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Effects of tyrosine kinase inhibitors on the contractility of rat mesenteric resistance arteries

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1 A pharmacological characterization of tyrosine kinase inhibitors (TKI) belonging to two distinct groups (competitors at the ATP-binding site and the substrate-binding site, respectively) was performed, based on their effects on the contractility of rat mesenteric arteries.

2 Both the ATP-site competitors (genistein and its inactive analogue, daidzein) and the substrate-site competitors (tyrphostins A-23, A-47 and the inactive analogue, A-1) reversibly inhibited noradrenaline (NA, (10 μM)) and KCl (125 mM) induced contractions, concentration-dependently. Genistein was slightly but significantly more potent than daidzein; the tyrphostins were all less potent than genistein, and there were no significant differences between the individual potencies. The tyrosine kinase substrate-site inhibitor *bis*-tyrphostin had no inhibitory effect.

3 Genistein, daidzein, A-23 and A-47 each suppressed the contraction induced by Ca^{2+} (1 μM) in α-toxin permeabilized arteries. A-1 and *bis*-tyrphostin had little or no effect on contraction of the permeabilized arteries.

4 Genistein was significantly more potent than daidzein with respect to inhibition of the contraction induced by 200 nM Ca^{2+} in the presence of NA (100 μM) and GTP (3 μM). The effect of A-23, A-47, A-1 and *bis*-tyrphostin was similar in permeabilized arteries activated with Ca^{2+} (200 nM) + NA (100 μM) + GTP (3 μM) and permeabilized arteries activated with 1 μM Ca^{2+}.

5 Genistein (30 μM) reduced the fura-2 measured intracellular calcium activity ($[Ca^{2+}]_i$) in arteries stimulated with NA but had no effect on $[Ca^{2+}]_i$ in arteries stimulated with KCl (125 mM).

6 The potent effect of the TKIs in this study is consistent with a role for tyrosine kinases in the mechanisms which regulate both cytoplasmic Ca^{2+} levels and the effect of Ca^{2+} on the contractile apparatus in smooth muscle cells in resistance arteries. However, the results must be interpreted cautiously because the enzyme inhibitors may have a poor specificity in intact tissues and because the presumed inactive analogues had potent effects.

Keywords: Tyrosine kinase inhibitors: vascular smooth muscle; tyrphostin; genistein; daidzein; rat mesenteric resistance arteries

Introduction

During the last decade it has become clear that tyrosine phosphorylation of proteins plays a major role in the diverse cellular signalling pathways which are involved in cell proliferation and transformation (Bishop, 1987; Draetta *et al.*, 1988), cellular interactions with the extracellular matrix (Schaller & Parsons, 1993) and the regulation of neurotransmitter receptors (O'Dell *et al.*, 1991): for review see Glenney (1991). Against this background, tyrosine kinase inhibitors (TKIs) have recently been developed. Two groups of TKIs have been described: compounds interacting with the ATP binding site, such as genistein, a quercetin derivative (Akiyama *et al.*, 1987; Casnellie, 1991) and those which interact with the substrate binding site, such as the tyrphostins, which are synthetic analogues of erbstatin (Levitzki & Gilon, 1991; Casnellie, 1991). Inactive analogues with respect to the epidermal growth factor receptor kinase are also now available for each group, namely daidzein and tyrphostin A-1, respectively.

In smooth muscle cells, growth factors activate tyrosine kinases (Auger *et al.*, 1989; Yang *et al.*, 1992; Weiss & Nuccitelli, 1992), and also have agonistic effects with respect to force development (Berk *et al.*, 1986; Yang *et al.*, 1992). Conversely, classical constrictor agonists like angiotensin II, vasopressin and carbachol have been shown to activate smooth muscle tyrosine kinase and cause tyrosine phosphorylation (Tsuda *et al.*, 1991), and have growth promoting effects. Thus, the tyrosine phosphorylation associated with the application of these hormones could be of importance for both the growth response and the contractile response of these cells.

Much of the evidence indicating that tyrosine kinase activity influences force development is based on the potent antagonistic effect of TKIs against growth factor-induced force development in smooth muscles (see, Hollenberg, 1994). For the classical constrictor agonists such evidence is less clear. For some agonists, like carbachol and bradykinin, the inhibitory potency of the TKIs is reported to be low (Yang *et al.*, 1992; 1993), while for others, like angiotensin II, the potency is high (Yang *et al.*, 1993). Moreover, in a recent study, TKIs were shown to have an inhibitory effect in moderately high concentrations against carbachol- and noradrenaline (NA)-induced contraction (DiSalvo *et al.*, 1993), while in another study they were without effect against phenylephrine- and phorbol ester-induced contraction (Sauro & Thomas, 1993). This prompted us to investigate a range of TKIs for their concentration-dependent effect on the calcium-dependent and independent regulation of tone in rat isolated small mesenteric arteries. Experiments were also performed to determine the effect of TKIs on the intracellular calcium activity ($[Ca^{2+}]_i$) in these vessels.

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Methods

Preparation

Mesenteric resistance arteries (second or third order branches of the superior mesenteric artery) from male Wistar rats (12–15 weeks old) killed with CO_2 were used for all experiments. The procedures used to isolate the arteries and the myograph for isometric force measurements have been described previously (Mulvany & Halpern, 1977). Briefly, the arteries were dissected free from the surrounding connective tissue. Segments, approximately 2 mm long, were mounted in an isometric myograph (JP-trading, Denmark), as ring preparations. The internal circumference of the vessels was set to 0.9 times the circumference the vessels have at 100 mmHg, based on the passive length-tension curve (Mulvany & Halpern, 1977). At this setting, the arteries had an internal diameter of about 200 μm and develop near maximal active tension during stimulation (Mulvany & Halpern, 1977). In all experiments, except those where the arteries were permeabilized, pretreatment with 6-hydroxydopamine (Aprigliano & Hermsmeyer, 1976), or 100 μM guanethidine for 20 min, was used to eliminate the effect of endogenous NA. N^G-nitro-L-arginine methyl ester (L-NAME, 10 μM) was added 30 min before the experimental procedure and during experiments to avoid possible interference from NO release from endothelial cells. All experiments were started by repetitively stimulating vessels with a solution of 125 mM K^+ (KPSS, for composition see below) containing 10 μM NA for 2 min with 10 min between stimulations, until reproducible contractions were elicited.

Experimental procedure

The effects of different TKIs on contractions induced by 10 μM NA or KPSS were assessed 10 min after inducing contractions. Cumulative concentration-response curves were obtained for tyrphostin A-1, A-23, A-47, *bis*-tyrphostin, genistein and daidzein. All TKIs were dissolved in dimethylsulphoxide (DMSO). Time control experiments where the vehicle was added, were made before and after the application of TKI. The maximal DMSO concentration in the bath was 1.27% (for 100 μM TKI).

Permeabilization with α-toxin

The procedure used to permeabilize these vessels with α-toxin was described by Jensen (1994). The arteries were mounted as described above and stimulated once with KPSS at room temperature (experiments with permeabilized preparations were always conducted at room temperature). The bubbling was changed to 100% O_2 and the arteries were incubated for 15–20 min in relaxing solution (for composition see below). Permeabilization was made by incubating the arteries 10–15 min in 10 μl relaxing solution with 1 μM free Ca^{2+} and 1000 u ml^{-1} α-toxin. After permeabilization, the arteries were held in relaxing solution. They were stimulated twice with 10 μM free Ca^{2+} before further experimental procedures. The effect of the different TKIs was assessed on contractions induced with 1 μM free Ca^{2+} to give near maximal contraction or with 200 nM free Ca^{2+} after pretreatment of the arteries with 100 μM NA and 3 μM GTP for 10 min, which gave the same contraction as 1 μM free Ca^{2+}. Time control experiments where the vehicle was added, were carried out before and after the application of TKI.

Simultaneous measurements of cytoplasmic free calcium and force

We have previously described this technique and the calibration of the fluorescence signal in detail (Jensen *et al.*, 1992; 1993). Briefly, arteries mounted in the myograph were loaded with fura-2. The arteries were stimulated every 10 s with 347 and 380 nm light. The emitted light was measured by a photomultiplier through a 500–530 nm filter and a cut-off (<720 nM) filter. The system was controlled by a computer, and both force and the two emission signals (F_{347} and F_{380}) were stored for subsequent analysis. At the end of the experiment fura-2 signals were calibrated, based on the determination of the maximal (R_{max}) and minimal (R_{min}) fluorescence ratio and the ratio (β) between the maximal and minimal fluorescence at 380 nM (Grynkiewicz *et al.*, 1985; Jensen *et al.*, 1992). Background fluorescence was determined after quenching with 20 mM $MnCl_2$. With this setup the only inhibitor we could use was genistein, because the other TKIs quenched more than 90% of the fura-2 signals.

Solutions

The physiological saline solution (PSS) had the following composition (mM): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 25, $CaCl_2$ 2.5, ethylenediaminetetraacetic acid (EDTA) 0.026 and glucose 5.5. The PSS was bubbled with 95% O_2 and 5% CO_2 (pH = 7.45–7.50 at 37°C). PSS containing high K^+ (KPSS, 125 mM K^+) was prepared by iso-osmolar substitution of NaCl with KCl. Relaxing solution had the following composition (mM): EGTA 2, potassium methane sulphonate 130, $MgCl_2$ 4, Tris maleate 20, Na_2ATP 4, creatine phoshate 10 and creatine phosphokinase 1 mg ml^{-1}. pH was adjusted to 7.15 with KOH and the solution was gassed with 100% O_2.

Chemicals

Fura-2AM and pluronic F127 (Molecular Probes, Oregon, U.S.A.), α-toxin (GIBCO, U.S.A.), tyrphostins, genistein and daidzein (Calbiochem, California, U.S.A.), cremophor EL, noradrenaline, nigericin, ionomycin, guanethidine, L-NAME and 6-hydroxydopamine (Sigma Chemicals, Poole, Dorset, U.K.) were used. The high purity EGTA (>99%) used in solutions for *in situ* calibration of fura-2 was from Fluka (Buchs, Switzerland). The TKIs were prepared as 10 mM stock solutions in DMSO.

Statistics

All results are shown as arithmetric means ± s.e.mean, n is the number of arteries, except for the mean $[Ca^{2+}]_i$ values, which are given as geometric means. Student's two-tailed t test or one way ANOVA test followed by Bonferroni test were used for statistical comparisons. $P<0.05$ was considered as a significant difference.

Results

Effects of TKIs on contraction induced with NA and KPSS

The effects of TKIs on tonic contractions induced with either 10 μM NA or KPSS were assessed. The results are presented in two main groups, according to the two different mechanisms for inhibition of the tyrosine kinases.

In the first group (Figure 1a and b), genistein and daidzein caused similar concentration-dependent relaxations of the active tension induced with both NA and KPSS. The pIC_{50}s ($-\log IC_{50}$, where IC_{50} is the concentration of the compounds causing a 50% inhibition of the contraction) of genistein and daidzein in arteries activated with KPSS were 4.65 ± 0.01 and 4.47 ± 0.03, $n = 4$, respectively ($P<0.05$). The pIC_{50}s of genistein and daidzein in arteries activated with 10 μM NA were 4.86 ± 0.10 and 4.56 ± 0.08, $n = 4$, respectively ($P<0.05$). A slight but significantly greater potency of genistein compared to daidzein was thus observed both in vessels activated with NA and with KPSS.

In the second group, the effects of four tyrphostins were assessed. Tyrphostin A-23, A-47 and A-1 caused concentration-dependent relaxations of contraction induced with both NA and KPSS (Figure 1c and d), but *bis*-tyrphostin had no significant effect on contractions. Tyrphostin A-23 in a concentration of 100 μM caused a complete inhibition of the force development and pIC_{50} was 4.30 ± 0.06, $n = 6$ and 4.43 ± 0.13, $n = 6$ for arteries activated with KPSS and 10 μM NA, respectively. The inhibitory effect of tyrphostin A-47 and A-1 was too small, in the concentration-range tested, to calculate the IC_{50}. After washout of genistein, daidzein, tyrphostin A-23, A-47 and A-1 the response to NA and KPSS almost completely recovered (data not shown except for genistein see Figure 2).

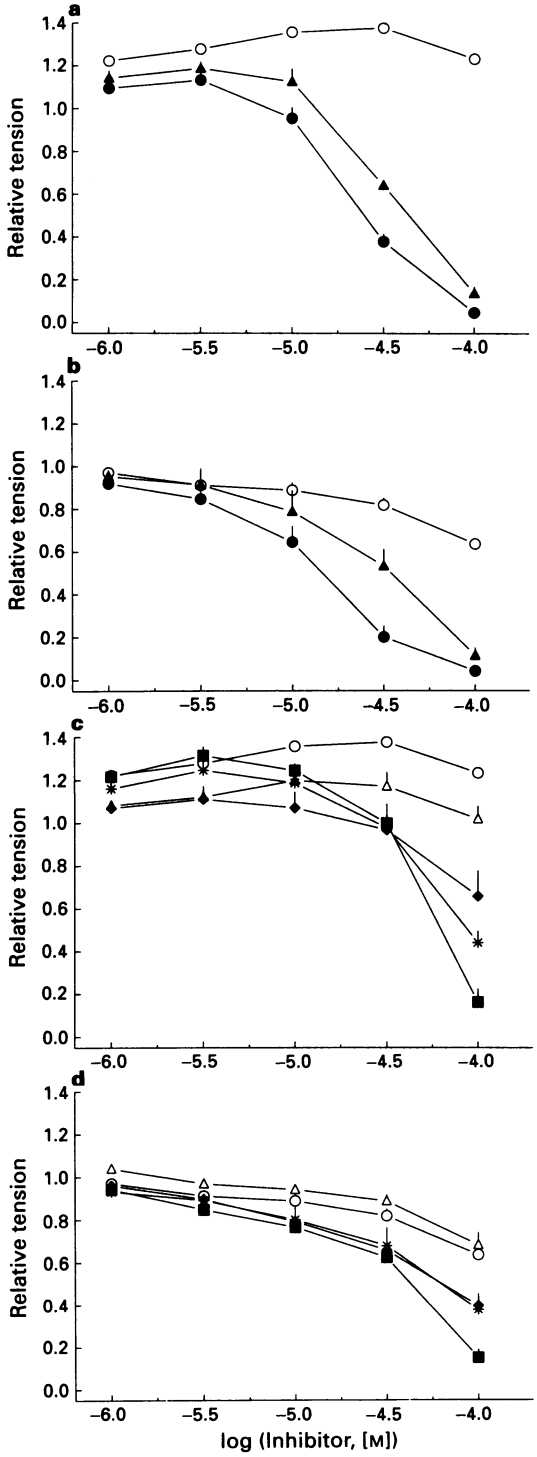

Figure 1 Effects of tyrosine kinase inhibitors (TKIs) on potassium-induced contraction (a and c) and noradrenaline-induced (10 μM) contraction (b and d) in intact arteries. The responses are expressed relative to the contraction immediately before the first concentration of drug or vehicle was added. Each point represents mean (with s.e.mean of 4–6 vessels where they exceed the size of the symbols). (a and b): (○) Vehicle; (●) genistein; (▲) daidzein. (c and d): (○) Vehicle; (△) *bis*-tyrphostin; (◆) A-47; (*) A-1; (■) A-23.

Effects of genistein on force and $[Ca^{2+}]_i$ during stimulation with NA and KPSS

Arteries were pretreated for 10 min with 30 μM genistein before stimulation with NA or KPSS. When genistein was added to the medium, both the F_{347} and the F_{380} signals

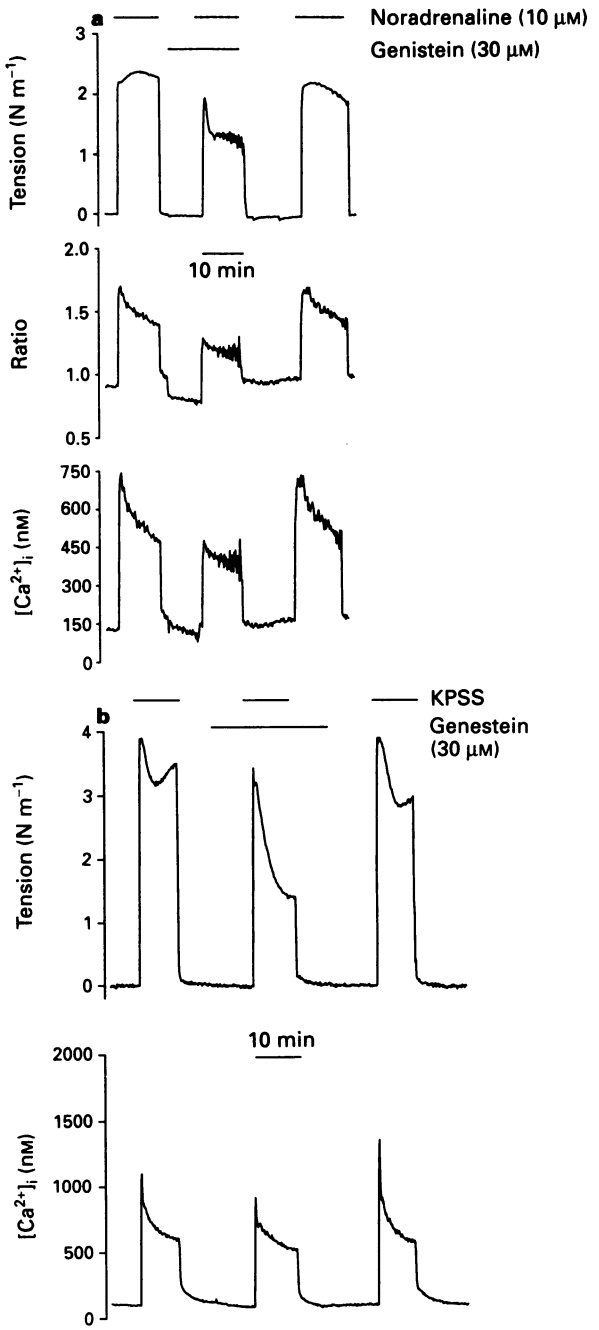

Figure 2 Recording of measurements of contraction and $[Ca^{2+}]_i$ during activation with noradrenaline (NA) (a) and high K^+ physiological saline solution (KPSS) (b). Where indicated 30 μM genistein was present. In (a) the middle trace shows the ratio of fura-2 fluorescence before correction for genistein-induced quenching of the fluorescence.

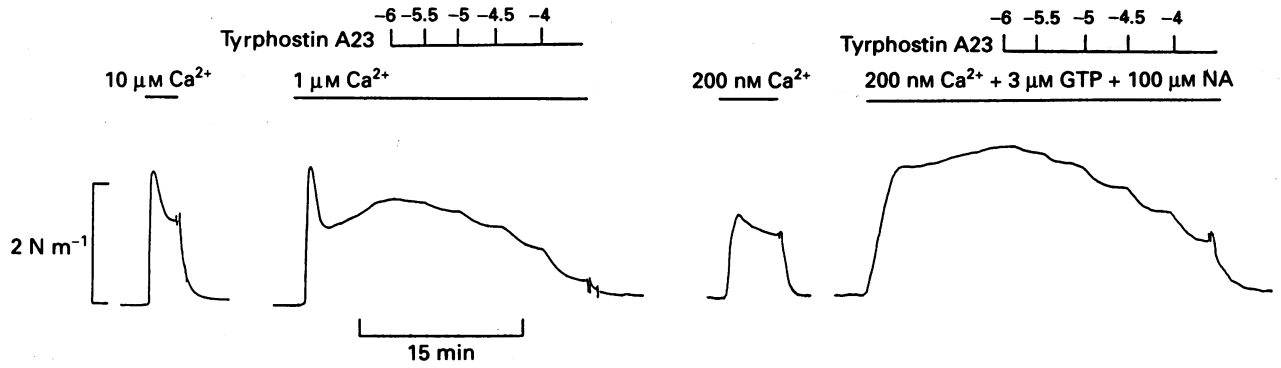

Figure 3 Trace showing the contraction in α-toxin-permeabilized mesenteric small arteries during stimulation with 1 μM Ca^{2+} and with 200 nM Ca^{2+} in the presence of 100 μM noradrenaline (NA) and 3 μM guanosine 5'-triphosphate (GTP). Where indicated, tyrphostin A-23 was added in increasing concentrations.

dropped significantly, and also the ratio was reduced (Figure 2a) as previously reported (Sargean *et al.*, 1993). The effect was quickly reversible after washout of genistein. At the end of experiments after fura-2 signals had been quenched by Mn^{2+}, 100 μM genistein had little effect on the remaining background signals (data not shown). This indicated that genistein quenched some of the Ca^{2+}-sensitive signals of fura-2, and could also reflect an effect of genistein on the K_d of fura-2 for $[Ca^{2+}]_i$. However, if 30 μM genistein was added to the acid form of fura-2 in PSS, there was little effect on fura-2 signals, indicating that genistein affected only the fura-2 signals from the cells. To circumvent these problems, the Ca^{2+}-sensitive fluorescence signals in the presence of genistein were calibrated using a R_{max}, R_{min} and β which were determined in the presence of genistein. Secondly, the K_d of the fura-2 Ca^{2+} complex in the presence of genistein was determined in the cells as described previously (Jensen *et al.*, 1993) and found not to be different from the K_d in the absence of genistein. For calibration of the fura-2 signals we therefore used the previously determined K_d of 342 nM (Jensen *et al.*, 1993).

Figure 2 depicts the effect of 30 μM genistein on active tension and $[Ca^{2+}]_i$ during stimulation with NA (Figure 2a) and during stimulation with KPSS (Figure 2b). In the presence of genistein, active tension after 10 min stimulation was significantly lower with both NA activation (2.35 ± 0.15 Nm^{-1} and 0.96 ± 0.21 Nm^{-1}, $n = 6$; control and genistein treated, respectively) and KPSS activation (2.72 ± 0.40 Nm^{-1} and 1.27 ± 0.32 Nm^{-1}, $n = 4$; control and genistein-treated, respectively). However, $[Ca^{2+}]_i$ at this time was significantly affected by genistein only during stimulation with NA ($p([Ca^{2+}]_i)$: 6.272 ± 0.048 and 6.592 ± 0.127, $n = 6$; control and genistein-treated, respectively); during KPSS activation no difference in $[Ca^{2+}]_i$ was observed ($p([Ca^{2+}]_i)$: 6.153 ± 0.037 and 6.163 ± 0.043, $n = 4$; control and genistein, respectively).

Effects of TKI on tension of α-toxin permeabilized arteries

Figure 3 illustrates the protocol used in the experiments with α-toxin permeabilized preparations. After α-toxin permeabilization, the arteries developed an active tension of 1.81 ± 0.07 N m^{-1} ($n = 50$) when maximally stimulated with 10 μM free Ca^{2+}. This response was $52 \pm 2\%$ of the response to KPSS (3.53 ± 0.12 N m^{-1}, $n = 50$) in the preparations before permeabilization. Since the response of rat mesenteric small arteries to KPSS is reduced to about 55% with denervation or in the presence of an α-adrenoceptor blocker (see e.g. Jensen *et al.*, 1992) this suggests that probably all of the smooth muscles in the preparation are activated by the 10 μM free Ca^{2+} and therefore presumably permeabilized. The effects of the two groups of TKIs on the contraction induced by a submaximal free Ca^{2+} concentration (1 μM) are shown in Figure 4a and b, and their inhibitory potencies are given in Table 1. Ca^{2+} (1 μM) elicited, in most preparations, a biphasic response with an initial transient and a second sustained phase which reached a plateau after 10–15 min (Figure 3). The plateau tension was 1.09 ± 0.09 N m^{-1} ($n = 28$), and this response was $60.1 \pm 0.5\%$ of the maximal tension elicited by 10 μM free Ca^{2+}.

Tyrosine kinase inhibitors relaxed in a concentration-dependent and reversible manner the contraction induced by Ca^{2+}. At the highest concentration used (100 μM), the maximal inhibitions elicited by genistein and daidzein were $93 \pm 2\%$ ($n = 5$) and $90 \pm 4\%$ ($n = 5$), respectively, of the Ca^{2+}-induced contraction (Figure 4a). The relaxations evoked by tyrphostin A-23 and tyrphostin A-47 were $62 \pm 8\%$ ($n = 6$) and $80 \pm 4\%$ ($n = 6$) (Figure 4b), respectively. In contrast, 100 μM tyrphostin A-1 induced only a slight inhibition of the contraction induced by Ca^{2+}, whereas the effect of *bis*-tyrphostin was not significantly different from that of the vehicle. There were no significant differences among the inhibitory potencies of the active compounds on the contractions induced by 1 μM free Ca^{2+} (Table 1).

Effects on contraction induced with Ca^{2+} in the presence of NA and GTP

Addition of 100 μM NA and 3 μM GTP 10 min before stimulation, increased the contraction to 200 nM Ca^{2+} from 0.54 ± 0.06 N m^{-1} ($n = 26$) to 1.31 ± 0.13 N m^{-1} ($n = 26$). These responses were $29 \pm 6\%$ and $70 \pm 3\%$ of the maximal tension elicited by 10 μM free Ca^{2+}, respectively. Figure 4c and d shows the effects of TKIs on contraction induced by Ca^{2+} in the presence of NA and GTP. The maximal relaxations evoked by genistein and daidzein were $96 \pm 2\%$ ($n = 5$) and $93 \pm 2\%$ ($n = 5$) of the initial contraction, respectively (Figure 4c). The maximal relaxations elicited by tyrphostin A-23 and tyrphostin A-47 were $68 \pm 2\%$ ($n = 4$) and $87 \pm 2\%$ ($n = 6$), respectively (Figure 4d). The effect of *bis*-tyrphostin on contraction to Ca^{2+} in the presence of NA and GTP was not significantly different from that of the vehicle. Tyrphostin A-1 evoked a slight inhibitory effect at the highest concentrations used (Figure 4d).

Genistein was more potent in inhibiting contractions elicited by Ca^{2+} in the presence of NA and GTP, than contraction elicited by Ca^{2+} alone (Table 1), while for the other TKIs no significant difference was observed.

Discussion

In the present study, the effects of two structurally and pharmacologically distinct groups of TKIs on active tension

in mesenteric small arteries have been characterized. In the discussion we first focus on the potency of these substances and secondly we discuss the possible role of tyrosine kinases for the excitation-contraction coupling in small arteries.

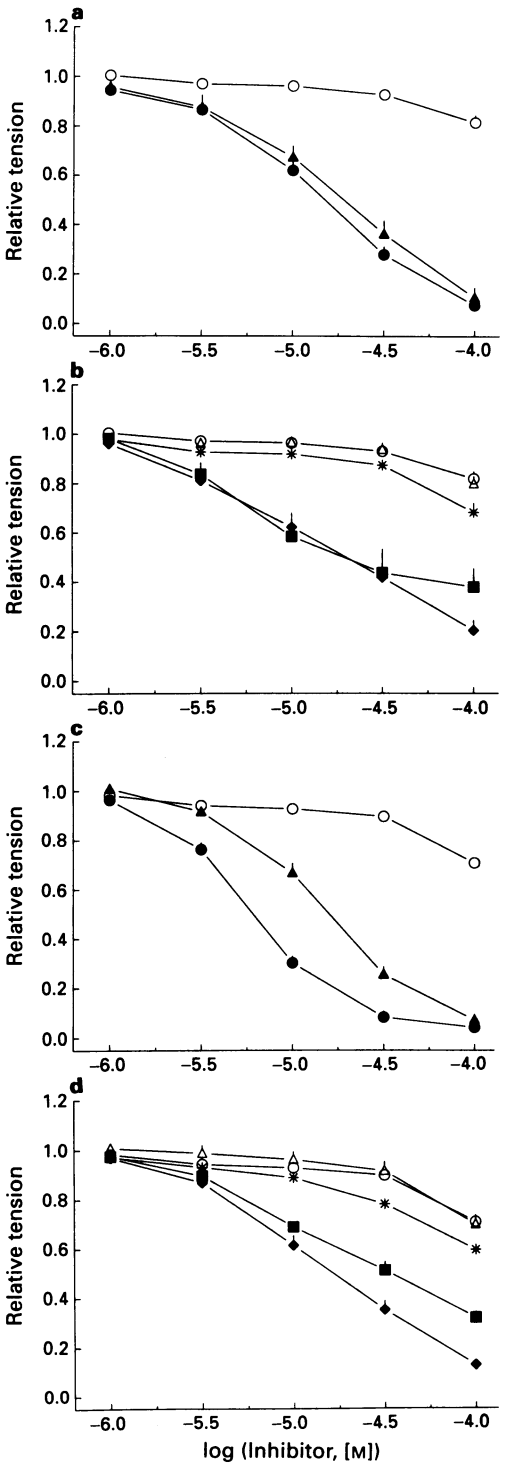

Figure 4 Effects of tyrosine kinase inhibitors (TKI) on Ca^{2+} (a, b) and Ca^{2+} + noradrenaline (NA) + guanosine 5'-triphosphate (GTP) (c, d) induced contraction of α-toxin-permeabilized mesenteric small arteries. Concentration-response curves for the inhibitory action of genistein and daidzein (a, c) and tyrphostins (b, d) are shown. Responses are expressed as percentage of the contraction just prior to the addition of the inhibitor or vehicle. Each point represents mean (with s.e.mean where they exceed the size of the symbols) of 4–10 vessels. (a and c): (○) Vehicle; (●) genistein; (▲) daidzein. (b and d): (○) Vehicle; (△) *bis*-tyrphostin; (◆) A-47; (*) A-1; (■) A-23.

Potency of tyrosine kinase inhibitors in resistance arteries

Genistein, which binds to the ATP binding site of tyrosine kinase, inhibited contraction with a potency that is consistent with it acting through inhibition of tyrosine kinase. Particularly in the α-toxin-permeabilized preparation, the potency of genistein (IC_{50} ca. 6 μM) is in the same range or lower than that reported for the inhibitory effect of genistein on angiotensin II (Yang *et al.*, 1993) or epidermal growth factor (EGF) induced contraction (Yang *et al.*, 1992) in guinea-pig stomach muscle. However, in the intact preparation, genistein was also potent with an IC_{50} of about 20 μM. On the other hand, the supposedly inactive analogue of genistein, daidzein, also potently inhibited the contraction both in the intact preparation and in the α-toxin-permeabilized preparation. Therefore, although the potency of daidzein was about two times less than that of genistein towards NA activated vessels, it is possible that genistein and daidzein also affect a tyrosine kinase independent pathway in these vessels, or that the tyrosine kinase in the smooth muscle cells of the resistance arteries is susceptible to daidzein in low concentrations.

Of the four tyrphostins tested in this study, A-1 is the supposedly inactive analogue and was indeed inactive in the permeabilized preparation in the sense that it had no effect at concentrations up to 100 μM. However, in the intact preparation 100 μM A-1 did cause an inhibition of both the K^+- and the NA-induced force development. This could be accounted for by an inhibitory effect of tyrphostin A-1 on the voltage-activated calcium channels, which we have previously reported (Wijetunge *et al.*, 1992). *Bis*-tyrphostin, which has a specificity towards EGF-receptor tyrosine kinase, had no effect on either K^+-induced or NA-induced responses, and was also inactive in the permeabilized preparation in concentrations up to 100 μM. This suggests that *bis*-tyrphostin may be used as an inactive analogue in the vessels studied here. In the rabbit ear artery though, one of us (Hughes, 1994) has shown, that 5 μM *bis*-tyrphostin inhibits the PDGF-induced tension. With respect to the active analogues, both A-23 and A-47 caused an inhibition of force development. In the α-toxin-permeabilized preparation, the IC_{50} of these two drugs was about 12–18 μM. This indicates a slightly less potent effect than reported for the EGF-activated guinea-pig gastric muscle (Yang *et al.*, 1992), for the EGF-activated rabbit

Table 1 Inhibitory potencies of tyrosine-kinase inhibitors on the contractions induced by 1 nM Ca^{2+} and by 200 nM Ca^{2+} in the presence of noradrenaline (NA) plus guanosine 5'-triphosphate (GTP) in α-toxin-permeabilized mesenteric resistance arteries

	Ca^{2+} (1 μM)		*Ca^{2+}* (0.2 μM) + *GTP* (3 μM) + *NA* (100 μM)	
	pIC_{50}	*n*	pIC_{50}	*n*
Genistein	4.82 ± 0.06	(5)	5.23 ± 0.03[1,2]	(5)
Daidzein	4.75 ± 0.08	(6)	4.83 ± 0.05	(5)
Tyr A-1	NC	(7)	NC	(6)
Tyr A-23	4.93 ± 0.19	(6)	4.74 ± 0.07	(4)
Tyr A-47	4.76 ± 0.13	(6)	4.76 ± 0.11	(6)
Bis-Tyr	NC	(4)	NC	(4)

Values are expressed as means ± s.e.mean. *n* indicates the number of vessels, NC means not calculated. The inhibitory potency of each compound is expressed as $pIC_{50} = -\log IC_{50}$, where IC_{50} is the concentration of the compounds causing a 50% inhibition of the contraction induced by either Ca^{2+} alone or Ca^{2+} plus NA and GTP. [1]Indicates significant differences between the pIC_{50} for genistein and daidzein (one-way ANOVA, posterio Bonferroni test). [2]Indicates significant differences between the pIC_{50} of genistein towards Ca^{2+} and Ca^{2+} plus NA and GTP activated vessels.

aorta (Merkel *et al.*, 1993) and for the PDGF-activated rat aorta (Sauro & Thomas, 1993), but in the same range as that reported for the angiotensin II-activated guinea-pig gastric muscle (Yang *et al.*, 1993) and probably consistent with these drugs acting through inhibition of tyrosine kinase. In the intact preparation, the potency of the TKIs was less than in the α-toxin-permeabilized preparations. It has been suggested (Lyall *et al.*, 1989) that it may take up to 16 h for the full effect of the tyrphostins to develop in intact cells. It is possible therefore that the lower apparent potency in the intact preparation, where the effect was assessed a few minutes after the TKIs were added, reflected poor penetration of the TKIs. This may point to difficulties in using TKIs for acute pharmacological experiments in membrane-intact preparations.

Effect of tyrosine kinase inhibitors on calcium-mediated contraction

One of the important aspects of this study was to assess which parts of the excitation-contraction coupling was potentially dependent on tyrosine phosphorylation. One way this was addressed was by comparing the effect of the TKIs on contraction induced by K^+ and by NA. It has previously been reported (DiSalvo *et al.*, 1993) that 50 μM tyrphostin A-47 inhibits the contraction of guinea-pig mesenteric small arteries to NA by about 80%, while the K^+-induced contraction was inhibited only by 20%. An apparent selectivity for the NA-induced contraction was not confirmed in this study, where the NA-induced response and the K^+-induced response was inhibited with almost the same potency and maximal effect by the tyrphostins. This observation was supported by the similar potency of the tyrphostins against the contraction induced by calcium and by calcium plus GTP and NA in the α-toxin-permeabilized preparation. It may not be surprising that agonist independent contraction (K^+-induced contraction of the intact arteries and calcium-induced contraction of the α-toxin permeabilized preparation) was inhibited by TKIs, since it has been shown in cultured vascular smooth muscle cells (Tsuda *et al.*, 1991) that the calcium ionophore, ionomycin, can induce tyrosine phosphorylation. In fact, the tyrosine phosphorylation pattern induced by the ionophore was not dissimilar to that induced by vasoconstrictors including NA (Tsuda *et al.*, 1991). There seems therefore good evidence that calcium can induce a tyrosine kinase activity in vascular smooth muscles. Also genistein had a potent effect on the KPSS-induced contraction and the calcium-induced contraction in the permeabilized arteries which supports this suggestion. In contrast to the tyrphostins, genistein was, however, more potent towards the NA-induced than to the KPSS-induced contraction. This would suggest that in addition to the calcium-induced tyrosine kinase activity, NA may induce a tyrosine kinase activity with a slightly higher susceptibility to genistein. The finding that genestein was also more potent towards the permeabilized arteries activated with calcium in the presence of NA and GTP compared to the arteries activated with calcium alone might suggest that the additional tyrosine kinase activity may play a role in the steps involved in agonist-induced modulation of the effectiveness of $[Ca^{2+}]_i$. On the other hand, the observation that the relaxant effect of genestein was associated with a decrease of $[Ca^{2+}]_i$ in NA-activated arteries, but not in potassium-activated arteries, indicates that the additional tyrosine kinase activated by NA may also be involved in regulation of $[Ca^{2+}]_i$ (see below).

Effect of genistein on the control of $[Ca^{2+}]_i$

We have previously shown that the calcium current in rabbit ear artery smooth muscle cells can be inhibited by genistein (IC_{50}, 36 μM), tyrphostin A-23 (IC_{50}, 88 μM) and tyrphostin A-1 (IC_{50}, 110 μM), although daidzein had no effect in concentrations below 300 μM (Wijetunge *et al.*, 1992). As pointed out above, the relaxant effect of tyrphostin A-1 in the non-permeabilized preparation could be explained by the inhibition of the potential sensitive calcium channels. However, it was unexpected that 30 μM genestein did not reduce $[Ca^{2+}]_i$ in potassium-activated vessels, in view of the above findings on the rabbit ear artery smooth muscle cells. Although we have no explanation for this, it is possible that the potency of genestein is different in the rabbit ear artery smooth muscle and in the rat mesenteric resistance arteries, either as a consequence of species differences or due to the fact that the voltage clamp experiments were done on isolated cells while the $[Ca^{2+}]_i$ measurements were made in whole tissue. In contrast, $[Ca^{2+}]_i$ was reduced by 30 μM genestein in NA-activated rat mesenteric arteries. In these vessels, the effect of NA on $[Ca^{2+}]_i$ is thought to be mediated almost exclusively by depolarization with a consequent opening of voltage-dependent calcium channels (Nilsson *et al.*, 1994). It is therefore a possibility that a tyrosine kinase is affecting the signal leading from stimulation of the adrenoceptor to membrane depolarization.

In conclusion, these data support a role for tyrosine kinases in the force development to classical vasoconstrictors. It is likely that the steps involved may include the NA-induced modulation of $[Ca^{2+}]_i$, the ability of calcium to induce force and perhaps also the agonist/GTP mediated modulation of the effectiveness of $[Ca^{2+}]_i$. On the other hand, these data also indicate that caution is needed in the interpretation of the effects of the tyrphostins in acute pharmacological experiments. Also the potent effect of the so called inactive analogues for the TKIs may indicate that the TKIs have potent effects on pathways unrelated to inhibition of tyrosine kinase, or that tyrosine kinases with a high susceptibility to the inactive analogues of the TKIs may play a role in excitation-contraction coupling.

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