, 1  $\mu$ M for nifedipine, 0.5  $\mu$ M for econazole, 5  $\mu$ M for SKF96365. Results are presented as the percentage of control (1st column) that is the area under the recording (25-200 s) of 100  $\mu$ M esculetin-provoked  $[Ca^{2+}]_i$  raises. Results were mean  $\pm$  standard deviation from three unrelated assays. \* $P < 0.05$  in comparison to 1st column.

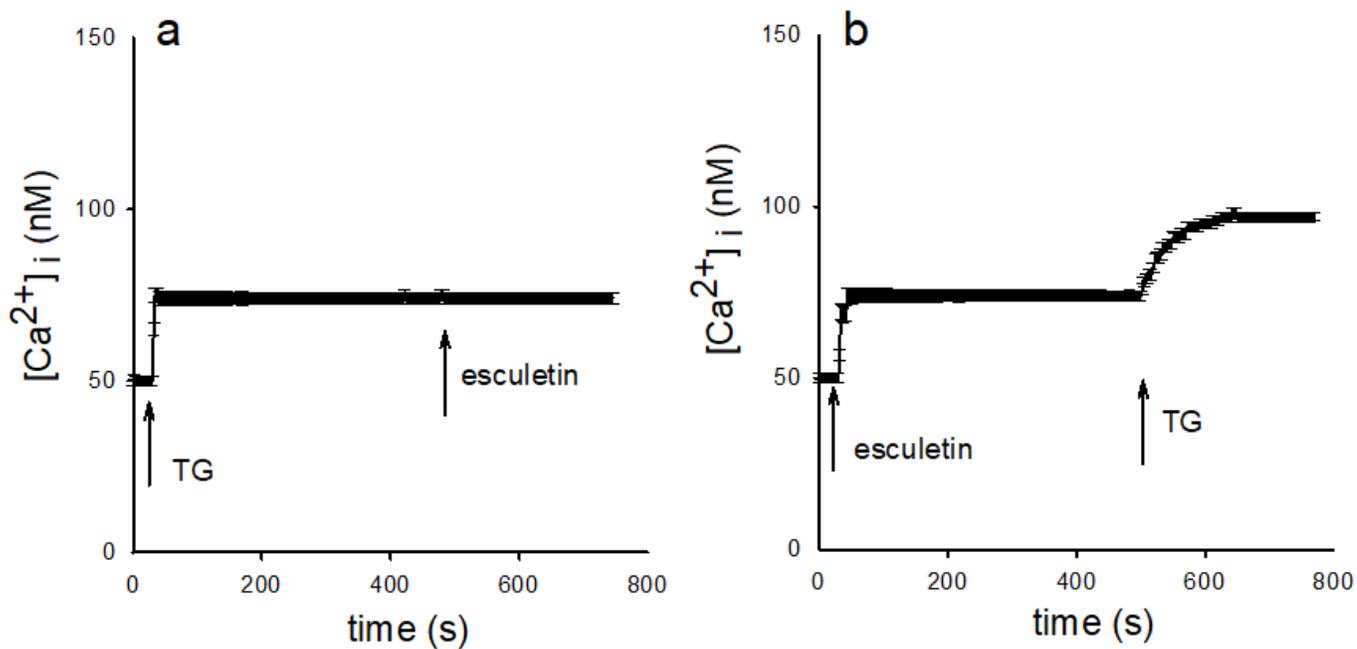


fig 5

### Figure 5

Action of thapsigargin on esculetin-provoked  $Ca^{2+}$  discharging. (A)(B) Thapsigargin (TG; 1  $\mu$ M) and esculetin (100  $\mu$ M) were administrated as indicated. Assays were performed in the absence of  $Ca^{2+}$ . Results were mean  $\pm$  standard deviation of three unrelated assays.

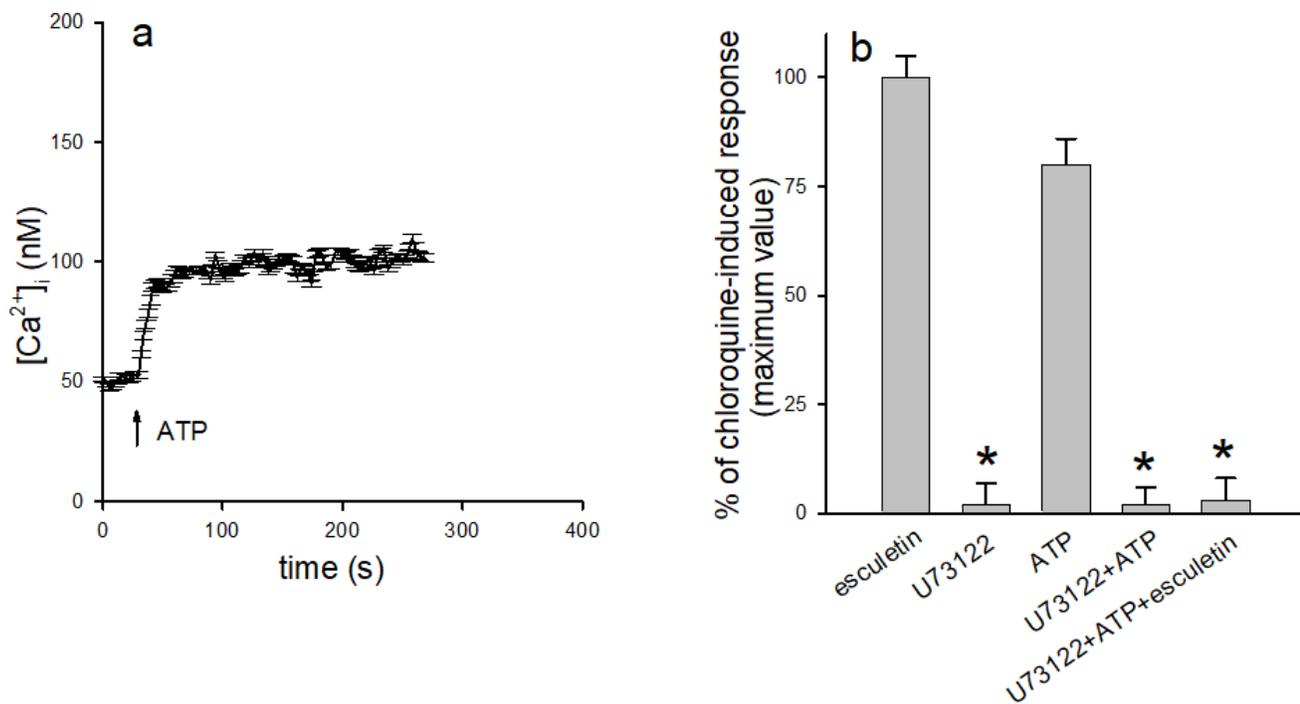
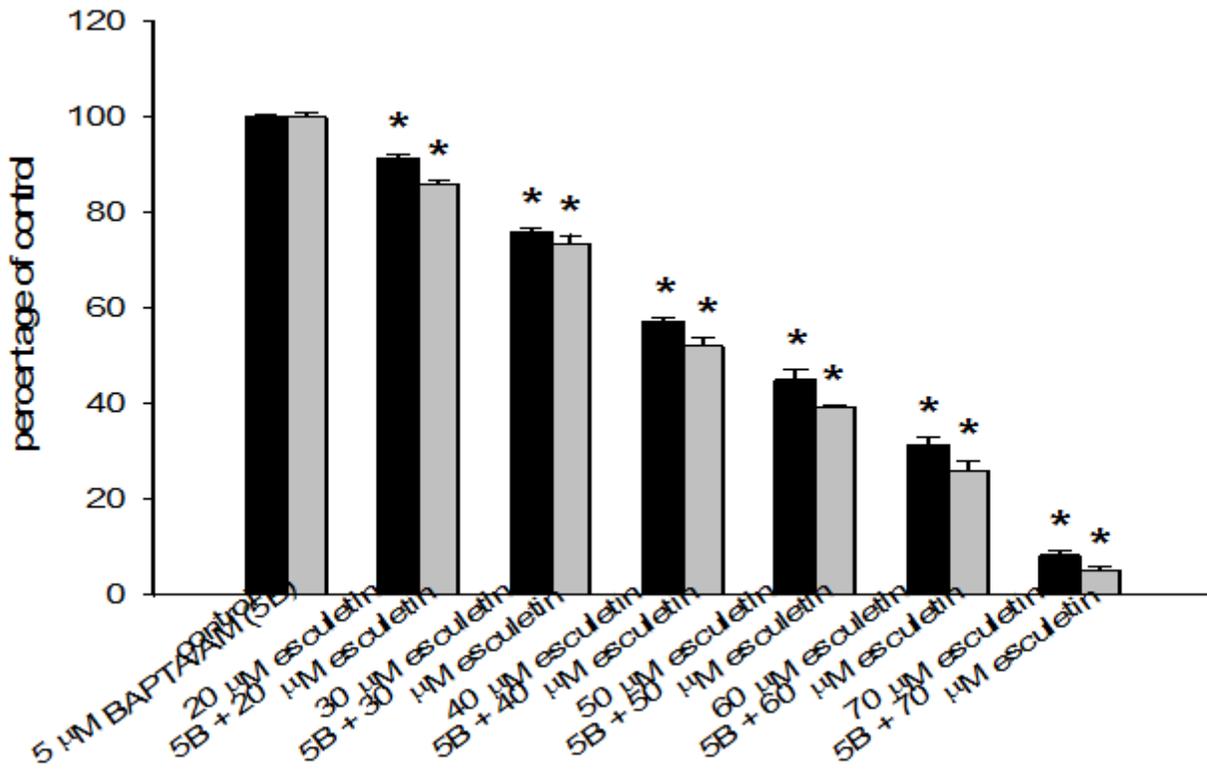


fig 6

### Figure 6

Action of U73122 on esculetin-provoked Ca<sup>2+</sup> discharging. Assays were conducted in the absence of Ca<sup>2+</sup>. (A) ATP (10 μM) was administered as shown. (B) 1st column is 100 μM esculetin-provoked [Ca<sup>2+</sup>]<sub>i</sub> raises. 2nd column depicts that 2 μM U73122 failed to change resting [Ca<sup>2+</sup>]<sub>i</sub>. 3rd column illustrated ATP-caused [Ca<sup>2+</sup>]<sub>i</sub> raises. 4th column confirms that U73122 treatment for 1 min totally suppressed ATP-provoked [Ca<sup>2+</sup>]<sub>i</sub> raises (\*P < 0.05 in comparison to 3rd column). 5th column depicts that U73122 (treatment for 1 min) and ATP (treatment for 30 s) treatment suppressed 100 μM esculetin-provoked [Ca<sup>2+</sup>]<sub>i</sub> raises. Results were mean ± standard deviation of three unrelated assays. \*P < 0.05 in comparison to 1st bar (control). Control is the area under the recording of 100 μM esculetin-provoked [Ca<sup>2+</sup>]<sub>i</sub> raises (25-220 s).



**Figure 7**

Action of esculentin on cytotoxicity. Cells were pretreated with 0-70  $\mu\text{M}$  esculentin overnight, and WST-1 assays were conducted. Results are presented as percentage of control that is the increase in cell numbers in esculentin-free groups. Control had  $10,811 \pm 180$  cells/well before assays, and had  $13,488 \pm 155$  cells/well after incubation overnight. In each group, the  $\text{Ca}^{2+}$  binding agent BAPTA/AM ( $5 \mu\text{M}$ ) was administrated to cells prior to pretreatment with esculentin in the presence of  $\text{Ca}^{2+}$ . Viability analyses were then conducted. Results are mean  $\pm$  standard deviation of three unrelated assays. \* $P < 0.05$  in comparison to control.