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European Journal of Pharmacology 277 (1995) 29–34

ejp

α_2-Adrenoceptor-mediated vasoconstriction requires a tyrosine kinase

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Received 18 July 1994; revised 18 January 1995; accepted 20 January 1995

Abstract

α_2-adrenoceptor-mediated contractions of the rabbit saphenous vein were previously found to be inhibited by wortmannin, a protein kinase inhibitor which blocks receptor-dependent phospholipase D activation. Since other studies have indicated that receptor-dependent phospholipase D activation required activity of a tyrosine kinase, we examined the influence of several tyrosine kinase inhibitors on both α_2-adrenoceptor-mediated contractions of rabbit saphenous vein and α_1-adrenoceptor-mediated contractions of rabbit aorta. Methyl 2,5-dihydroxycinnamate, genistein and erbstatin each caused non-competitive inhibition of rabbit saphenous vein contractions elicited by the α_2-adrenoceptor-selective agonist 5-bromo-6-[2-imidazolin-2-yl-amino]-quinoxaline (UK14304), yielding complete inhibition at 100 μM and IC_{50} values of 15, 35 and 40 μM respectively. By contrast, phenylephrine-induced dose-response curves in rabbit aorta were largely unaffected by tyrosine kinase inhibitors at 50 μM. In a separate analysis of intracellular Ca^{2+}-dependent and extracellular Ca^{2+}-dependent α_1-adrenoceptor responses of rabbit aorta, genistein (50 μM) did partially reduce the initial intracellular Ca^{2+}-dependent response, but did not reduce maximal response. Methyl 2,5-dihydroxycinnamate (25 μM) had no effect on intracellular or extracellular Ca^{2+} responses in rabbit aorta. High K^+-induced contractions of both rabbit saphenous vein and aorta were unaffected by up to 100 μM of the tyrosine kinase inhibitors. These results indicate an obligatory requirement for tyrosine kinase activity in α_2-adrenoceptor-mediated but not α_1-adrenoceptor-mediated vasoconstriction.

Keywords: α_2-Adrenoceptor; Tyrosine kinase inhibitor; Phospholipase D

1. Introduction

Postjunctional α_1- and α_2-adrenoceptors both mediate pressor responses via the contraction of vascular smooth muscle. The varied distribution of these postjunctional adrenoceptors (α_1 and α_2) in the vasculature makes them suitable for differentially regulating vascular tone. Larger arterioles and venules are regulated by both α_1- and α_2-adrenoceptors while terminal vessels are subserved primarily by α_2-adrenoceptors (Faber, 1988). The efficacy of α_2-adrenoceptors to elicit contractility is much lower than that of α_1-adrenoceptor even in vascular preparations where α_2-adrenoceptor density is high (Daniel et al., 1991). It has been suggested that efficacy and potency of α_2-adrenoceptor contractile response can be facilitated by co-stimulation with angiotensin II (Dunn et al., 1991a), Bay K 8644, endothelin-1 and KCl (Shimamoto et al., 1992) possibly by augmenting Ca^{2+} influx. Similarly Daly et al. (1988) found that co-stimulation of α_1-adrenoceptors augmented α_2-adrenoceptor efficacy in rabbit saphenous vein. The α_2-adrenoceptor contractile response is almost totally dependent on extracellular Ca^{2+} influx, unlike the α_1-adrenoceptor response which initially relies on the release of intracellular Ca^{2+} from internal stores. However, sensitivity of this Ca^{2+} entry during α_2-adrenoceptor response to voltage-dependent Ca^{2+} channel blockers has been reported to be variable in different vascular preparations (Dunn et al., 1991b). Dependence on extracellular Ca^{2+} entry may reflect a facilitation of receptor coupling rather than direct promotion of myosin phosphorylation (Aburto et al., 1995).

Although α_2-adrenoceptors have been linked to inhibition of adenylyl cyclase via pertussis toxin-sensitive G-proteins (G_i), in many instances there is no causal relationship between the decrease in cAMP levels and the biologic response (Andrade and Aghajanian, 1985;

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SSDI 0014-2999(95)00053-4

Limbird et al., 1985). Recent studies in our laboratory have shown that α_2-adrenoceptor stimulation in rabbit saphenous vein generates phosphatidic acid and diacylglycerol, possibly through a phospholipase D pathway (Aburto et al., 1995). The cloned α_{2A}-adrenoceptor expressed in rat 1 fibroblasts has been shown to stimulate phosphatidylcholine hydrolysis via phospholipase D (MacNulty et al., 1992). Further, wortmannin, a kinase inhibitor which interferes with receptor-mediated phospholipase D activity, inhibits α_2-adrenoceptor-induced contractile response of rabbit saphenous vein in a dose-dependent manner (Waen-Safranchik and Deth, 1994). It therefore seems that phospholipase D may be the downstream effector for α_2-adrenoceptor in vascular tissues.

Receptor-mediated phospholipase D activation by chemoattractants via the activation of a pertussis toxin-sensitive G-protein has been reported to be associated with tyrosine phosphorylation and tyrosine kinase inhibitors inhibit receptor-induced phospholipase D activity in human neutrophils (Uings et al., 1992). Phospholipase D activity can also be stimulated by receptors with intrinsic tyrosine kinase activity such as the epidermal and platelet-derived growth factor receptor (Ben-Av and Liscovitch, 1989; Fisher et al., 1991) and tyrosine phosphatase inhibitors such as vanadate can facilitate phospholipase D activity in granulocytes by a receptor-independent manner (Bourgoin and Grinstein, 1992). IgE receptor-mediated phospholipase D activation has also been shown to be sensitive to tyrosine kinase inhibitors (Kumada et al., 1993). Thus the present study was designed to investigate the possible involvement of tyrosine kinase activity in α_2-adrenoceptor-mediated vasoconstrictor response in rabbit saphenous vein as compared to α_1-adrenoceptor response in rabbit aorta using a series of tyrosine kinase inhibitors. These inhibitors differ structurally from each other and act at either the ATP binding site (genistein) or at the peptide binding site of the tyrosine kinase (erbstatin, methyl 2,5-dihydroxycinnamate). The results of this study suggest that tyrosine kinase activity is necessary for α_2-adrenoceptor-mediated vasoconstrictor response.

2. Materials and methods

2.1. Tissue preparation

Male New Zealand rabbits (2–3 kg) were killed by cervical dislocation and the saphenous veins and thoracic aorta were quickly excised and placed in normal Krebs-Henseleit bicarbonate buffer of the following composition (mM): NaCl 118, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11.1, equilibrated with 95% O_2 and 5% CO_2 to final pH of 7.4 (Alabaster et al., 1985). Tissues were cleaned of adhering fat and connective tissue and cut into strips for contraction studies.

2.2. Contraction studies

Spiral strips from saphenous vein or transverse strips of aorta were mounted in a 20 ml jacketed water bath maintained at 37° C. One end of the tissue was fixed while the other was connected to a force transducer. Tissues were stretched to a passive tension of 2 g and were allowed to equilibrate for 60 min. After this equilibration period, only when a high concentration (1 μM) of the appropriate agonist (i.e. UK14304 for rabbit saphenous vein and phenylephrine for rabbit aorta) showed two successive responses within 10% of each other was the experimental protocol started. Isometric contractions were recorded on a polygraph recorder.

To provide optimal α_2-adrenoceptor responses in rabbit saphenous vein, experiments were conducted in a buffer with elevated KCl (15 mM) also containing the α_1-adrenoceptor antagonist prazosin (0.1 μM) as described by Shimamoto et al. (1992). This buffer was prepared by replacing an equimolar amount of NaCl with KCl. In order to generate Ca^{2+} concentration-response curves in rabbit saphenous vein or aorta the normal Krebs-Henseleit bicarbonate buffer was replaced with Ca^{2+}-free buffer containing 1 mM EGTA for 5 min followed by two washes in Ca^{2+}-free buffer during a further 10 min period before addition of cumulative concentration of $CaCl_2$. Treatment with different tyrosine kinase inhibitors was achieved by incubating the tissues for 30 min before and during the contraction curve. A depolarizing concentration of KCl (60 mM) was used to determine maximal contractile ability of the tissue.

2.3. Drugs

UK14304 was provided by Research Biochemicals International as part of the Chemical Synthesis Program of the National Institute of Mental Health, Contract 278-90-007 (Natick, MA, USA). Phenylephrine was purchased from Sigma Chemicals (St. Louis, MO, USA). Tyrosine kinase inhibitors were kindly provided to us by Dr. Alan Hudson of the Wellcome Foundation (Beckenham, Kent, UK). UK14304 and tyrosine kinase inhibitors were dissolved in DMSO whose final bath concentration (0.1%) did not affect tissue contractility.

2.4. Statistical analysis

Data means were analyzed for Student's t-test. A probability of $P \leq 0.05$ was selected as a criterion for statistical significance.

3. Results

We tested the activity of tyrosine kinase inhibitors under conditions of elevated K^+ in the rabbit saphenous vein using the α_2-adrenoceptor-selective agonist UK14304. Fig. 1 shows the ability of tyrosine kinase inhibitors to inhibit α_2-adrenoceptor contractile response in a dose-dependent and non-competitive manner. Methyl 2,5-dihydroxycinnamate, a cinnamate derivative, was the most potent inhibitor followed by the bioflavonoid genistein while erbstatin, a formamide, was the least potent. IC_{50} values were 15, 35 and 40 μM respectively with a complete loss of α_2-adrenoceptor response at 50 or 100 μM. α_2-Adrenoceptor contractile rssponse of rabbit saphenous vein is thus entirely dependent on tyrosine kinase activity.

α_2-Adrenoceptor contractile response in rabbit saphenous vein and other blood vessels is highly dependent upon extracellular Ca^{2+} (Dunn et al., 1991a; Aburto et al., 1995). In order to test the relationship between tyrosine kinase activity and Ca^{2+} influx, we obtained UK14304 (1 μM) response in Ca^{2+}-free buffer. α_2-Adrenoceptor response was completely inhibited under these conditions, while subsequent incremental addition of Ca^{2+} upto 10 mM increased tone to a level similar to the maximal UK14304 response in normal buffer (Fig. 2). This response, referred to as the extracellular Ca^{2+}-dependent response, was inhibited by methyl 2,5-dihydroxycinnamate (25 μM) and genistein (50 μM) by 30% and 50%, respectively (Fig. 2A,B). Thus elevation of $[Ca^{2+}]$ to 4-fold above normal was only able to partially overcome the effects of tyrosine kinase inhibition.

We also evaluated if these tyrosine kinase inhibitors affect α_1-adrenoceptor responses in rabbit aorta using the α_1-adrenoceptor-selective agonist phenylephrine in a dose-response protocol. All three tyrosine kinase inhibitors tested were ineffective in blocking α_1-adrenoceptor response even at concentrations higher than their IC_{50} values in rabbit saphenous vein (data not shown). Thus α_1-adrenoceptor contractile response is much less sensitive to tyrosine kinase inhibitors than is α_2-adrenoceptor response.

In studies similar to those for α_2-adrenoceptors we investigated if these tyrosine kinase inhibitors could separately influence either the initial intracellular Ca^{2+} release phase or the slow extracellular Ca^{2+} entry-dependent phase in rabbit aorta. Genistein (50 μM) inhibited the initial phase by about 55% but did not reduce the influx-dependent phase while methyl 2,5-dihydroxycinnamate (25 μM) was essentially without effect on either phase (Fig. 3). This difference may be due to different mechanisms of inhibition by these two structurally distinct tyrosine kinase inhibitors.

In order to rule out inhibition by these tyrosine kinase inhibitors at the level of contractile elements, we tested their effects on contractile response induced by 60 mM KCl (data not shown). High K^+-induced contractions of both rabbit saphenous vein and aorta were unaffected by up to 100 μM of tyrosine kinase inhibitors. This indicates the ability of tyrosine kinase inhibitors to selectively interfere with receptor-mediated vasoconstrictor responses, especially the mechanism activated by α_2-adrenoceptors.

4. Discussion

Kinase-dependent protein phosphorylation is an important step in activation of excitation-contraction coupling in vascular smooth muscle cells at the level of contractile elements. Among these groups of kinases,

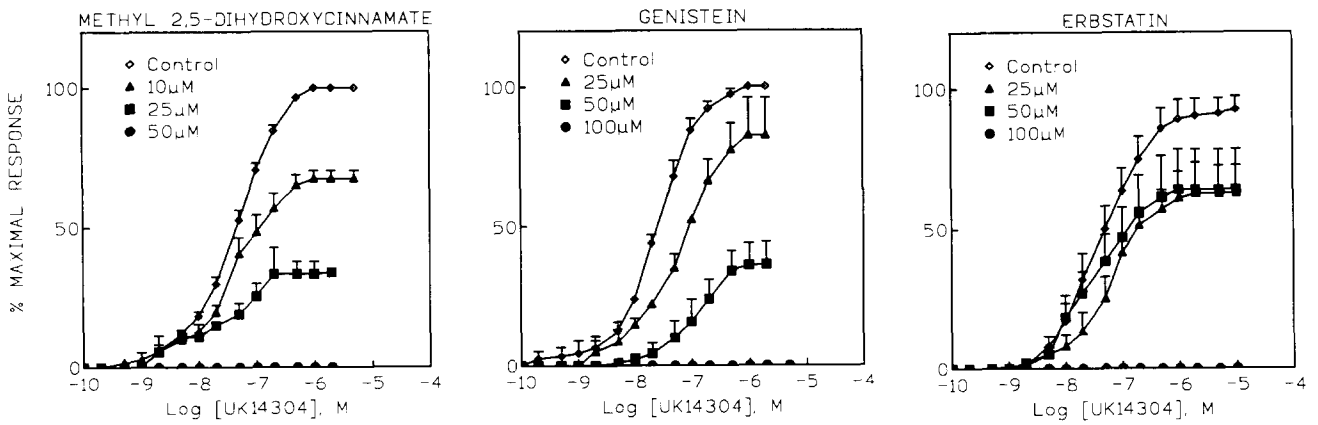

Fig. 1. Dose-dependent non-competitive effect of tyrosine kinase inhibitors on UK14304 responses in rabbit saphenous vein. Tissues were exposed to different concentrations of methyl 2,5-dihydroxycinnamate, genistein, erbstatin for 30 min prior to eliciting the dose-response curve. Responses have been expressed as a percentage of the maximum response to UK14304 and each data point shown is the mean ± S.E.M. of at least four or more determinations.

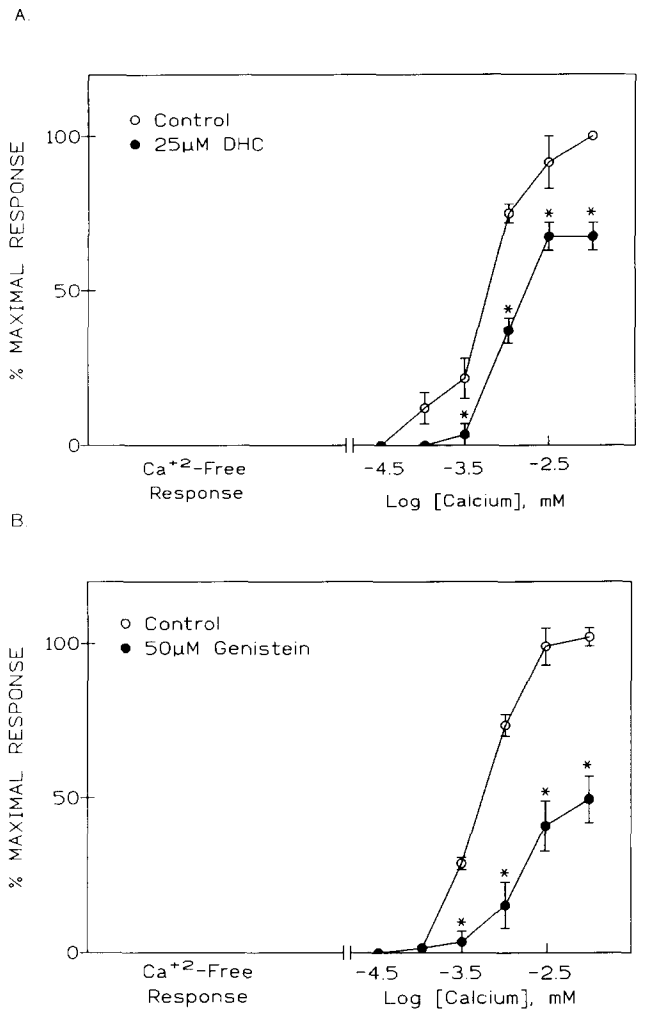

Fig. 2. Effect of methyl 2,5-dihydoxycinnamate (DHC;A) and genistein (B) Ca^{2+}-free and Ca^{2+}-dependent responses to the α_2-adrenoceptor agonist UK14304 in rabbit saphenous vein. Tissues were exposed to tyrosine kinase inhibitors for 30 min prior to eliciting the dose-response curve. Responses have been expressed as a percentage of the maximum response to UK14304 and each data point shown is the mean ± S.E.M. of at least four or more determinations. * Statistical significance at $P \leq 0.05$ from untreated tissues.

protein tyrosine kinases are one of the most abundant in the cell and includes not only receptor tyrosine kinases but also cytosolic tyrosine kinases. The low levels of in vivo tyrosine phosphorylation, when compared with the rapid and pleiotropic cellular responses observed, argues that the cellular targets of protein tyrosine kinases are likely to be amplifiers (Cantley et al., 1991; Pan et al., 1992). The exact nature and physiological significance of tyrosine kinase activity in vascular smooth muscle cells has yet to be identified. Historically, tyrosine kinase activity has been associated with cell growth, proliferation and transformation. More recently, the observation that receptors which stimulate growth encompass not only the classical insulin-like receptors with intrinsic tyrosine kinase activity but also G-protein-coupled receptors, such as the endothelin-1 receptor, suggests the possible involvement of cytosolic tyrosine kinases such as $pp60^{c\text{-}src}$ as the common link between the two types of growth factor receptors (Hadcock et al., 1992; Simonson and Herman, 1993).

We sought to investigate the importance of tyrosine kinase activity in the contractile response of two vascular preparations, each containing mainly one α-adrenoceptor subtype. Rabbit saphenous vein was used as a model for α_2-adrenoceptor and rabbit aorta for α_1-adrenoceptor responses. A series of structurally distinct tyrosine kinase inhibitors were examined for their effects on vasoconstriction responses induced by α_2- or α_1-adrenoceptor agonist. Our results indicate a critical role for tyrosine kinase activity in α_2-adrenoceptor but not in α_1-adrenoceptor responses.

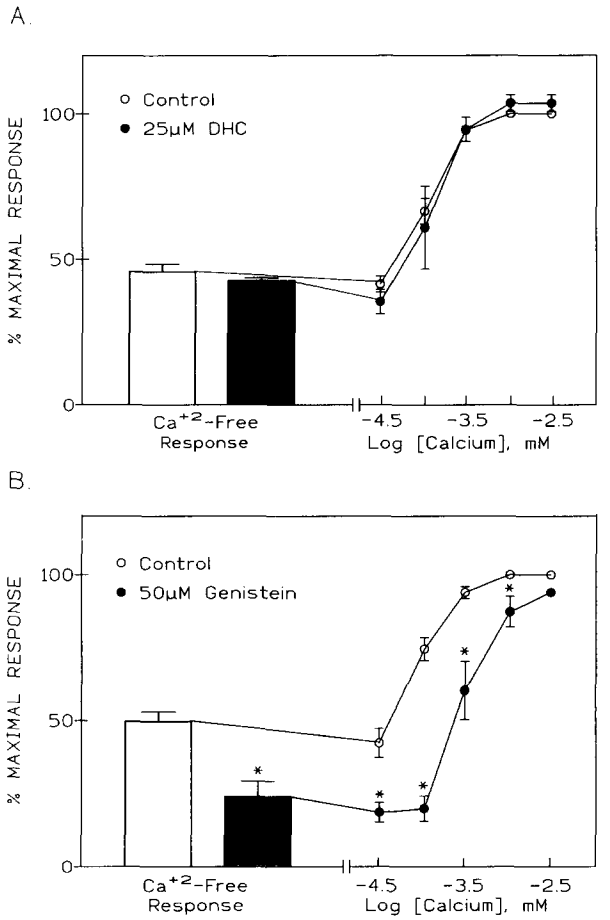

Fig. 3. Effect of methyl 2,5-dihydoxycinnamate (DHC;A) and genistein (B) Ca^{2+}-free and Ca^{2+}-dependent responses to the α_2-adrenoceptor agonist UK14304 in rabbit aorta. Tissues were exposed to tyrosine kinase inhibitors for 30 min prior to eliciting the dose-response curve. Responses have been expressed as a percentage of the maximum response to UK14304 and each data point shown is the mean ± S.E.M. of at least four of more determinations. * Statistical significance at $P \leq 0.05$ from untreated tissues.

Genistein is an isoflavone compound isolated from fermentation broth of *Pseudomonas* sp. and is a highly specific inhibitor of tyrosine kinases. Its inhibition is competitive with respect to ATP and non-competitive for the phosphate acceptor (i.e. protein substrate) such as histone H2B (Akiyama et al., 1987). Genistein inhibits EGF-receptor kinase activity with an IC_{50} value of 22 μM (Akiyama et al., 1987) and inhibited α_2-adrenoceptor contractile response in rabbit saphenous vein with an IC_{50} value of approximately 35 μM. Methyl 2,5-dihydroxycinnamate and erbstatin belong to another class of tyrosine inhibitors which bind to peptide recognition sites and inhibit tyrosine phosphorylation (Powis, 1991). Methyl 2,5-dihydroxycinnamate and erbstatin inhibit EGF receptor-kinase activity with IC_{50} values of 0.77 and 3.3 μM, respectively (Isshiki, 1987; Umezawa et al., 1990) while their IC_{50} for inhibition of α_2-adrenoceptor-mediated contraction of rabbit saphenous vein were 15 and 40 μM, respectively. The lower potency might reflect involvement of a different tyrosine kinase; however, the relationship between contraction and kinase activity may not be direct so that IC_{50} values for each would not be expected to be the same.

Although the observed rank order potency for inhibition in the rabbit saphenous vein was different than that for inhibition of growth factor receptor tyrosine kinase activity, these tyrosine kinase inhibitors were clearly effective in inhibiting vasoconstrictor response to an α_2-adrenoceptor agonist in rabbit saphenous vein, providing complete inhibition at 50–100 μM. This indicates the obligatory involvement of a non-receptor tyrosine kinase in α_2-adrenoceptor-mediated vasoconstrictor response. Similar inhibition of agonist-induced contractions by tyrosine kinase inhibitors have also been demonstrated by Di Salvo et al. (1993) in vascular and visceral smooth muscle, however the subtype of the α-adrenoceptor involved was not identified. In the same study tyrosine kinase inhibitors caused inhibition of $pp60^{c\text{-}src}$ tyrosine kinase activity while myosin light chain kinase and cAMP-dependent protein kinase activity was unaffected.

Recently numerous reports suggest that chemoattractant receptor, α_2-adrenoceptor and angiotensin II receptor are coupled through pertussis toxin sensitive G-proteins to phospholipase D activity (Lasseque et al., 1991; Kessels et al., 1991; MacNulty et al. 1992). Receptor-mediated phospholipase D activity has been shown to be dependent upon tyrosine kinase activity such that tyrosine kinase inhibitors block phospholipase D activation (Thompson et al., 1991; Uings et al., 1992). Our previous findings indicate involvement of a phospholipase D pathway in vascular α_2-adrenoceptor signal transduction which is wortmannin-sensitive (Aburto et al., 1995; Waen-Safranchik and Deth, 1994). We suggest that tyrosine activity might be essential for α_2-adrenoceptor-mediated phospholipase D activity. Inhibition of this requisite tyrosine kinase activity (such as that provided by $pp60^{c\text{-}src}$ for example) can thus completely eliminate the α_2-adrenoceptor-vasoconstrictor response.

Although the exact relationship between a tyrosine kinase and phospholipase D activation remains to be established, we view the role of a tyrosine kinase to be permissive in this pathway. A specific association between the alpha subunit of G_i and protein tyrosine kinase $pp60^{c\text{-}src}$ has been reported by Torti et al., 1992). In fact, Jiang et al. (1994) suggest that v-src-induced phospholipase D activity is mediated via cholera and pertussis toxin-insensitive G-proteins. Since src-like tyrosine kinases regulate the activity of monomeric G-proteins, it is therefore possible that α_2-adrenoceptor-mediated phospholipase D activity can be regulated by the combined obligatory effect of a tyrosine kinase both at the level of heterotrimeric and monomeric G-proteins (Bowman et al., 1993; Cockcroft et al., 1994). While this is a hypothetical mechanism, it serves to illustrate a potential link between tyrosine kinase activity and α_2-adrenoceptor-induced phospholipase D stimulation.

In rabbit aorta, genistein caused partial inhibition of initial Ca^{2+} release-dependent tension whereas methyl 2,5-dihydroxycinnamate was without effect (Fig. 3A,B). Total response was, however, completely recovered after genistein. Genistein is known to act at both the ATP and substrate binding site, unlike methyl 2,5-dihydroxycinnamate which inhibits only the substrate binding site (Akiyama et al., 1989). Thus genistein may exhibit a broader range of inhibition than methyl 2,5-dihydroxycinnamate, perhaps extending to some Ca^{2+}-related process in the rabbit aorta. Since tyrosine kinase activity (Vostal et al., 1991) and low molecular weight G-proteins (Bird and Putney, 1993; Fasolato et al., 1993) have been implicated in the process of refilling releasable Ca^{2+} stores, genistein inhibition may possibly reflect an impairment of such refilling.

Tyrosine kinase inhibitors did not inhibit excitation-contraction coupling non-specifically since it had no effect on high K^+-buffer-induced contraction induced in either rabbit saphenous vein or aorta. In these rabbit blood vessels tyrosine kinase activity seems therefore to be of paramount importance in α_2-adrenoceptor-mediated response and less so in α_1-adrenoceptor vasoconstrictor response, but not at all involved at the level of contractile elements.

In conclusion, the present study demonstrates for the first time the critical and selective role of tyrosine kinase activity in α_2-adrenoceptor-mediated vasoconstrictor response. Tyrosine kinase activity may be essential for coupling of α_2-adrenoceptors to phospholipase D activation, although this mechanism requires additional verification.

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