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Differential action of a protein tyrosine kinase inhibitor, genistein, on the positive inotropic effect of endothelin-1 and norepinephrine in canine ventricular myocardium

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1 Experiments were carried out in isolated canine ventricular trabeculae and acetoxymethylester of indo-1-loaded single myocytes to elucidate the role of protein tyrosine kinase (PTK) in the inotropic effect of endothelin-1 (ET-1) induced by crosstalk with norepinephrine (NE). The PTK inhibitor genistein was used as a pharmacological tool.

2 Genistein but not daidzein inhibited the positive inotropic effect and the increase in Ca^{2+} transients induced by ET-1 by crosstalk with NE at low concentrations.

3 Genistein and daidzein antagonized the negative inotropic effect and the decrease in Ca^{2+} transients induced by ET-1 by crosstalk with NE at high concentrations, but genistein did not affect the antiadrenergic effect of carbachol.

4 Genistein but not daidzein enhanced the positive inotropic effect and the increase in Ca^{2+} transients induced by NE *via* β-adrenoceptors, while the enhancing effect of genistein was abolished by the protein tyrosine phosphatase inhibitor vanadate.

5 These findings indicate that genistein (1) induces a positive inotropic effect in association with an increase in Ca^{2+} transients, (2) inhibits the positive inotropic effect of ET-1 induced by crosstalk with NE, and (3) enhances the positive inotropic effect of NE induced *via* β-adrenoceptors by inhibition of PTK. In addition, genistein inhibits the negative inotropic effect of ET-1 induced by crosstalk with NE through a PTK-unrelated mechanism. PTK may play a crucial role in the receptor-mediated regulation of cardiac contractile function in canine ventricular myocardium.
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Abbreviations: cAMP, 3′,5′-cyclic adenosine monophosphate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; CaT, Ca^{2+} transient; CRC, concentration–response curve; DMSO, dimethyl sulfoxide; ET-1, endothelin-1; $I_{(Ca)L}$, L-type Ca^{2+} current; indo-1/AM, acetoxymethylester of indo-1; ISO, isoproterenol; ISO_{max}, maximal response to ISO; NE, norepinephrine; NIE, negative inotropic effect; pD_2, $-\log_{10}$ (concentration to induce 50% of the maximal response); PIE, positive inotropic effect; PKA, protein kinase A; PKC, protein kinase C; PP, protein phosphatase; PTK, protein tyrosine kinase; PTPase, phosphotyrosine phosphatase

Introduction

Endothelin-1 (ET-1) is a potent vasoactive peptide of 21 amino acids that was originally isolated from a culture medium of porcine aortic endothelial cells (Yanagisawa *et al*., 1988). It has been demonstrated that ET-1 plays a crucial role in the regulation of cardiovascular function in various cardiovascular disorders (Wei *et al*., 1994; Sakai *et al*., 1996). ET-1 exerts a positive inotropic effect (PIE) in association with a negative lusitropic effect in most mammalian species but not in canine ventricular myocardium (Takanashi & Endoh, 1991). However, in the presence of low concentrations of β-adrenoceptor agonists, including norepinephrine (NE) and isoproterenol (ISO), ET-1 induces a PIE, and elicits a negative inotropic effect (NIE) in the presence of high concentrations of β-adrenoceptor agonists (Zhu *et al*., 1997; Chu & Endoh, 2000; Takahashi *et al*., 2001; Chu *et al*., 2003b). Stimulation of ET receptors is coupled to divergent signal transduction pathways, including the activation of protein kinase C (PKC) and protein kinase G that leads to subsequent activations of various types of ion channels and ion transporters. Activation of these processes results in an increase or decrease in intracellular Ca^{2+} transients and/or an increase in myofilament Ca^{2+} sensitivity in the final step of cardiac contractile regulation (Watanabe & Endoh, 2000; Chu *et al*., 2003b).

Protein tyrosine kinase (PTK) mediates actions of a variety of hormones and neurotransmitters on a wide range of cellular processes that regulate cell growth and differentiation (Van der

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Geer *et al*., 1994), ion channel conductance (Hunter & Cooper, 1985; Siegelbaum, 1994) and cardiovascular function (Di Salvo *et al*., 1993; Akaishi *et al*., 2000; Liew *et al*., 2003). It has been established that receptors that belong to a family involved in the regulation of growth factors have PTK activity in their intracellular domain and phosphorylate tyrosine residues of their own receptors, regulatory proteins or structural proteins, and thus activate a cascade of intracellular signaling (Fantl *et al*., 1993). In addition to receptor-associated PTKs, cytosolic PTKs play also an important role in mediating signal transduction induced by heterotrimeric G protein-coupled receptors (Hollenberg, 1994). Recent studies have revealed that activation of PTK is involved in smooth muscle contraction and relaxation induced by various vasoactive agents, such as angiotensin II (Tsuda *et al*., 1991; Molloy *et al*., 1993), ET-1 (Ohanian *et al*., 1997), α_1- and α_2-adrenoceptor agonists (Di Salvo *et al*., 1993; Jinsi & Deth, 1995), muscarinic receptor agonists (Di Salvo *et al*., 1993), nitroglycerin (Satake *et al*., 1999) and β-adrenoceptor agonists (Satake & Shibata, 1999; Satake *et al*., 2000). PTKs are also present in the heart (Maher, 1991) and have been implicated in the regulation of L-type Ca^{2+} channels (Chiang *et al*., 1996; Shuba *et al*., 1996; Yokoshiki *et al*., 1996; Hool *et al*., 1998; Wang & Lipsius, 1998; Boixel *et al*., 2000; Liew *et al*., 2003) and atrial contractility (Akaishi *et al*., 2000). In addition, it has been reported that the PTK inhibitor genistein (Tsiani & Fantus, 1997) enhanced an increase in L-type Ca^{2+} current ($I_{(Ca)L}$) induced by β-adrenoceptor stimulation, which indicates that activation of PTKs exerts an inhibitory action on $I_{(Ca)L}$ by antagonizing the β-adrenoceptor-mediated response (Hool *et al*., 1998). In contrast, we found that genistein exerted an inhibitory action on the ET-1-induced PIE and an increase in Ca^{2+} transients in rabbit ventricular myocytes (Wang & Endoh, 2001). While these findings imply that activation of PTKs may be involved in the regulation of cardiac contractile function, relatively little is known about that role.

In the present study, we studied the effects of genistein, which has been reported to specifically inhibit PTK activities and is widely used as a pharmacological tool, to elucidate the role of PTK signaling in a variety of systems (Tsiani & Fantus, 1997). The influence of genistein on the regulation of cardiac contractility and Ca^{2+} transients elicited *via* different signaling processes, that is, the effects induced by crosstalk of ET-1 and NE, and those mediated by β-adrenoceptor stimulation was investigated in canine ventricular myocardium. The effects of daidzein, which is structurally similar to genistein but does not inhibit PTK activity (Peterson & Barnes, 1993), and vanadate, which enhances tyrosine phosphorylation by inhibiting phosphotyrosine phosphatase (PTPase) activities (Gordon, 1991), were also investigated to clarify the selectivity of the effect of genistein mediated by PTK inhibition.

Experiments were performed in both isolated canine ventricular trabeculae and in myocytes to confirm that the effects observed in the former preparation that includes various types of cells, including endothelial and nervous cells, could be reproducible in single myocytes. It has been shown that in rabbit, the effects of ET-1 and receptor antagonists in single ventricular myocytes are quantitatively different from but qualitatively similar to those in ventricular papillary muscles (Talukder *et al*., 2001; Yomogida *et al*., 2004). Part of this study has been presented in an abstract form (Chu & Endoh, 2001).

Methods

This study was conducted in accordance with Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society and the Guidance for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication no. 85-23, revised 1996). The approval for the animal experiments was obtained from the Committee for Animal Experimentation, Yamagata University School of Medicine prior to the experiments, and the study was carried out in accordance with the Declaration of Helsinki.

Measurements of inotropic effects in trabeculae

The experimental procedures employed were essentially similar to those in previous studies (Talukder *et al*.,. 2001; Chu *et al*., 2003b). Briefly, mongrel dogs (7–10 kg) of either sex were used in the experiments. Two to four ventricular trabeculae were excised from the right ventricle and then mounted in 20-ml organ baths that contained Krebs–Henseleit solution (with 0.057 mM ascorbic acid and 0.027 mM EDTA, disodium salt) bubbled with 95% O_2 and 5% CO_2 at 37°C (pH 7.4). Muscle preparations were electrically stimulated with a pulse of 5-ms duration and a voltage 20% above the threshold (approximately 0.4 V) at 0.5 Hz. Isometric force of contraction was detected with strain gauge transducers and recorded on a thermal pen writing oscillograph. Muscle preparations had an average dimension of 13.31 ± 3.09 mm length and 6.27 ± 1.37 mm^2 cross-sectional area ($n = 212$, from 41 dogs). In all experiments, α-adrenoceptor antagonist prazosin (300 nM) was allowed to act for 30 min before the addition of NE and was present in the organ bath throughout the experiments. Genistein was administered 30 min before the addition of NE and was present in the organ bath throughout the experiments.

In a series of experiments, the concentration–response curve (CRC) for NE was determined in the absence and presence of genistein at different concentrations. After CRC for NE was completed, NE was washed out for 2 h and the maximal response to ISO (ISO_{max}) was then obtained. The NE-induced inotropic response was calculated as a percentage of ISO_{max}.

Influence of genistein on the NIE of ET-1 in the presence of NE at 100 nM was investigated by single administration. In these experiments, the first response to NE was determined for 60 min, NE was washed out for 60 min, and it was then added again and allowed to act for 15 min before the addition of ET-1. The PTPase inhibitor vanadate was administered 15 min before the addition of genistein.

Isolation of cardiac myocytes

Dog ventricular cardiomyocytes were obtained by means of a procedure that has been described previously (Watanabe & Endoh, 2000). Briefly, a portion of the left ventricular free wall that is supplied with the branch of left anterior descending artery was excised, and the artery was then cannulated and perfused for approximately 2 min at 37°C by means of a Langendorff apparatus with Tyrode's solution. The tissue was then perfused with nominally Ca^{2+} free Tyrode's solution for 8 min at a rate of about 40 ml min^{-1}. Tyrode's solution contained (in mM) 136.5 NaCl, 5.4 KCl, 0.53 $MgCl_2$, 1.8

$CaCl_2$, 0.33 NaH_2PO_4, 5.0 glucose, and 5.0 HEPES (pH 7.4), and was bubbled continuously with 100% O_2. The perfusion solution was changed to nominally Ca^{2+}-free Tyrode's solution that contained 1.0 mg ml^{-1} collagenase and 0.1 mg ml^{-1} protease, and the perfusion was continued for 15–20 min at a perfusion rate of 20 ml min^{-1} by use of a recirculating system. Finally, the muscle piece was perfused with Tyrode's solution that contained 0.2 mM $CaCl_2$ and then cut into small pieces with a scalpel. The cell suspension was rinsed several times with gradual increases in the Ca^{2+} concentration up to 1.8 mM.

Loading of myocytes with indo-1/AM

Myocytes were loaded with acetoxymethylester of indo-1 (indo-1/AM) by incubating them in 5 μM indo-1 solution for about 3 min at room temperature (25°C). After loading, they were centrifuged at $5 \times g$ for 1 min. The pellet was resuspended in HEPES-Tyrode solution. Myocytes were then laid in the chamber superfused with bicarbonate buffer for about 10 min. The bicarbonate buffer contained (in mM) 116.4 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 1.8 $CaCl_2$, 1.0 NaH_2PO_4, 5.0 glucose and 23.8 $NaHCO_3$ (pH 7.4) and had been equilibrated with 95% O_2 and 5% CO_2.

Simultaneous measurements of cell shortening and Ca^{2+} transients

Myocytes were laid in a perfusion chamber placed on the stage of an inverted microscope (Diaphot TMD 300, Nikon, Tokyo, Japan). After 10 min when the cells settled down to attach loosely to the bottom of chamber, perfusion was started with bicarbonate buffer containing 1.8 mM $CaCl_2$ at a rate of 1 ml min^{-1} at room temperature (25°C) and cells were stimulated electrically by square-wave pulses with voltage about 30–40% above the threshold at a frequency of 0.5 Hz.

Fluorescence of indo-1 was excited with light from a xenon lamp (150 W) at a wavelength of 355 nm, reflected by a 380 nm long-pass dichroic mirror, and detected by a fluorescence spectrophotometer (CAM-230, Japan Spectroscopic Co., Tokyo, Japan). Excitation light was applied to myocytes intermittently through a neutral density filter to minimize the photobleaching of indo-1. The emitted fluorescence was collected by an objective lens (CF Fluor DL40, Nikon, Tokyo, Japan) and then separated by a 580 nm long-pass dichroic mirror to permit simultaneous measurements of light at both 405 and 500 nm wavelengths through band-pass filters.

A fluorescence ratio of 405/500 nm was used as an indicator of $[Ca^{2+}]_i$ (Grynkiewicz *et al*., 1985). Cells were simultaneously illuminated with red light (wavelength above 620 nm) through the normal bright-field illumination optics of the microscope, and a myocyte's bright-field images were collected by an objective lens and then separated by a 580-nm long-pass dichroic mirror (Omega Optical, Brattleboro, VT, U.S.A.). A bright-field cell image was projected onto a photodiode array of the edge detector (C6294-01, Hamamatsu Photonic KK, Hamamatsu, Japan) with 5 ms temporal resolution.

Experimental protocols

When the response of myocytes to the applied agent reached a stable level, indo-1 fluorescence was measured and the perfusion then switched to a solution that contained an additional agent. In the current study, an increase or decrease in cell shortening is considered to qualitatively reflect the PIE or NIE in isometric contractions, and is often referred to as PIE or NIE interchangeably without explanation. Prazosin (300 nM) and genistein were allowed to act for 20 min before the application of NE or ET-1, and were present throughout the experiments.

Data recordings and analysis

Cell length and fluorescence of indo-1 were stored and displayed by means of a computer (Power Macintosh 8100/100AV, Apple Computer Inc., Cutertino, CA, U.S.A.) equipped with an A/D converter (MP-100A, BIOPAC Systems Inc., Santa Barbara, CA, U.S.A.) at 200 Hz and analyzed after low-pass filtering (cutoff frequency of 20 Hz). The data used for statistical analysis were obtained by signal averaging of five successive tracings of cell shortening and Ca^{2+} transients. In the analysis of data, the diastolic cell length and indo-1 fluorescence ratio prior to the first application of the agent were regarded as basal values for each myocyte.

Drugs

The drugs used were ET-1 (Peptide Institute, Osaka, Japan); prazosin hydrochloride (Pfizer Taito, Tokyo, Japan); norepinephrine hydrochloride (Nakarai Chemicals Ltd, Kyoto, Japan); carbamylcholine chloride (carbachol), genistein, daidzein, vanadate, (-)-isoproterenol and protease type XIV (Sigma, Chemical Co., St Louis, MO, U.S.A); pentobarbital sodium (Tokyo Kasei, Tokyo, Japan); indo-1/AM (Dojindo Chemical, Kumamoto, Japan); collagenase type II (Worthington Biochemical, Freehold, NJ, U.S.A.); and dimethyl sulfoxide (DMSO; Wako Pure Chemicals, Osaka, Japan).

Statistics

Experimental values are presented as means $\pm$ s.e.mean. Significant differences between mean values were estimated by a repeated-measures analysis of variance and/or by Student's *t*-test with the analytic software STATVIEW J-4.5 (Abacus Concepts, Berkeley, CA, U.S.A.). A P value <0.05 was considered to indicate a significant difference between two means.

Results

Effects of genistein on cardiac contractility and Ca^{2+} transients

Inotropic effects of genistein, daidzein and vanadate in isolated ventricular trabeculae are shown in Figure 1. Genistein at 10–30 μM did not affect the basal force of contraction (10 μM: $99.5 \pm 1.81\%$, $n=5$; 30 μM: $104.8 \pm 2.39\%$, $n=12$). At 100 μM, it induced a significant long-lasting PIE ($181.0 \pm 11.06\%$, $n=8$, $P<0.001$). Unlike genistein, daidzein (30 μM) and vanadate (30 μM) exhibited an NIE (daidzein: $80.0 \pm 3.04\%$, $n=12$, $P<0.001$; vanadate: $80.2 \pm 2.64\%$, $n=7$, $P<0.001$).

Figure 2a shows actual tracings of the effect of genistein at 30 μM on cell shortening in ventricular myocytes loaded with indo-1. Genistein at 30 μM increased cell shortening in association with an increase in indo-1 fluorescence ratio, which returned to control levels by washout (Figure 2b). On average,

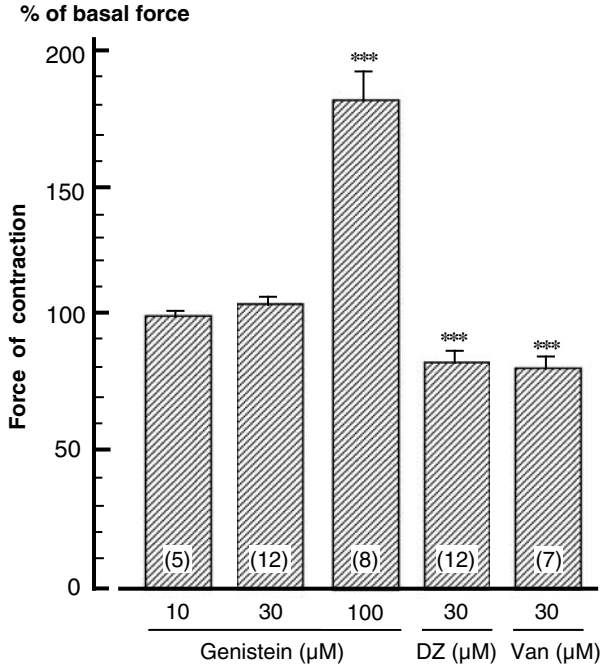


Figure 1 Effects of genistein on the basal force of contraction in isolated canine ventricular trabeculae. DZ: daidzein; Van: vanadate. Values are means±s.e.mean. Average basal force of contraction prior to the administration of drugs was $6.09 \pm 1.33\,mN\,mm^{-2}$ ($n=44$). Numbers in parentheses indicate the numbers of muscle preparations examined. ***$P<0.001$ vs the respective basal values.

genistein induced a significant increase in indo-1 ratio at 30 μM (130.7±8.75%, $n=7$, $P<0.05$) in association with an increase in cell shortening (128.1±2.09%, $n=7$, $P<0.01$) (Figure 2c).

Influence of genistein on the PIE of ET-1 induced by crosstalk with NE at low concentrations

In isolated ventricular trabeculae, ET-1 alone at 10 nM (Figure 3a) and 100 nM (Figure 3b) did not induce any PIE (10 nM: 101.9±0.51%, $n=8$; 100 nM: 99.6±1.85%, $n=6$). In the presence of NE at a subthreshold concentration of 1 nM, however, ET-1 elicited a long-lasting PIE in a concentration-dependent manner (10 nM: 117.03±2.70%, $n=5$, $P<0.001$; 100 nM: 154.4±6.97%, $n=7$, $P<0.001$) (Figure 3a and b).

Genistein at 30 μM inhibited almost completely the PIE of ET in the presence of 1 nM NE (10 nM: 101.3±2.85%; $n=8$; 100 nM: 106.6±5.74%; $n=8$) (Figure 3a and b). Daidzein had no inhibitory action of the PIE of ET-1 at 100 nM (146.3±3.09%; $n=7$) (Figure 3b). While ET-1 at 100 nM elicited a small transient NIE preceding the long-lasting PIE, genistein and daidzein did not have significant influence on NIE (Figure 3b). Summarized data determined 30–40 min after administration of ET-1 are presented in Figure 3c: genistein but not daidzein inhibited significantly the PIE of ET-1 in the presence of 1 nM NE.

In ventricular myocytes, ET-1 alone at 10 nM did not significantly affect the indo-1 ratio or cell shortening by itself, but in the presence of NE at 0.1 nM, which did not affect indo-1 ratio or cell shortening at all (98.1±4.25 and 103.0±3.27%), ET-1 induced a significant increase in cell shortening (140.3±5.93%) in association with a small but significant increase in the indo-1 ratio (112.4±4.78%) (Figure 4a, left

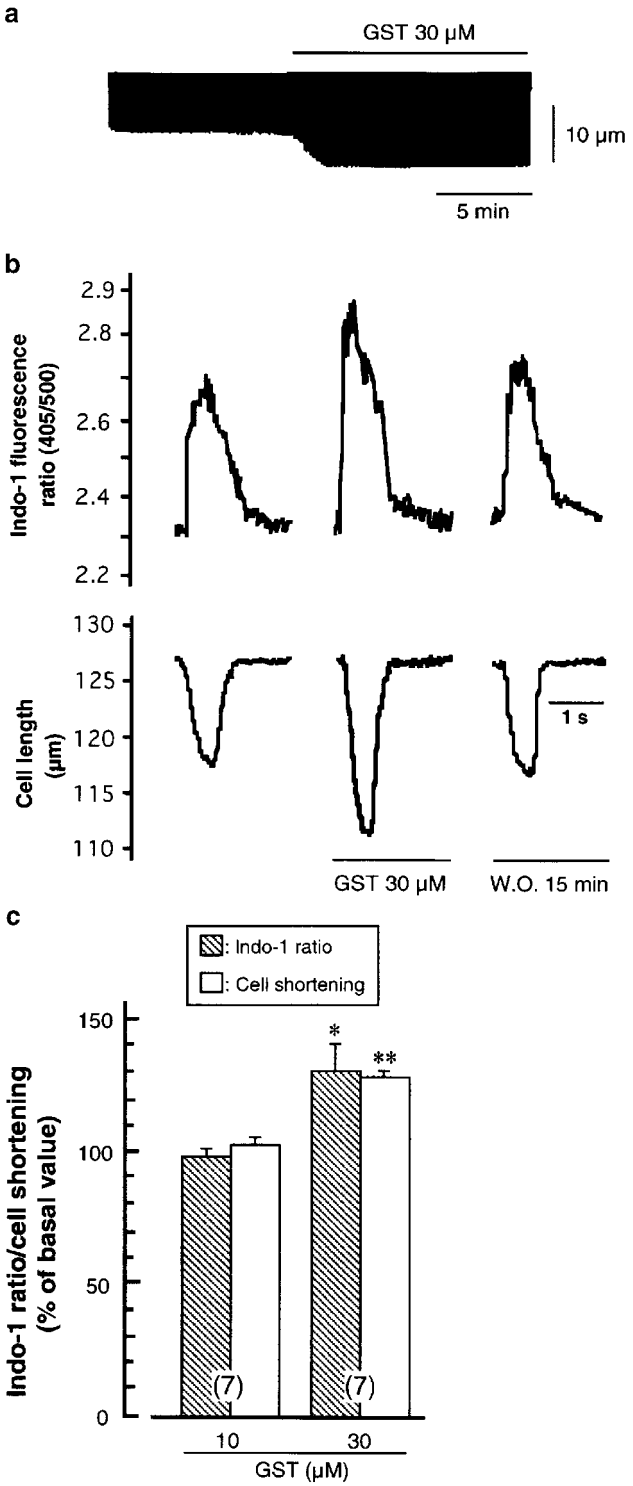
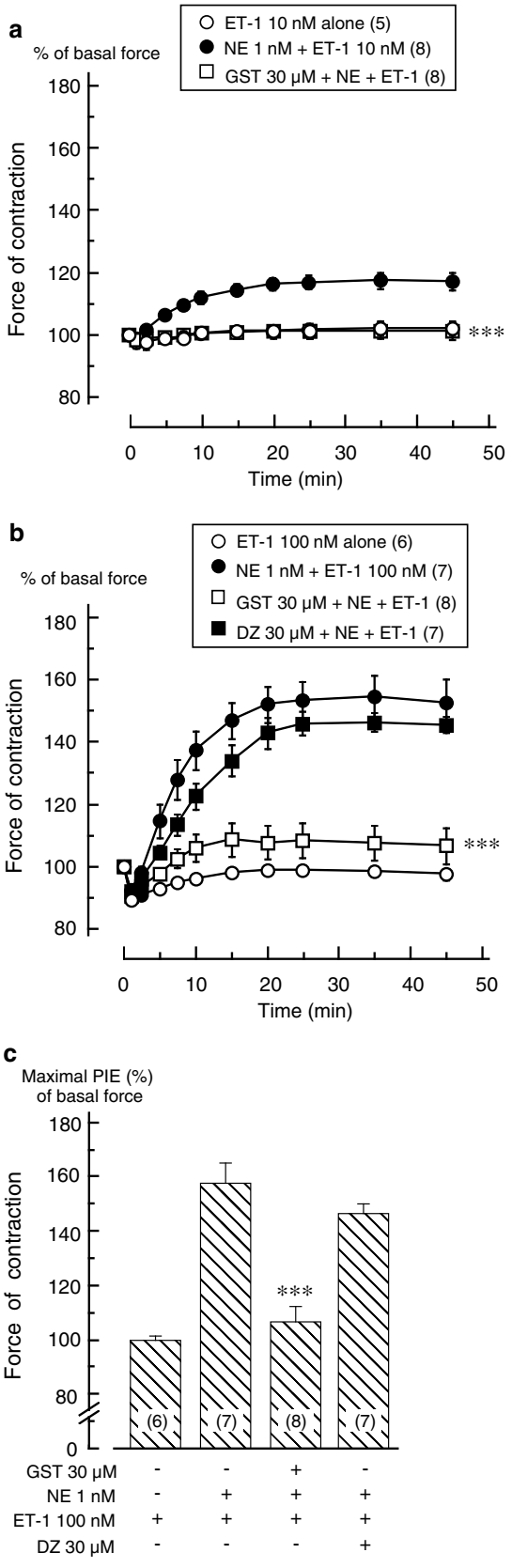


Figure 2 Effects of genistein on Ca^{2+} transients and cell shortening in isolated canine ventricular myocytes. (a) Actual tracings of the effects of 30 μM genistein (GST) in a myocyte. (b) Individual signals of indo-1 ratio (upper tracings) and cell shortening (lower tracings) recorded prior to, during application and after washout of genistein. Individual tracings were obtained by means of signal averaging of five successive signals. (c) Summarized data on the effects of genistein at 10 and 30 μM on indo-1 ratio and cell shortening. Basal values (amplitudes of Ca^{2+} transients and cell shortening) prior to the administration of genistein were 0.73±0.18 (indo-1 ratio) and 8.27±0.68 μm ($n=14$) respectively. Numbers in parentheses indicate the numbers of cells. *$P<0.05$; **$P<0.01$ vs the respective basal values.

panel). Genistein at 30 μM almost completely inhibited the ET-1-induced increase in the amplitude of indo-1 ratio and cell shortening to 101.8 $\pm$ 2.32 and 99.1 $\pm$ 6.30% (Figure 4a, right panel). Summarized data are shown in Figure 4b. Neither ET-1 ($n=8$) nor NE ($n=7$) significantly affected the indo-1 ratio and cell shortening, but ET-1 and NE in combination increased significantly the indo-1 ratio (112.4 $\pm$ 4.78%, $n=11$, $P<0.05$) and cell shortening (140.3 $\pm$ 5.93%, $n=11$, $P<0.001$). Genistein abolished these increases induced by crosstalk of ET-1 with NE ($n=8$).



Influence of genistein on the PIE of NE induced via β-adrenoceptors

In isolated ventricular trabeculae, the influence of genistein on the CRC for the PIE of NE mediated by β-adrenoceptors was investigated and the results are presented in Figure 5a. While genistein at 3 μM did not affect the CRC for NE, it shifted the CRC for NE at 10 and 30 μM to the left, an indication that genistein enhances the PIE mediated by β-adrenoceptors. The pD_2 values for NE were significantly increased by genistein at 10 and 30 μM, while the maximal response to NE was unaffected by genistein (Table 1). Daidzein at 30 μM did not affect the CRC for NE (Figure5b and Table 1).

Figure 6 shows the influence of genistein at 30 μM on the time course of the PIE of 100 nM NE. NE at 100 nM increased the contractile force by 60–80% of the basal force at 20 min after administration. While DMSO (the solvent of genistein) and genistein at 3 μM did not significantly affect the PIE of NE (Figure 6a and b), genistein at 10 and 30 μM prominently enhanced the PIE of NE in a concentration-dependent manner (Figure 6c and d). On average, in the presence of genistein at 3, 10 and 30 μM, NE increased the contractile force by 72.9 $\pm$ 8.28% ($n=7$; control: 64.3 $\pm$ 7.00%), 106.4 $\pm$ 12.39% ($n=5$; $P<0.01$ vs control: 73.0 $\pm$ 9.18%) and 144.4 $\pm$ 21.47% ($n=14$; $P<0.001$ vs control: 63.1 $\pm$ 9.19%). DMSO did not affect the PIE of NE (NE alone: 72.2 $\pm$ 5.01%; DMSO plus NE: 79.1 $\pm$ 7.57%; $n=7$).

Vanadate at 30 μM abolished the enhancing effect of genistein at 30 μM on the PIE of NE in isolated ventricular trabeculae (Figure 6e). In the presence of genistein at 30 μM with vanadate at 30 μM, the PIE of NE was 95.3 $\pm$ 13.69% ($n=11$), which was not significantly different from the control response (78.7 $\pm$ 4.35%).

It was confirmed in isolated ventricular trabeculae that the PIE induced by elevation of $[Ca^{2+}]_o$ (5 mM) was not affected by genistein (Figure 6f). The PIE induced by elevation of $[Ca^{2+}]_o$ (5 mM) was 119.4 $\pm$ 13.74% ($n=6$), which was not significantly different from the control response (97.5 $\pm$ 7.52%).

Figure 3 Influence of genistein on the ET-1-induced PIE in the presence of 1 nM NE in isolated canine ventricular trabeculae. (a) Influence of genistein (GST) at 30 μM on the PIE of 10 nM ET-1 in the presence of 1 nM NE. Basal force of contraction in this series of experiments was 7.31 $\pm$ 1.38 mN mm^{-2} ($n=15$). Values are means $\pm$ s.e.mean; where they are not shown, s.e.mean is smaller than the symbol. (b) Influence of genistein at 30 μM and daidzein (DZ) at 30 μM on the PIE of 100 nM ET-1 in the presence of 1 nM NE. Basal force of contraction in this series of experiments was 6.85 $\pm$ 1.14 mN mm^{-2} ($n=7$). (c) Summarized data on the influence of genistein and daidzein at 30 μM on the PIE induced by crosstalk of ET-1 and NE. Numbers in parentheses indicate the numbers of muscle preparations. ***$P<0.001$ vs the control value (ET-1 + NE) by repeated-measures analysis of variance. Experiments were carried out in the presence of 300 nM prazosin.

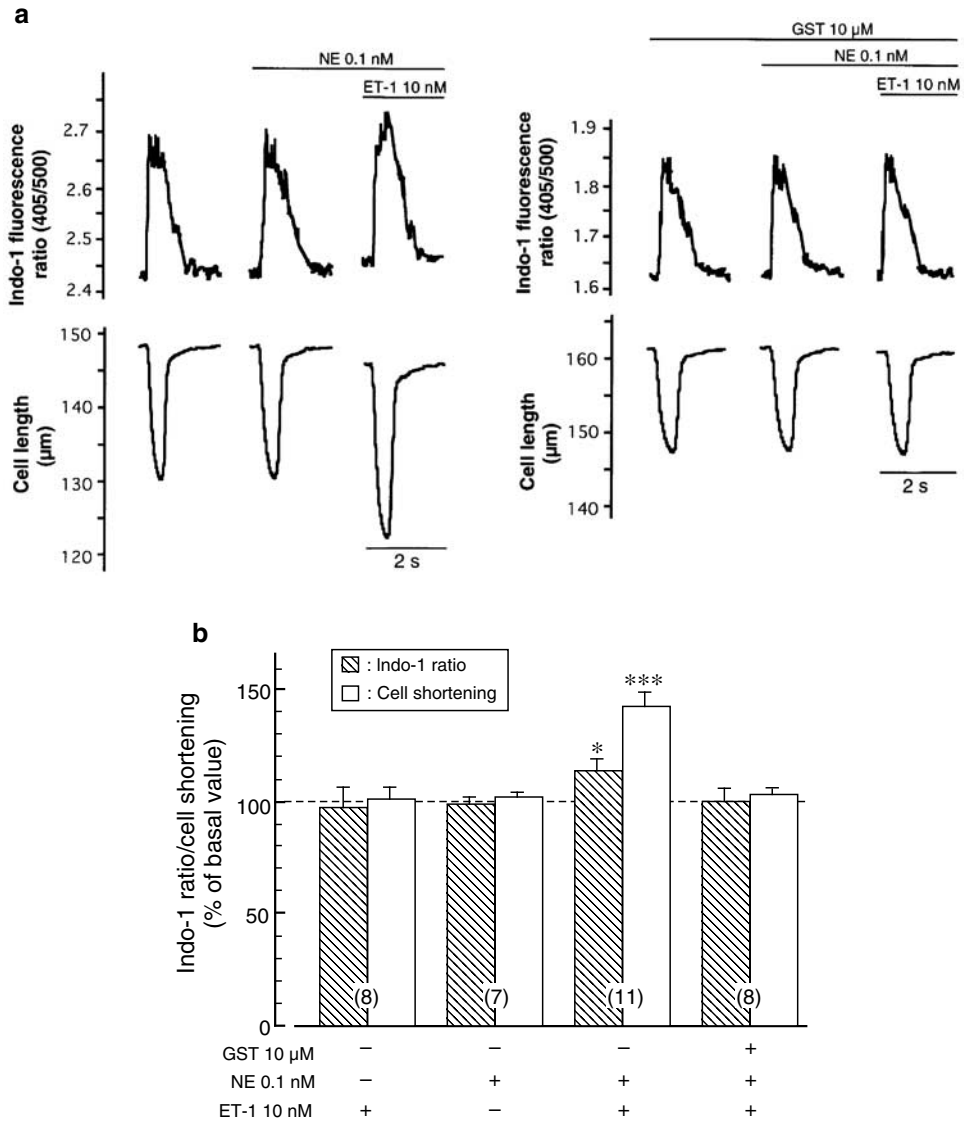


Figure 4 Influence of genistein on the ET-induced increase in Ca^{2+} transients and cell shortening in the presence of NE in isolated canine ventricular myocytes. (a) Actual tracings of the effects of 10 nM ET-1 in the presence of 0.1 nM NE in the absence (left panel) and the presence (right panel) of genistein (GST) at 10 μM in a myocyte. Signals were obtained by means of signal averaging of five successive signals. Upper tracings: indo-1 fluorescence ratio; lower tracings: cell shortening. (b) Summarized data on the influence of genistein at 10 μM on the PIE induced by crosstalk of ET-1 (10 nM) and NE (0.1 nM). Basal values (amplitudes of Ca^{2+} transients and cell shortening) prior to the administration of drugs were 0.44 ± 0.10 (indo-1 ratio) and 9.13 ± 0.98 μm ($n = 34$), respectively. Numbers in parentheses indicate the numbers of cells. $^{*}P < 0.05$; $^{***}P < 0.001$ vs the respective basal values. Experiments were carried out in the presence of 300 nM prazosin.

Genistein enhanced the increase in the indo-1 ratio and cell shortening induced by NE in ventricular myocytes as shown by the actual tracings in Figure 7a and b, and summarized in Figure 7c. The increases in the indo-1 ratio ($58.9 \pm 9.81\%$) and cell shortening ($84.7 \pm 17.23\%$) induced by 30 nM NE were enhanced significantly by genistein at 10 μM to $143.8 \pm 23.33\%$ (indo-1 ratio) and $177.3 \pm 19.17\%$ (cell shortening) ($n = 7$, each).

Influence of genistein on the NIE of ET-1 induced by crosstalk with NE at high concentrations

Neither ET-1 nor carbachol affected the basal force of contraction in isolated canine ventricular myocardium or myocytes (data not shown), but during β-adrenoceptor stimulation induced by NE at 100 nM, ET-1 at 10 nM (Figure 8a(i)) and carbachol (Figure 8a(iii)) elicited a definite NIE. The PIE of NE alone declined spontaneously to $87.9 \pm 4.16\%$ of the maximal response after 60 min in control ($P < 0.05$). The PIE of NE was reproducible after 60-min washout. Carbachol (30 nM) elicited an NIE to an extent similar to that of ET-1 (10 nM) in the presence of NE at 100 nM when they were administered 15 min after the application of NE (Figure 8a). Genistein at 10 μM suppressed the NIE of ET-1 (10 nM) as shown in Figure 8a(ii), whereas the absolute extent of NIE of carbachol (30 nM) was unaffected by genistein (Figure 8a(iv)). Because genistein enhanced the β-adrenoceptor-mediated PIE of NE, this effect alone partially overcame

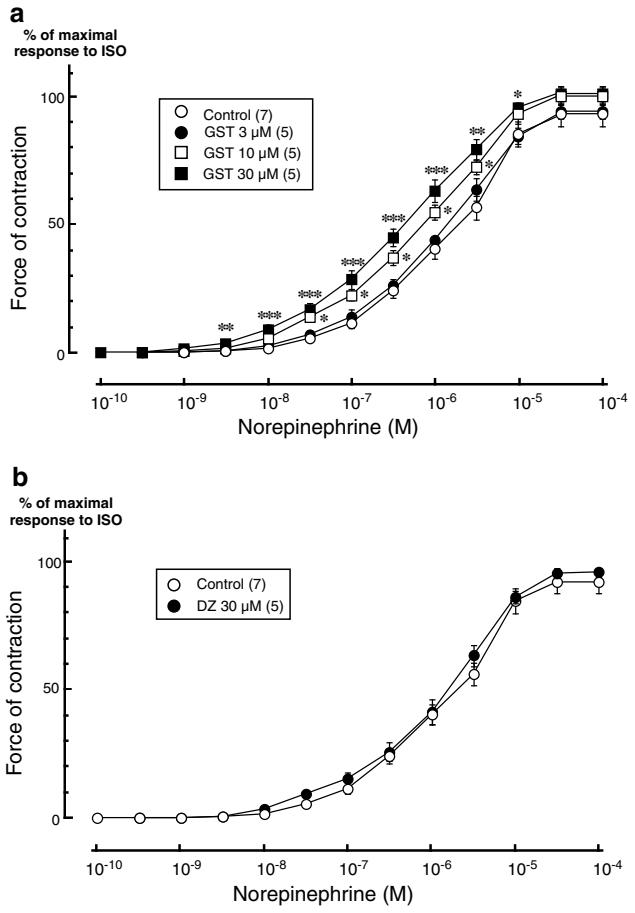


Figure 5 Influence of various concentrations of genistein (a) and daidzein (b) on the CRC for the PIE of NE mediated by β-adrenoceptors in isolated canine ventricular trabeculae. Ordinate: the PIE expressed as a percentage of the ISO_{max}; abscissa: concentration of NE. Open circle: control CRC for NE in the absence of genistein (GST) or daidzein (DZ). Basal forces of contraction prior to the administration of NE and ISO_{max} were 4.33 ± 0.80 and $15.38 \pm 2.64\,mN\,mm^{-2}$ ($n = 27$), respectively. Numbers in parentheses indicate the numbers of muscle preparations examined. Presented are means$\pm$s.e.mean; where they are not shown, s.e.mean is smaller than the symbol. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ vs the corresponding control response to NE. Experiments were carried out in the presence of 300 nM prazosin.

Table 1 Effects of genistein (GST) and daidzein (DZ) on pD_2 values and maximal responses induced by NE in canine ventricular trabeculae

Agents (μM)	n	*pD_2 values*	P	*Maximal response (% of ISO_{max})*	P
Control	7	5.83 ± 0.10	—	94.0 ± 4.28	—
GST (3)	5	5.92 ± 0.07	NS	93.4 ± 2.86	NS
GST (10).	5	6.14 ± 0.09	<0.05	100.3 ± 3.37	NS
GST (30)	5	6.32 ± 0.12	<0.01	101.5 ± 2.76	NS
DZ (30)	5	5.88 ± 0.07	NS	96.1 ± 1.77	NS

Numbers in parentheses indicate the concentrations of GST on DZ at μM; values presented are means$\pm$s.e.mean; ISO_{max}: maximal response to isoproterenol; NS: not significantly different from the control.

the inhibitory effect of carbachol. The NIE 45 min after adding carbachol at 30 nM was $66.3 \pm 6.93\%$ in the absence of genistein, while it was $43.7 \pm 3.31\%$ in the presence of genistein at 10 μM ($n = 9$ each, $P<0.01$).

Summarized data with different concentrations of genistein at 3, 10 and 30 μM are shown in Figure 8d. Genistein at 3 μM did not affect the NIE of ET-1, but at 10 and 30 μM it inhibited the NIE of ET-1 almost completely. In the presence of genistein at 10 and 30 μM, the NIEs 45 min after the addition of 10 nM ET-1 were 12.4 ± 0.40 and $9.10 \pm 0.50\%$ of the maximal response to NE, respectively, which were significantly less than the respective control responses of 64.7 ± 7.33 and $63.8 \pm 22.1\%$.

In isolated ventricular trabeculae, daidzein at 30 μM suppressed likewise markedly the NIE of ET-1 as shown in Figure 8b and c (maximal response: $11.8 \pm 0.50\%$ with daidzein vs control of $71.7 \pm 17.4\%$; $n = 6$; $P<0.001$) and Figure 8b (time course), an indication that the PTK inhibition induced by genistein may not be responsible for the inhibitory action of genistein on the NIE of ET-1.

In ventricular myocytes, NE at 30 nM induced a pronounced increase in cell shortening associated with a marked elevation of the peak indo-1 ratio and a remarkable attenuation of both signals. As shown in Figure 9, decreases in the indo-1 ratio ($51.4 \pm 12.1\%$) and cell shortening ($63.5 \pm 15.9\%$) induced by ET-1 at 10 nM in the presence of NE at 30 nM were significantly attenuated by genistein at 10 μM to $29.5 \pm 2.43\%$ (indo-1 ratio) and $23.8 \pm 1.77\%$ (cell shortening) (Figure 9a), whereas the decreases in the indo-1 ratio ($58.2 \pm 14.9\%$) and cell shortening ($72.0 \pm 27.1\%$) induced by carbachol at 30 nM in the presence of NE at 30 nM were unaffected by genistein at 10 μM. The corresponding values in the presence of genistein were $59.4 \pm 13.1\%$ (indo-1 ratio) and $57.2 \pm 16.2\%$ (cell shortening), respectively.

Discussion

Important findings in the present study obtained utilizing the PTK inhibitor genistein are that PTK activation may be involved in both inhibitory and facilitatory regulation of contractile function and Ca^{2+} transients induced by receptor activation in canine ventricular myocardium. In addition, basic PTK activity may exert a tonic inhibitory action on cardiac contractile function.

Influence of genistein on the PIE of ET-1 induced by crosstalk with NE at low concentrations

In canine ventricular myocardium, ET-1 elicited a PIE in the presence of low concentrations of β-adrenoceptor agonists, for example, NE at 0.1–1 nM (Chu & Endoh, 2000; Takahashi *et al*., 2001; Chu *et al*., 2003b). The PIE of ET-1 induced by crosstalk with NE was inhibited almost completely by genistein. At concentrations used in the current study, genistein may inhibit the PTK activity without any significant effects on activities of other enzymes, including myosin light chain kinase (Di Salvo *et al*., 1993), protein kinase A (PKA) (Akiyama *et al*., 1987; Gazit *et al*., 1989; Di Salvo *et al*., 1993) or PKC (Akiyama *et al*., 1987; Gazit *et al*., 1989). Since daidzein, an inactive derivative of genistein (Peterson & Barnes, 1993), did not affect the PIE of ET-1, it is postulated

that activation of PTK may be responsible for the signal transduction triggered by crosstalk of ET-1 and NE at low concentrations to lead to PIE. It has been shown that the PIE induced by crosstalk of ET-1 and NE is due to the combination of an increase in Ca^{2+} transients and of myofilament Ca^{2+} sensitivity, and requires simultaneous activation of both PKC and PKA (Chu *et al*., 2003b). The inhibitory action of genistein may be ascribed to the signal process involving the activation of PKC, since the cAMP/PKA signaling process was enhanced by genistein. Observations that increases in both Ca^{2+} transients and Ca^{2+} sensitivity were inhibited by genistein imply that the signaling process leads to the final modulation of ion channels and/or contractile proteins may be susceptible to PTK inhibition, and that tyrosine phosphorylation of enzyme and/or regulatory proteins may be an important step in the signaling pathway subsequent to activation of ET receptors. In this context, it is noteworthy that the activation of PTK leads to phosphorylation of phospholipase C, to the resultant stimulation of hydrolysis of phosphoinositides, and the subsequent activation of PKC and mobilization of intracellular Ca^{2+} ions (Auger *et al*., 1989; Homma *et al*., 1993). Angiotensin II and phenylephrine, which stimulate heterotrimeric G_q protein-linked receptors, have been shown to activate PTK, as ET-1 does in cardiac myocytes (Sadoshima *et al*., 1993; Thorburn & Thorburn, 1994). Thus, the pathway leading to the activation of the PTK may involve activation of G_q proteins. The finding that the angiotensin II-induced generation of inositol phosphate is mediated by activation of the PTK pathway in cardiomyocytes (Goutsouliak & Rabkin, 1997) implies a crucial role for the phosphorylation of phospholipase C in the inhibitory action of genistein on the signaling process of G_q protein-linked receptors. The present observations indicate that an increase in myofilament Ca^{2+} sensitivity induced by ET-1 may involve the activation of PTK and are consistent with similar findings in rabbit ventricular myocardium (Wang & Endoh, 2001) and rat vascular smooth muscle (Ohanian *et al*., 1997).

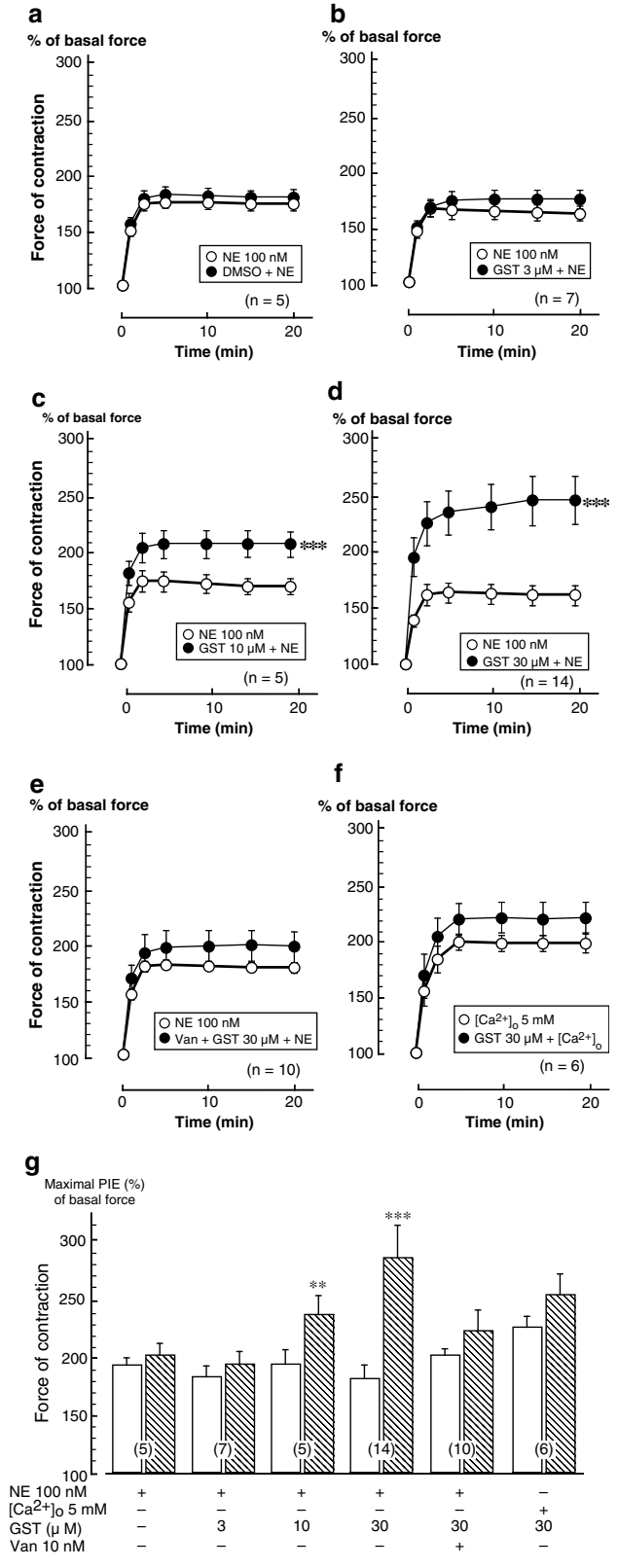


Figure 6 Influence of genistein on the time course of the PIE induced by NE, and by the elevation of $[Ca^{2+}]_o$ to 5 mM in isolated canine ventricular trabeculae. (a) The PIE of 100 nM NE in the absence and presence of DMSO (solvent of genistein). (b–d) Influence of genistein (GST) at 3–30 μM on the PIE of NE. (e) Influence of vanadate (Van) on the effect of genistein (30 μM) on the PIE of NE. (f) Influence of genistein at 30 μM on the PIE of 5 mM $[Ca^{2+}]_o$. Open circle: the control PIEs of NE and $[Ca^{2+}]_o$ in the absence of genistein; closed circle: the PIEs in the presence of genistein. Ordinates: increases in force of contraction expressed as a percentage of the basal value prior to addition of NE and elevation $[Ca^{2+}]_o$; abscissa: time after the addition of NE and elevation $[Ca^{2+}]_o$. (g) Summarized data of (a–f) determined at the maximal steady levels in each series. Open columns: the control PIE in the absence of genistein; hatched columns: the respective PIE in the presence of genistein. Average basal force of contraction before the addition of NE and elevation $[Ca^{2+}]_o$ was 4.98 ± 1.04 mN mm^{-2} ($n = 47$). Numbers in parentheses indicate the numbers of muscle preparations examined. $^{**}P < 0.01$; $^{***}P < 0.001$ vs the respective control values by repeated-measures analysis of variance. Experiments were carried out in the presence of 300 nM prazosin.

Influence of genistein on the PIE of NE mediated via β-adrenoceptors

In contrast to the PIE induced by crosstalk of ET-1 with NE, genistein enhanced the PIE and an increase in Ca^{2+} transients induced by NE *via β*-adrenoceptors. The observations, that (1) the enhancing effect of genistein on the PIE of NE was not shared by the inactive analog daidzein, (2) the PTKase

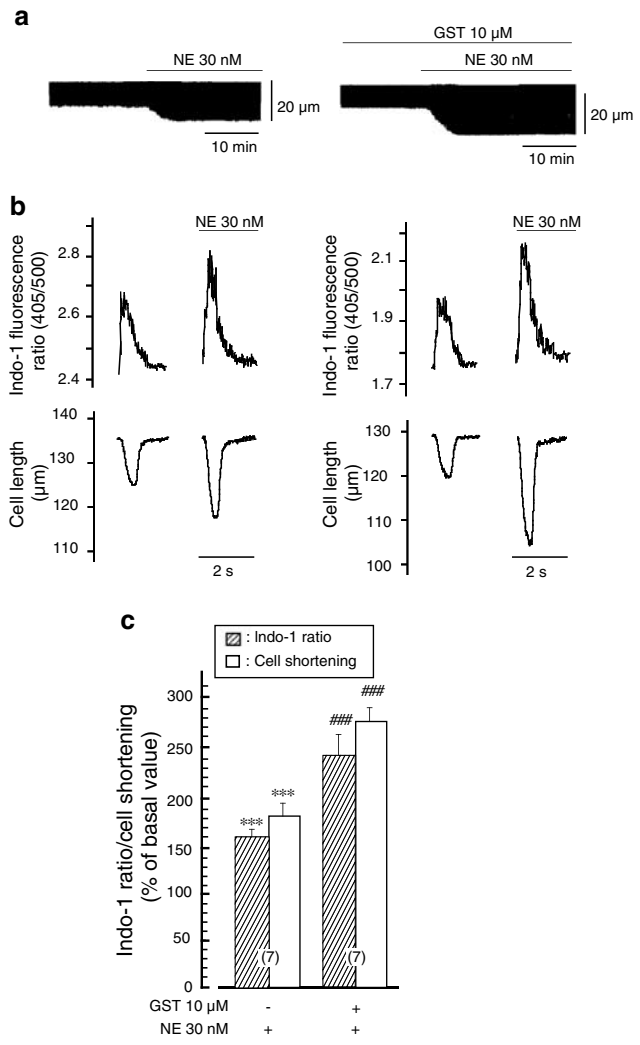


Figure 7 Influence of genistein on the NE-induced increase in Ca^{2+} transients and cell shortening in canine ventricular myocytes. (a) Actual tracings of the effect of NE in the absence (left panel) and in the presence (right panel) of 10 μM genistein (GST) in a myocyte. (b) Individual signal tracings of the indo-1 ratio (upper tracings) and cell shortening (lower tracings) in the same experiments presented in (a). Individual tracings were obtained by means of signal averaging of five successive signals. (c) Summarized data of the influence of genistein on the NE-induced increases in the indo-1 ratio and cell shortening. Basal values (amplitudes of Ca^{2+} transients and cell shortening) prior to the administration of NE were 0.81 ± 0.09 (indo-1 ratio) and 8.51 ± 1.20 μm ($n = 14$), respectively. Numbers in parentheses indicate the numbers of cells. $^{***}P < 0.001$ vs the basal values; $^{\#\#\#}P < 0.001$ vs the respective control responses to NE at 30 nM in the absence of genistein. Experiments were carried out in the presence of 300 nM prazosin.

inhibitor vanadate reversed the enhancing action of genistein on the PIE of NE, (3) genistein at 30 μM as applied in the current study was within the concentration range for specific PTK inhibition (Akiyama *et al*., 1987) and (4) genistein did not affect the PIE induced by elevation of $[Ca^{2+}]_o$ (5 mM), imply that the enhancing action of genistein on the PIE of NE mediated by activation of β-adrenoceptors may be ascribed to the inhibition of PTK.

A question arises about the mechanism and the role of PTK activation in β-adrenoceptor-mediated PIE and regulation of Ca^{2+} transients. Under physiological conditions in the absence of β-adrenoceptor stimulation, constitutive activation of the PTK may exert a tonic inhibitory influence on some processes of the cAMP-mediated signaling pathway, and genistein may potentiate PIE induced *via* β-adrenoceptors due to the suppression of tonic inhibition (Hool *et al*., 1998). The results that genistein alone exerted a PIE, that is, increased the basal force of contraction, support this possibility. It is noteworthy that receptor-mediated signaling is more susceptible than basal contractility to genistein. The effect of genistein on β-adrenoceptors is also probable: the tyrosine phosphorylation of β_1- (Ho *et al*., 1995) as well as β_2-adrenoceptors (Hadcock *et al*., 1992; Karoor *et al*., 1995) suppressed the coupling of receptors to stimulation of adenylyl cyclase and subsequent accumulation of cAMP. The view that tyrosine kinase activity directly inhibits the β-adrenoceptor-mediated function in cardiac myocytes is supported by the previous findings on β-adrenoceptor-mediated regulation of L-type Ca^{2+} channels by Sims *et al*. (2000) and Belevych *et al*. (2001).

Effects of genistein on basal contractility and Ca^{2+} transients

The PTK inhibitor genistein at the high concentrations of 30–100 μM induced a PIE in canine ventricular trabeculae and a significant increase in cell shortening in association with an increase in Ca^{2+} transients in canine ventricular myocytes. These effects of genistein may be due to the inhibition of the PTK activity, because (1) the concentrations used are in the range to inhibit PTK activity (Akiyama *et al*., 1987) and (2) daidzein, an inactive analog of genistein (Peterson & Barnes, 1993), did not exert stimulatory effects. These results are essentially consistent with those in isolated perfused rat heart (Taskinen *et al*., 1994), and that genistein stimulated $I_{Ca(L)}$ by inhibition of PTK in human and cat atrial myocytes (Wang & Lipsius, 1998; Boixel *et al*., 2000), and strengthen the view that PTK may play a crucial role in the regulation of $I_{Ca(L)}$ in various types of cells, including myometrial (Kusaka & Sperelakis, 1995), vascular smooth muscle (Wijetunge *et al*., 1992; Huang *et al*., 1997), pituitary cells (Cataldi *et al*., 1996) and cardiac myocytes (Hool *et al*., 1998; Wang & Lipsius, 1998; Boixel *et al*., 2000). It has been reported that PTK regulates $I_{Ca(L)}$ by direct phosphorylation of the α-subunit of L-type Ca^{2+} channels, as is the case with nonreceptor PTK c-Src and focal adhesion kinase in smooth muscle cells (Hu *et al*., 1998). It is postulated that PTK inhibits L-type Ca^{2+} channels and disinhibition by genistein increases $I_{Ca(L)}$ in human and cat atrial myocytes (Wang & Lipsius, 1998; Boixel *et al*., 2000). On the other hand, it has been reported that in guinea-pig ventricular myocytes, genistein increases Ca^{2+} transients by an increase in the SR Ca^{2+} store and inhibition of Na^+/Ca^{2+} exchange in spite of a decrease in $I_{Ca(L)}$ (Liew *et al*., 2003).

Influence of genistein on the NIE of ET-1 induced by crosstalk with NE at high concentrations

ET-1 induced a pronounced NIE, apparently very similar to the 'antiadrenergic effect' of muscarinic M_2-receptor agonists such as acetylcholine and carbachol (Endoh, 1999). In ventricular myocytes, ET-1 and carbachol at the concentra-

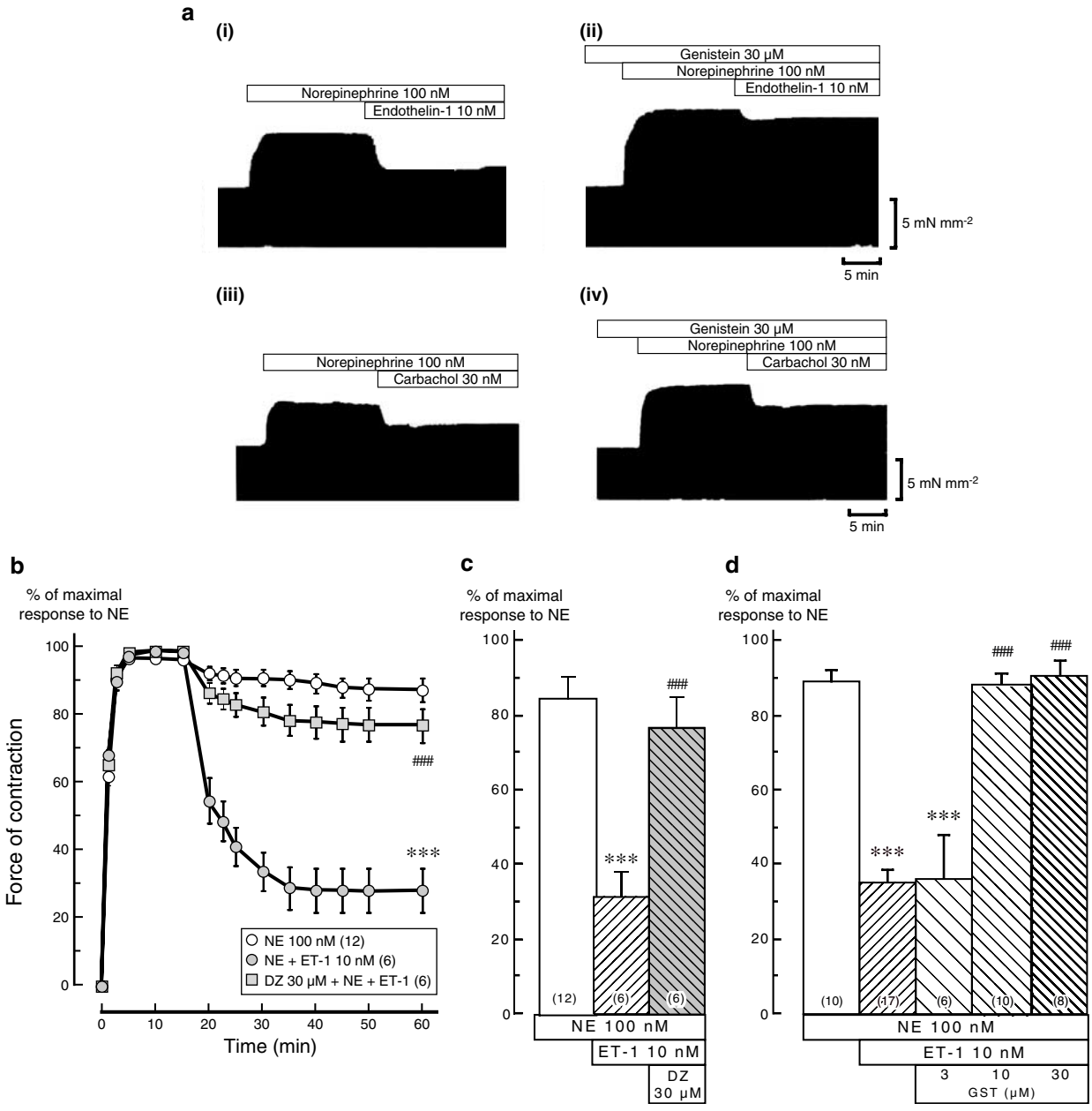


Figure 8 Influence of genistein on the NIE of 10 nM ET-1 in the presence of 100 nM NE in isolated canine ventricular trabeculae. (a) Actual tracings of the NIE of 10 nM ET-1 (upper panel: (i) and (ii)) and 30 nM carbachol (CCh) (lower panel: (iii) and (iv)) in the absence (left tracings) and the presence (right tracings) of genistein (GST) at 30 μM. (b, c) Influence of daidzein (DZ) at 30 μM on the NIE of 10 nM ET-1 in the presence of 100 nM NE (b: time course; c: extent). (d) Influence of genistein at various concentrations on the ET-1-induced NIE in the presence of 100 nM NE. The maximal response to 100 nM NE before the addition of ET-1 was assigned to 100% for each preparation, and the changes in force of contraction are expressed as the percentage of the control NE-induced response. Baseline force of contraction and maximal increase in force induced by 100 nM NE were 6.18 ± 1.03 and 10.32 ± 2.27 mN mm^{-2} ($n = 40$), respectively. Presented are means $\pm$ s.e.mean. Numbers in parentheses indicate the numbers of muscle preparations examined. ***$P < 0.001$ vs the control response to NE; $^{\#\#\#}P < 0.001$ vs the response to ET-1 + NE in the absence of genistein or daidzein. Experiments were carried out in the presence of 300 nM prazosin.

tions employed decreased the NE-induced increase in contractility and Ca^{2+} transients almost to a similar extent. The NIE of ET-1 may be due to the ET-1-induced inhibition of facilitation of $I_{Ca(L)}$ mediated by β-adrenoceptors *via* pertussis toxin-sensitive G (G_i)-dependent signal pathway in canine ventricular myocytes (Zhu *et al*., 1997; Watanabe & Endoh, 2000; Chu *et al*., 2003b). The NIE of ET-1 takes place without a detectable lowering of cAMP levels in dog (Zhu *et al*., 1997) and human myocardium (Walker *et al*., 2001). The effect of 8-bromo-cAMP, which activates directly the PKA activity, was also suppressed by ET-1 (Reid *et al*., 1991; Watanabe & Endoh, 1999). In dog ventricular myocardium, the NIE of ET-1 has been shown to be inhibited by guanylyl cyclase and PKG inhibitors (Chu *et al*., 2003b), and by protein phosphatase (PP)

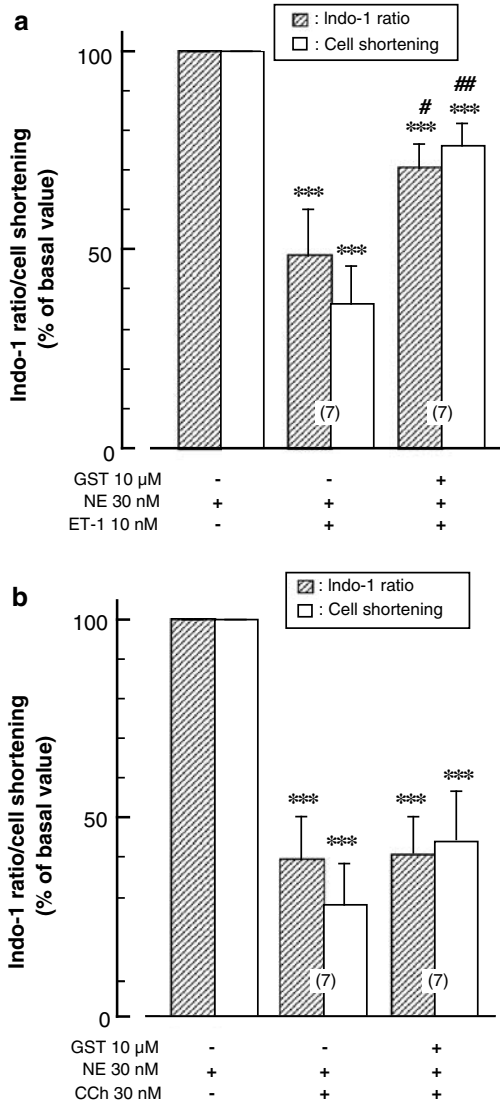


Figure 9 Influence of genistein on the ET-1-induced decrease in the indo-1 ratio and cell shortening in the presence of NE in canine ventricular myocytes. (a) Influence of genistein (GST) at 10 μM on the effects of 10 nM ET-1 in the presence of NE at 30 nM. (b) Influence of genistein at 10 μM on the effects of 30 nM carbachol (CCh) in the presence of NE at 30 nM. Basal and the maximal values of Ca^{2+} transients and cell shortening induced by NE were 0.94 ± 0.07 and 1.48 ± 0.01 (indo-1 ratio), and 9.44 ± 0.58 and 18.4 ± 1.09 μm (cell shortening) ($n = 28$), respectively. Numbers in parentheses indicate the numbers of cells. $^{***}P < 0.001$ vs 100 nM NE alone; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs the respective values with ET-1 + NE. Experiments were carried out in the presence of 300 nM prazosin.

inhibitor (Chu *et al*., 2003a, b), an indication that the NIE of ET-1 is mediated *via* the G_i/cGMP/PKG/PP signal pathway (Chu *et al*., 2003b).

Genistein at 10–30 μM inhibited the NIE of ET-1 in the presence of NE. While attenuation of the inhibitory action by genistein could be due to an enhancement of the PIE of NE that occurred over the same concentration of genistein, this is unlikely because the effect of carbachol was unaffected by genistein. The absence of effects of genistein on the NIE of carbachol is consistent with previous findings that the PTK does not contribute to the inhibitory regulation induced by carbachol (Yang *et al*., 1992; 1993; Fleichman *et al*., 2004). While the NIEs of ET-1 and carbachol in the presence of NE appear to be similar, the findings in the current study together with previous observations (Endoh, 1999; Chu *et al*., 2003a, b) imply that the subcellular mechanisms involved are not the same. Namely, susceptibility of the ET-1-induced effect to the PP inhibitor cantharidin is much higher than that of carbachol (Chu *et al*., 2003a).

Daidzein showed almost the same inhibitory action as genistein on the NIE of ET-1. While the possibility that different PTK isoforms are involved cannot be completely excluded, it appears more likely that the PTK-unrelated but structurally related mechanism may contribute to the inhibitory action of daidzein. Similarity of the action induced by genistein and daidzein has also been reported in earlier studies. In murine mammary carcinoma cells, genistein and daidzein inhibited cell growth with similar potencies (Scholar & Toews, 1994). In rat ventricular cells, genistein and daidzein both inhibited $I_{Ca(L)}$ (Yokoshiki *et al*., 1996). Genistein and daizein have been shown to be partial agonists of estrogen receptors with identical affinities (Han *et al*., 2002; Murata *et al*., 2004), although the role of such effects in cardiac functional regulation has not yet been known and remains for future study. These observations, however, together with the current findings imply that genistein possesses an additional action unrelated to PTK inhibition, which is shared by daidzein.

In summary, the current study indicates that in canine ventricular myocardium and myocytes, genistein exerts actions as a PTK inhibitor and the action is unrelated to the PTK inhibition. Genistein induced (1) inhibition of the PIE and Ca^{2+} signal induced by crosstalk of ET-1 and NE, (2) enhancement of the PIE and Ca^{2+} transients induced by NE *via* β-adrenoceptors, and (3) a direct facilitatory action on basal contractility and Ca^{2+} transients through the former mechanism. In addition, genistein inhibited the NIE of ET-1 in the presence of a high concentration of NE, which was mimicked by an inactive analog, daidzein. The present findings indicate that the activity of PTK may play a crucial role in cardiac contractile function by modulation of basal as well as receptor-mediated control of Ca^{2+} signaling under physiological and pathophysiological conditions.

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