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Genistein inhibits contractile force, intracellular Ca^{2+} increase and Ca^{2+} oscillations induced by serotonin in rat aortic smooth muscle

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The soy-derived isoflavones genistein and daidzein affect the contractile state of different kinds of smooth muscle. We describe acute effects of genistein and daidzein on contractile force and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in *in situ* smooth muscle of rat aorta. Serotonin (5-HT) (2 µM) or a depolarizing high K^+ solution produced the contraction of aortic rings, which were immediately relaxed by 20 µM genistein and by 20 µM daidzein. Accordingly, both 5-HT and a high K^+ solution increased the $[Ca^{2+}]_i$ in in situ smooth muscle cells. Genistein strongly inhibited the $[Ca^{2+}]_i$ increase evoked by 5-HT (74.0 ± 7.3%, n=11, $p<0.05$), and had a smaller effect on high K^+ induced $[Ca^{2+}]_i$ increase (19.9 ± 4.0%, n=7, $p<0.05$). The K^+ channels blocker tetraethylammonium (TEA) (0.5 mM) diminished genistein effects on 5-HT-induced $[Ca^{2+}]_i$ increase. Interestingly, during prolonged application of 5-HT, the $[Ca^{2+}]_i$ oscillated and a short (90 s) preincubation with genistein (20 µM) significantly diminished the frequency of the oscillations. This effect was totally abolished by TEA. In conclusion, in rat aortic smooth muscle, genistein is capable of diminishing the increase in $[Ca^{2+}]_i$ and in force evoked by 5-HT and high K^+ solution, and of decreasing the frequency of $[Ca^{2+}]_i$ oscillations induced by 5-HT. The short time required by genistein, and the relaxing effect of daidzein suggest that tyrosine kinases inhibition is not involved. The small inhibiting effect of genistein on the $[Ca^{2+}]_i$ increase evoked by high K^+ and the effect of TEA point to the activation by genistein of calcium-activated K^+ channels.

Key words: Genistein, Rat aorta, Serotonin, Intracellular Ca^{2+}.

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Genistein and daidzein are two isoflavones present in soy-derived products which have been shown to posses both estrogenic activity (they are classified like selective estrogen receptor modulators) and antioxidant activity (5, 13). Genistein is also a well-known tyrosine kinase inhibitor, while its structural analog daidzein is not (1, 2). The vasoactive effects of these isoflavones have been tested in different kinds of smooth muscles (6, 14), showing that they are able to regulate the contractile state of these cells through different mechanisms of action. For instance, in rat mesenteric artery genistein and daidzein are able to induce a vasorelaxant effect, probably mediated by the activation of BK_{Ca} channels (10). The voltage-operated Ca^{2+} channels are also targets for these substances, since in patch-clamp studies in rat portal vein, genistein has been shown to decrease Ca^{2+} currents through these channels both in the whole-cell configuration (8) and at the single channel level (7).

Serotonin (5-HT) is an important vasoactive agent. SAINI *et al*. (12) demonstrated that the contraction induced by 5-HT in rat aorta is mediated by the $5\text{-}HT_{2a}$ receptor, which produces an intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increase due to both extracellular and intracellular sources of Ca^{2+}. The purpose of this work was to study genistein effects on 5-HT-induced contraction in rat aortic rings using $[Ca^{2+}]_i$ measurements in *in situ* smooth muscle cells. Moreover, we focused our investigation on acute effects of genistein, likely independent of its tyrosine kinase inhibitor effect, and in particular we explored genistein effects mediated by K^+ channels activation. Aortic ring contraction strength was also evaluated.

Material and Methods

Isometric tension measurements.– Male Wistar rats (45-60 days old) were anesthetized with ethyl ether and the thoracic aorta was rapidly removed and placed in a modified Krebs solution (KS) continuously bubbled with a mixture of 5% CO_2 and 95% O_2. The KS composition was in mM: 130 NaCl, 4.7 KCl, 24 $NaCO_3H$, 1.17 Na_2PO_4H, 1.16 $MgSO_4$, 1.6 $CaCl_2$ and 11 glucose. Adherent connective tissue was removed carefully and rings 2-3 mm long were cut. The endothelium was carefully removed by gentle rubbing the intima with a stainless steel wire. Each ring was isometrically attached to a force transducer (Letica TRI-201) whose output signal was amplified and recorded using a data acquisition software (Labtech Notebook Pro, Laboratory Technology Corp.) and stored for further analysis. The preparations were placed in a 20 ml plastic chamber filled with KS continously bubbled with 5% CO_2 and 95% O_2, and thermostatized at 37 °C. A passive tension of 2 g was applied, and the rings were allowed to stabilize for 1 hour, renewing the solution in the chamber every 20 minutes. For some experiments, a high K^+ KS was used, prepared by elevating KCl in the KS to 80 mM and accordingly reducing NaCl in order to preserve the osmolarity.

Measurement of $[Ca^{2+}]_i$ by microfluorimetry in rat aortic smooth muscle cells.– Rat aorta strips were incubated for 150 min at room temperature in a physiological saline solution (PSS) of the following composition (in mM): 150 NaCl, 6 KCl, 1 $MgCl_2$, 1.6 $CaCl_2$, 10 HEPES and 10 glucose, containing 16 µM fura-2 AM (1 mM fura-2 AM in DMSO stock solution). They were afterwards washed with PSS without fura-2 AM and fixed to the

bottom of a Petri dish. In situ smooth muscle cells were visualized by an upright epifluorescence microscope (Zeiss, Axiolab) equipped with a 100-W Hg lamp and a Zeiss 63 x Achroplan water immersion objective (0.75 numerical aperture). The cells were excited alternately at 340 or 380 nm and the emitted light was detected at 510 nm. A neutral density filter (0.3 optical density) reduced the overall intensity of the exciting light and a second neutral density filter (optical density = 0.3) was coupled to the 380 nm filter to approach the intensity of the 340 nm light. The exciting filters were mounted on a filter wheel (Lambda 10, Sutter Instrument, Novato, Calif., USA). Custom software, working in the LINUX environment, was used to drive the camera (Extended-ISIS Camera, Photonic Science, Millham, UK), the filter wheel, and to measure and plot on-line the fluorescence from about 5 rectangular regions of interest (ROIs), which enclosed 1 to 5 single cells each. Variations in the $[Ca^{2+}]_i$ were monitored by evaluating for each ROI the ratio of the fluorescence signal emitted at 510 nm when exciting at 340 and 380 nm. Experiments were carried out at room temperature (20–23 °C), and ratio measurements were performed every 2 seconds.

For some experiments a high K^+ PSS was prepared by increasing KCl in the PSS up to 80 mM and reducing NaCl accordingly in order to preserve osmolarity.

Statistics.– The data are expressed as mean ± 1 standard error. Differences between means were evaluated with the Student's *t* test for paired or unpaired samples, and ANOVA followed by an appropriate test in the case of multiple comparisons. Signification was accepted when $p < 0.05$.

Reagents.– 5-HT, genistein, daidzein, and tetraethylammonium (TEA) were purchased from Sigma Chemical Company (St Louis, MS, USA). Fura-2-AM was obtained from Molecular Probes (Eugene, OR, USA). All other drugs were purchased from local vendors and were of analytical grade.

Results

Genistein effects on 5-HT and high K^+ induced contractions.– Fig. 1A shows the recordings obtained in a typical mechanical experiment, where rat aortic rings were contracted by the vasoactive agonist 5-HT, producing a maximal force of 48.8 ± 4.6 gF/gW (n=32). Genistein or daidzein were applied 8 min after the beginning of the contraction, and they both quickly relaxed the rings (Fig. 1A and 1B, relaxation measured 10 min after the addition of genistein or daidzein).

A set of aortic rings were contracted by depolarization with a high K^+ KS (80 mM KCl) in order to study if genistein and daidzein were able to relax contractions induced by a mechanism not involving the binding of a vasoconstrictor to its membrane receptor, and also because the relaxant effects induced by K^+ channels activation are blunted in this case by the rise in extracellular K^+ concentration. The force evoked by the high K^+ KS was greater than that produced by 5-HT (293.9 ± 30.5 gF/gW, n=29, $p<0.001$ *vs.* 5-HT). Although genistein and daidzein relaxed the high K^+-induced contractions (Fig. 1C and 1D), the relaxing effect was significantly lower than that on the 5-HT-induced contractions (compare Fig. 1B and 1D).

Dimethylsulfoxide (DMSO), the vehicle of genistein and daidzein solution, added 8 min after 5-HT or high K^+ KS,

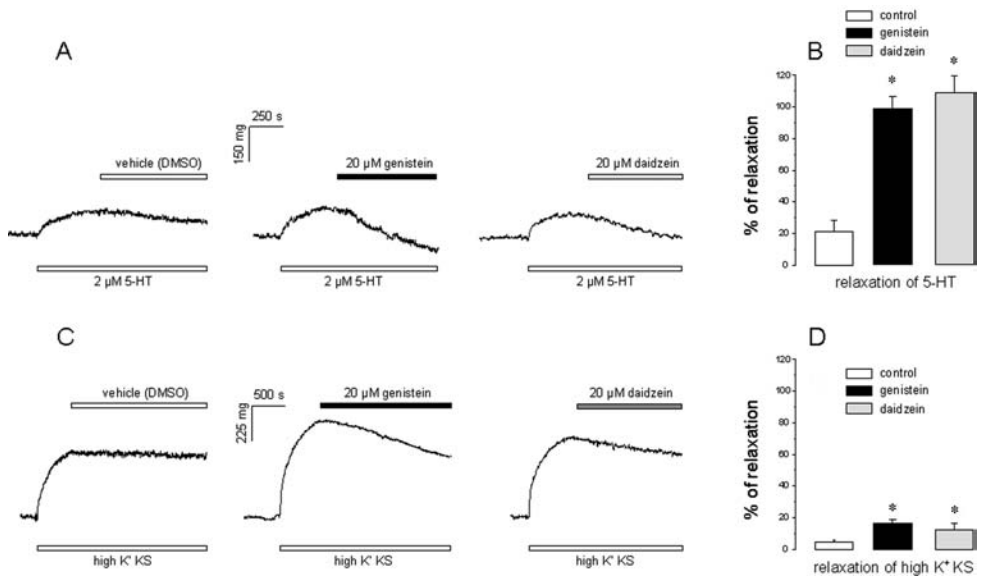

Fig. 1. A: Typical recordings of the relaxing effect of 20 µM genistein and 20 µM daidzein on aortic rings precontracted 8 min previously with 2 µM 5-HT. Also shown are control contractions on top of which the vehicle (DMSO) of genistein and daidzein was added. **B:** Mean values of the spontaneous relaxation of 5-HT contractions (control, n=7, measured 18 min after the beginning of contraction) and of the relaxing effects of genistein (n=10) and daidzein (n=8), both measured 10 min after their addition. **C:** Typical recordings of the relaxing effect of 20 µM genistein and 20 µM daidzein on aortic rings precontracted with high K^+ KS. **D:** Mean values of the spontaneous relaxation of high K^+ KS contractions (control, n=3, measured 18 min after the beginning of contraction) and of the relaxing effects of genistein (n=7) and daidzein (n=7), both measured 10 min after their addition. The symbol * denotes statistically significant differences from controls ($p<0.05$).

had not induced, after 10 min, any significant relaxant effects on the contractions (32.3 ± 6.4 %, n=8, for 5-HT; and 4.4 ± 2.8 %, n=3, for high K^+ KS), because its addition did not modify the slight spontaneous decay of force observed in the controls measured after an equivalent time.

Genistein effects on $[Ca^{2+}]_i$ increases induced by 5-HT and high K^+.– $[Ca^{2+}]_i$ measurements performed in smooth muscle cells of desendothelized rat aortic segments showed that the depolarizing high K^+ KS and 2 µM of 5-HT induced an $[Ca^{2+}]_i$ increase and a second application of 5-HT or high K^+ PSS produced $[Ca^{2+}]_i$ increases which did not differ from the first application (Fig. 2C and 2D, open bars).

Genistein decreased the amplitude of $[Ca^{+2}]_i$ increase induced by a second application of 2 µM 5-HT and high K^+ PSS, by approximately 75 % and 20 %, respectively (Fig. 2). The effect was totally reversible in the case of high K^+ (after 5 min of washout of genistein, the original response was restored), but only partially reversible in the case of 5-HT (data not shown). The fact that genistein had less effect on the high K^+ response, compared to the effect produced when the cells were stimulated with 5-HT, could be due to the involvement of K^+ channels activation in its relaxing mechanism. This

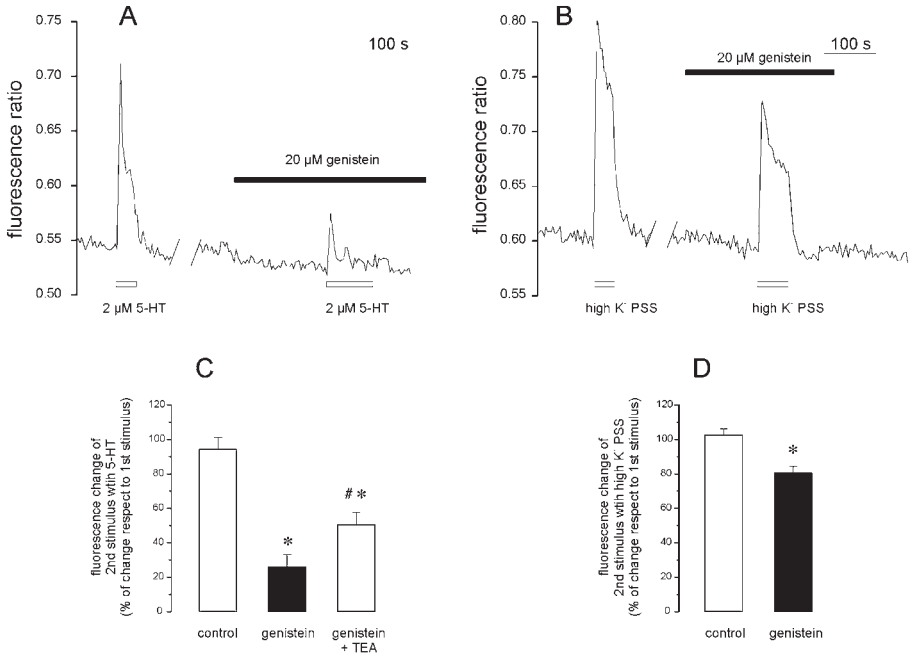

Fig. 2. Typical recording of $[Ca^{2+}]_i$ increase in rat aortic smooth muscle cells (measured as changes in fluorescence ratio) caused by 2 μM 5-HT (**A**) or high K^+ PSS (**B**), in the absence or in the presence of genistein. **C:** mean values of $[Ca^{2+}]_i$ increase of a second stimulus with 5-HT (expressed as % of the value obtained with a first stimulus) either in control conditions, in the presence of 20 μM genistein or a combination of genistein and 0.5 mM TEA. The symbols * and # indicate statistically significant differences from the control bar and from the genistein bar, respectively. **D**: inhibition of high K^+ PSS -evoked increase in $[Ca^{2+}]_i$ by 20 μM genistein. Inhibition is evaluated by comparing the second stimuli to the first one. The symbol * indicates statistically significant differences from the control bar.

hypothesis is based in the fact that K^+ channels opening cannot readily produce hyperpolarization in the presence of high extracellular K^+ due to the change this produces in the K^+ electrochemical potential. Hence, we tested the effect of genistein on 5-HT-induced $[Ca^{2+}]_i$ increase in the presence of a low concentration of TEA (0.5 mM). Genistein inhibition of $[Ca^{2+}]_i$ increase induced by 5-HT was significantly lowered upon the addition of the K^+ channels blocker (Fig. 2C).

Genistein effects on $[Ca^{2+}]_i$ oscillations induced by 5-HT.– When 5-HT was maintained in the bath for a longer time (150-180 seconds) in most cases there appeared $[Ca^{2+}]_i$ oscillations overlapping the plateau phase, with a frequency of 9.0 ± 0.3 cycles/min (n=27). Interestingly, these oscillations were synchronized in all the cells enclosed in the different ROIs observed at the same time in a given experiment. When 5-HT was washed, the $[Ca^{2+}]_i$ returned to basal levels, and a second application of 5-HT (after 4 min of washing) produced a response equal to the first one (Fig. 3A). If the second application of 5-HT was added after 90 s of preincubation with 20 μM genistein, it

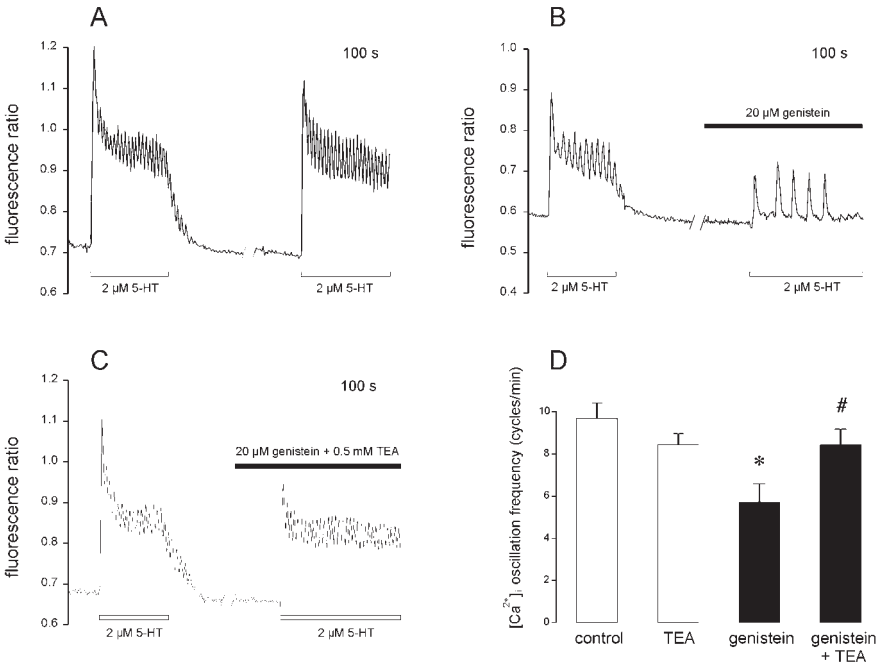

Fig. 3. Typical recording of $[Ca^{2+}]_i$ increases and oscillations in rat aortic smooth muscle cells (measured as changes in fluorescence ratio) caused by 5-HT. **A**: two stimulations with 2 µM 5-HT. **B**: a control stimulation with 5-HT, followed by a stimulation in the presence of genistein. **C**: a control stimulation with 5-HT, followed by a stimulation in the presence of genistein plus 0.5 mM TEA. **D**: Mean values of the frequency of $[Ca^{2+}]_i$ oscillation in control conditions and in the presence of TEA (0.5 mM), genistein (20 µM) and genistein + TEA.

was observed that the isoflavone significantly diminished the frequency of $[Ca^{2+}]_i$ oscillations (from 9.71 ± 0.75 cycles/min, n=7 to 5.74 ± 0.93 cycles/min, n=8; $p<0.05$) (Fig. 3B). This inhibitory effect on $[Ca^{2+}]_i$ oscillations was abolished by 0.5 mM TEA (Fig. 3C), while the K^+ channel blocker alone did not modify the response. Mean results are presented in Fig. 3D.

Discussion

We present results showing that genistein induced an immediate relaxation of 5-HT-induced contractions after a relatively short time and this effect was mimicked by the same concentration of daidzein, a hidroxy genistein analogue without effect on tyrosine kinase activity. In accordance with these results, genistein inhibited the $[Ca^{2+}]_i$ increases induced by 5-HT in smooth muscle cells in situ. Unfortunately, daidzein is a fluorescent molecule, and its effect on $[Ca^{2+}]_i$ cannot be tested using epifluorescence techniques, as it was also reported by WHEELER-JONES *et al.* (16). Our results can be compared to those of NELSON *et al.* (9) who report that a long preincubation (45 min) with genistein produced a decrease of the magnitude of the change in $[Ca^{2+}]_i$ in cultured rat aortic smooth muscle cells stimulated with a

much higher 5-HT concentration (148 μM) than that used in our experiments. In rat aortic rings, FLORIAN *et al.* (4) demonstrated that pretreatment for 60 min with genistein (5 μM) shifted to the right dose-response curves to 5-HT. Our report differs from this latter results in that we show acute genistein relaxant effects on top of a stable 5-HT elicited contraction.

In the case of aortas depolarized with high extracellular K^+ i) genistein relaxed high K^+-contracted rings, and ii) attenuated the $[Ca^{2+}]_i$ changes induced by this treatment in aortic segments. However, genistein had more effect on $[Ca^{2+}]_i$ changes and aortic rings contractions when the cells were challenged with 5-HT than with high K^+, so it may be possible that activation of membrane K^+ channels, with a concomitant cell hyperpolarization and inhibition of voltage operated Ca^{2+} channels, is one of the mechanisms involved in genistein actions. This hypothesis is based in the fact that K^+ channels opening cannot readily produce hyperpolarization in the presence of high extracellular K^+ due to the change in K^+ electrochemical potential when extracellular K^+ is increased. Accordingly, genistein effects on $[Ca^{2+}]_i$ changes induced by 5-HT were partially inhibited by TEA, a K^+ channel blocker, which is in agreement with our proposal that K^+ channels are important for its relaxant effects. Furthermore, given the TEA concentration we used (0.5 mM), the K^+ channels involved are most likely high-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}). A similar proposal was advanced by NEVALA *et al.* (10) who studied genistein effects on force development of rat mesenteric arteries rings. Moreover, patch clamp data show that genistein is actually able to activate BK_{Ca}, both at the whole-cell and single-channel level, in rat tail artery cultured smooth muscle cells (17). In contrast, genistein has also been shown to inhibit another type of K^+ channels, namely delayed rectifier ones, through a tyrosine-kinase independent mechanism in guinea pig ventricular myocytes (15).

On the other hand, about half of the inhibitory effect of genistein we saw on $[Ca^{2+}]_i$ increases induced by 5-HT still remained in the presence of TEA, and the isoflavone also had a partial inhibitory effect on force development and $[Ca^{2+}]_i$ increase following application of a high K^+ solution. It is possible, then, that there exists an additional genistein vasodilator effect accompanying the activation of K^+ channels. Although we did not explore this possibility, genistein may be inhibiting voltage activated Ca^{2+}channels, as it has been previously seen in rat (18) and guinea pig (3) cardiomyocytes, and in rat portal vein (7, 8).

We also observed that exposure to 5-HT for a prolonged time induced $[Ca^{2+}]_i$ oscillations which were synchronized. This synchronicity probably reflects the presence of electrical communication between neighboring cells through low-resistance membrane structures such as gap junctions. NELSON *et al.* (9), however, do not report oscillations in $[Ca^{2+}]_i$ in rat aortic smooth muscle cells stimulated with 5-HT, but this differences may be attributed to the fact that they work with cultured cells whereas our experiments were performed in intact segments, where the tissue architecture is intact and hence the cells retain whatever inter-cellular communications there may be.

We found that genistein significantly reduced the frequency of $[Ca^{2+}]_i$ oscillations in rat aorta, again, probably, through the activation of BK_{Ca} channels, since 0.5 mM TEA abolished its inhibitory effects. It should be noted that in this case TEA

completely reverted genistein actions, while it only had a partial inhibitory effect on genistein reduction of the amplitude of $[Ca^{2+}]_i$ increases. Moreover, genistein effect on $[Ca^{2+}]_i$ oscillations is an interesting result, because through this mechanism, genistein may diminish aortic smooth muscle cells sensitivity to 5-HT, because it has been demonstrated that the frequency of the oscillations produced by certain agonists is a direct function of its concentration (11).

Summarizing, in rat aorta, genistein is able to diminish the contraction, the $[Ca^{2+}]_i$ increase and the frequency of the $[Ca^{2+}]_i$ oscillations (when present), evoked by 5-HT. Genistein probably activates BK_{Ca} channels, which hyperpolarizes the smooth muscle cells and causes voltage-activated Ca^{2+} channels to close. These effects do not need prolonged incubation of genistein, suggesting that tyrosine kinase inhibition is not involved. The inhibitory effect of daidzein on force development supports this hypothesis.

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F. SPERONI, A. REBOLLEDO, S. SALEMME, M.C. AÑÓN, F. TANZI y V. MILESI. *Inhibición por genisteína de la fuerza contráctil y la concentración citosólica de Ca^{2+} inducida por 5-HT en aorta de rata.* J. Physiol. Biochem., **63** (2), 143-152, 2007.

Genisteína y daidzeína, dos isoflavonas presentes en la soja, afectan el estado contráctil de diferentes tipos de músculo liso. Describimos aquí efectos agudos de estos compuestos sobre la fuerza contráctil y la concentración intracelular de Ca^{2+} ($[Ca^{2+}]_i$) en músculo liso aórtico de rata *in situ*. La serotonina (5-HT) (2 µM) o una solución despolarizante de alto K^+ produjeron la contracción de anillos de aorta de rata, que fueron relajados inmediatamente por genisteína (20 µM) y daidzeína (20 µM). En concordancia con esto, tanto la 5-HT como el alto K^+ incrementaron la $[Ca^{2+}]_i$ en células de músculo liso aórtico in situ. La genisteína inhibió el aumento de $[Ca^{2+}]_i$ producido por 5-HT (74,0 ± 7,3%, n=11, p<0,05) y tuvo un efecto menor sobre aquel debido al alto K^+ (19,9 ± 4,0%, n=7, p<0,05). El bloqueante de canales de K^+ tetraetilamonio (TEA) (0,5 mM) disminuyó los efectos de genisteína sobre el aumento de $[Ca^{2+}]_i$ debido a 5-HT. Durante la aplicación prolongada de 5-HT, la $[Ca^{2+}]_i$ comenzó a oscilar y una preincubación corta con genisteína (90 s) disminuyó significativamente la frecuencia de estas oscilaciones, siendo este efecto totalmente bloqueado por TEA. En conclusión, en el músculo liso aórtico de rata la genisteína es capaz de atenuar el aumento de $[Ca^{2+}]_i$ y de la fuerza inducidos por 5-HT, así como de disminuir la frecuencia de las oscilaciones en la $[Ca^{2+}]_i$. El corto tiempo requerido por la genisteína, así como la relajación inducida por daidzeína sugieren que esto no se debe a inhibición de tirosín quinasas. El menor efecto de genisteína sobre el aumento de $[Ca^{2+}]_i$ debido al alto K^+, así como los efectos del TEA apuntan a la inhibición por parte de genisteína de canales de K^+ activados por Ca^{2+}.

Palabras clave: Genisteína, Aorta, Serotonina, Ca^{2+} citosólico.

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