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Multiple effects of tyrosine kinase inhibitors on vascular smooth muscle contraction

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Abstract

The effects of three tyrosine kinase inhibitors: genistein, quercetin and psi-tectorigenin, were investigated on contractions evoked in de-endothelised rat aortic rings, either by phenylephrine or 70 mM K^+. A dose-dependent inhibition of both contractions by all three compounds was observed, the phenylephrine-mediated contractions being more sensitive to genistein. No differences between genistein or quercetin effects in pre-treatment or post-treatment protocols were found. Ca^{2+} store refilling, expressed in terms of phenylephrine-induced tension in Ca^{2+}-free medium, was dose-dependently blocked by quercetin and genistein. Sodium orthovanadate, an inhibitor of tyrosine phosphatase, contracted the rat aortic rings with an IC_{50} of 0.66 μM. Its presence during the refilling period after exposure to Ca^{2+}-free medium completely prevented the subsequent response to phenylephrine. One can conclude that the use of the above-mentioned protein tyrosine kinase inhibitors in the rat aorta blocks a step involved in Ca^{2+} entry and Ca^{2+} store refilling. A definite conclusion regarding the vanadate effects is not possible due to the fact that this compound also affects Ca^{2+} ATP-ases.

Keywords: Tyrosine kinase inhibitor; Aorta, rat; Contraction; Ca^{2+} store refilling; Vanadate

1. Introduction

Since 1991, when Vostall et al. showed that tyrosine phosphorylation is involved in the modulation of stored and cytosolic calcium ($[Ca^{2+}]_i$) in platelets, tyrosine kinase phosphorylation/dephosphorylation became an exciting new candidate to explain Ca^{2+} entry triggered by emptying of Ca^{2+} stores (Vostall et al., 1991). Studies with platelets (Sargeant et al., 1993) and in fibroblasts (Lee et al., 1993) showed that tyrosine kinase inhibitors selectively inhibit Ca^{2+} entry.

Two different studies (Di Salvo et al., 1993a; Yang et al., 1993) have shown that tyrosine kinase inhibitors alter smooth muscle contraction. In this tissue contractions can be evoked by two different mechanisms: pharmaco-mechanical coupling which implicates Ca^{2+} entry from extracellular space through receptor-operated Ca^{2+} channels (ROC) without change in the membrane potential, and electro-mechanical coupling involving the Ca^{2+} entry through voltage-operated Ca^{2+} channels (VOC), activated by changes in membrane potential (Somlyo, 1985). It was suggested (Di Salvo et al., 1993a) that tyrosine kinase-induced phosphorylations are involved in pharmaco-mechanical coupling more than in electro-mechanical coupling.

The present work investigated the effects of three different tyrosine kinase inhibitors on force development in rat aortic smooth muscle, a widely used in vitro model of vascular smooth muscle. The tyrosine kinase inhibitor effects on the refilling of internal Ca^{2+} stores were also investigated. This last mechanism is still an obscure aspect of the events accompanying smooth muscle activity. Earlier studies proposed a direct communication between extracellular space and internal Ca^{2+} stores (Casteels and Droogmans, 1981; Putney, 1986). More recently it was demonstrated that L-type Ca^{2+} channel blockers and thapsigargin could inhibit the refilling of internal Ca^{2+} stores (Low et al., 1993; Bourreau et al., 1993), whereas inositol 1,3,4,5-tetrakis-

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phosphate can promote Ca^{2+} entry (Brailoiu et al., 1993). To our knowledge the effects of tyrosine kinase inhibitors upon refilling of internal Ca^{2+} stores in vascular smooth muscle have not so far been assessed. The effects of vanadate, a proposed tyrosine phosphatase inhibitor, were also tested.

2. Materials and methods

2.1. Tissue preparation

Male Wistar rats (180–220 g) were killed by decapitation and their thoracic descending aorta was carefully removed and rapidly immersed in Krebs-Henseleit solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.6, $NaHCO_3$ 24.9, glucose 5.55 (pH 7.3 adjusted with NaOH). The adherent connective tissue was removed using fine scissors and 6–8 rings (2 mm wide) were obtained. After removal of the endothelium by gently rubbing the intimal surface with a smooth softwood stick, the rings were mounted between wire hooks in a 10 ml organ bath containing Krebs-Henseleit solution warmed at 37° C and continuously aerated with 95% O_2 and 5% CO_2. The lower wire hook was fixed in the organ bath and the upper to an isometric force transducer (type IMF 002). Isometric force development was recorded by using commercially available pen recorders.

A resting tension of 2 g was imposed on each ring and allowed to equilibrate for 90–120 min (bath solution changed every 15 min). Thereafter a control contraction was elicited by phenylephrine 10^{-5} M and after the peak was reached carbachol 10^{-5} M was added to confirm the lack of a functional endothelium. Rings that did not develop at least 8 mN contractile active force or that relaxed in response to carbachol were discarded. After washing and re-equilibration, repetitive contractions were elicited with phenylephrine 10^{-5} M until two successive contractions different by less than 5% were obtained. The amplitude of the last contraction was considered as 100% for further comparisons. The experimental procedure was started with the rings randomly distributed along different experimental series in order to minimize influences of anatomical location on the final results.

2.2. Experimental procedure

In the first series of experiments the effects of tyrosine kinase inhibitors on the contractions induced by either phenylephrine 10^{-5} M or K^+ 70 mM (isosmotic replacement of NaCl with KCl) in Ca^{2+}-containing medium were tested. For post-treatment protocols, after a stable contraction (at least 15 min of plateau force level with less than 1% variation) had been obtained, increased concentrations of tyrosine kinase inhibitors were added each time after the tension reached a new stable level (considered as such if lasting for more than 10 min). As in the case of phenylephrine a maximal volume of 100 μl per dissolved drug was added in such a way that the solvent amount added into the bath never exceeded 250 μl. In pretreatment protocols, relaxed preparations were equilibrated for 10 min with the appropriate concentration of tyrosine kinase inhibitors. Prolongation of the incubation period to 20, 30 or 60 min or shortening to 5 min did not affect quercetin or genistein effects ($n = 3$ in each case). Thereafter contractions to phenylephrine or K^+ were obtained and the final amplitudes were compared with the control.

In experiments using sodium orthovanadate this drug was added from a stock solution 100 times more concentrated without determination of osmotic change. The final concentration of sodium orthovanadate was too small to affect the osmotic behaviour of the smooth muscle, taking into account that 10 mM sucrose also had no effect (not shown).

In experiments testing the refilling of internal Ca^{2+} stores, the control contractions were first obtained, then the preparations were immersed in Ca^{2+}-free solution (no $CaCl_2$ and 2 mM EGTA added) for 5 min and the response to 10^{-5} M phenylephrine was measured. A second application of the same concentration of phenylephrine after washout of the first dose did not elicit any contractile response, showing that the phenylephrine-sensitive internal Ca^{2+} stores were depleted. Incubation for 10 min at 37° C in normal Ca^{2+}-containing solution fully restored the response to phenylephrine in Ca^{2+}-free medium ($97 \pm 4\%$, $n = 8$). In order to test the tyrosine kinase inhibitor effects on internal Ca^{2+} store refilling, different concentrations of tyrosine kinase inhibitors were added during the refilling period and the second response elicited by phenylephrine in Ca^{2+}-free medium was compared to the first (see Fig. 3A).

2.3. Drugs

Genistein (4′,5,7-trihydroxyisoflavone), quercetin (3,3′,4′,5,7-pentahydroxyflavone), phenylephrine, carbachol, EGTA (ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid were from Sigma, psitectorigenin was from Hammya, sodium orthovanadate (Na_3VO_4) and methoxyverapamil (D600) were from Merck and dimethyl sulfoxide (DMSO) was from Fluka. All others compounds were of the best grade commercially available.

Phenylephrine, carbachol, sodium orthovanadate and D600 were dissolved in bidistilled water on the morning of the experiment. Addition of up to 1 ml bidistilled water to relaxed or contracted rings had no effects on tension.

Psi-tectorigenin was dissolved in absolute ethanol (purity greater than 98%) as stock solution (50 mM). The final concentration of ethanol in the bath never exceeded 0.1% (v/v) and was without effects upon the resting or maximal tension.

Genistein and quercetin were dissolved in DMSO as stock solution (50 mM). Concentrations of pure DMSO between 1% and 3% (v/v) induced a small relaxation of phenylephrine-precontracted rings (94 ± 2%, extremes between 99% and 82%, $n = 8$). As will be shown in the Results section these relaxations were clearly of reduced amplitudes as compared to those induced by tyrosine kinase inhibitors at doses corresponding to the final DMSO concentrations.

2.4. Statistics

The unpaired Student t-test was used to test the significance of differences between different series. IC_{50} values were calculated using a computer program based upon the least-squares method.

3. Results

3.1. Effects of tyrosine kinase inhibitors upon contractions in Ca^{2+}-containing medium

In Fig. 1 concentration-effect curves are shown for tyrosine kinase inhibitors on aortic rings precontracted with 10^{-5} M phenylephrine. All three compounds

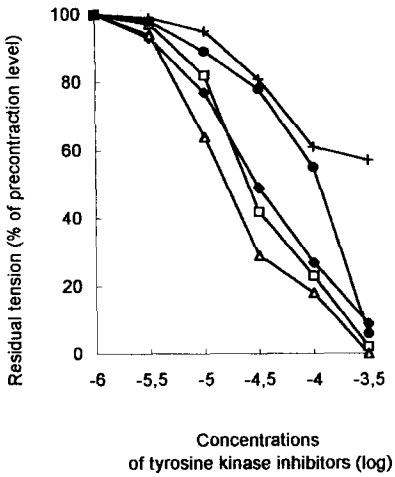

Fig. 1. Concentration-effect curves for the tyrosine kinase inhibitors in phenylephrine- and K^+-precontracted rings (percentage of the tension level before inhibitor addition). Squares indicate genistein effects on phenylephrine-induced contraction, diamonds indicate quercetin effects on phenylephrine-induced contraction and triangles indicate the same for psi-tectorigenin. Circles represent the effects of genistein on K^+-induced contraction and crosses the same for quercetin ($n = 6$ in each case). S.E.M. was always less than 7% and is not represented in the figure for reasons of clarity.

Table 1
IC_{50} values for psi-tectorigenin (PSI), genistein (GEN) and quercetin (QUE) on phenylephrine- (PHE) and K^+-induced rat aortic contraction

IC_{50} (μM)	PHE	K^+
PSI	27 ± 4	ND
GEN	69 ± 15	131 ± 12
QUE	57 ± 18	101 ± 27

Genistein values are statistically different ($P = 0.038$), whereas quercetin values are not ($P = 0.071$). ND – not determined.

tested nearly suppressed, although at relatively high concentrations, these contractions. The IC_{50} values showed psi-tectorigenin as being more potent than genistein or quercetin (Table 1). The effects were irreversible even after 60 min washing. None of these compounds interfered with the relaxation induced by D600 on phenylephrine- or K^+-induced contractions (data not shown).

In order to find out upon which type of contraction (phenylephrine- or K^+-induced) the tyrosine kinase inhibitors' relaxation acts preferentially, the effects of genistein and quercetin on both types of contractions were compared. As shown in Fig. 1, even though genistein also blocked the K^+-induced contractions, this inhibition appeared at significantly higher concentrations (see also Table 1). For quercetin, preferential inhibition of phenylephrine-induced over K^+-induced contractions was significant only at relatively low concentrations and disappeared at higher concentrations. The IC_{50} values for quercetin were not significantly different (Table 1).

The effects of genistein and quercetin upon phenylephrine-induced and K^+-induced contractions were also compared in pre-treatment versus post-treatment protocols. No significant differences were found (Fig. 2). If phenylephrine was washed out in the continuous presence of tyrosine kinase inhibitors and the contractile effects of phenylephrine were tested again 15 min later, the amplitude of this second contraction was not significantly different from the first ($n = 4$, data not shown).

3.2. Effects of tyrosine kinase inhibitors on internal Ca^{2+} store refilling

The extent of internal Ca^{2+} store refilling was assessed as shown before. After exposure of the preparation to a Ca^{2+}-free EGTA medium for 5 min, administration of 10^{-5} M phenylephrine resulted in a rapidly developing transient contraction of 27.7 ± 3.1% ($n = 16$) of the control contraction in Ca^{2+}-containing solution. This contraction returned to the basal tension level in less then 3 min. As already mentioned in section 2.2, the second phenylephrine challenge in Ca^{2+}-free medium did not result in any contractile

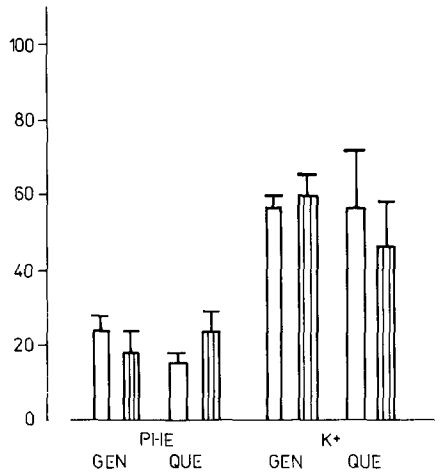

Fig. 2. Phenylephrine (the left-hand columns) and K^+ (the four right-hand columns) inhibition by genistein (100 μM) and quercetin (100 μM) as indicated below the figure. Comparisons between the effects of these two inhibitors as post-treatment (open columns) or pre-treatment (hatched columns). The ordinate shows the amplitude of the response in the absence of inhibitor ($n = 4$ in each case).

response. A 10 min incubation period at 37° C in Ca^{2+}-containing solution was enough to fully restore the first response, which could be considered as an indicator of the degree of internal Ca^{2+} store refilling (Bourreau et al., 1993) (Fig. 3a, upper trace).

Administration of genistein or quercetin during the refilling period reduced the subsequent response to phenylephrine (Fig. 3a, middle trace). As shown in Fig. 3b, this inhibition is concentration-dependent. In order to further confirm that the inhibition of the phenylephrine-induced response in Ca^{2+}-free medium was due to inhibition of internal Ca^{2+} store refilling we tested the effects of genistein and quercetin administered (pre-treatment) during the Ca^{2+} depletion period (5 min in Ca^{2+}-free solution) (Fig. 3a, lower trace). Even if the phenylephrine response obtained after 10 min in normal Ca^{2+} (no tyrosine kinase inhibitors in Ca^{2+}-containing medium but present in Ca^{2+}-free medium) was still inhibited (Fig. 3a, lower trace and Fig. 3b), this inhibition was significantly less than that observed when tyrosine kinase inhibitors were present during the refilling period ($P = 0.031$).

The effects of the tyrosine kinase inhibitors on the Ca^{2+}-induced Ca^{2+} release were not investigated in our preparation because, at 37° C, caffeine, a known activator of Ca^{2+}-induced Ca^{2+} release, induces in rat aortic rings a contractile response only at very high, toxic concentrations ($> 2 \times 10^{-1}$ M). This lack of caffeine-induced contraction in Ca^{2+}-free medium in rat aorta from Wistar rats at 37° C has been reported (Noguera and D'Ocon, 1992).

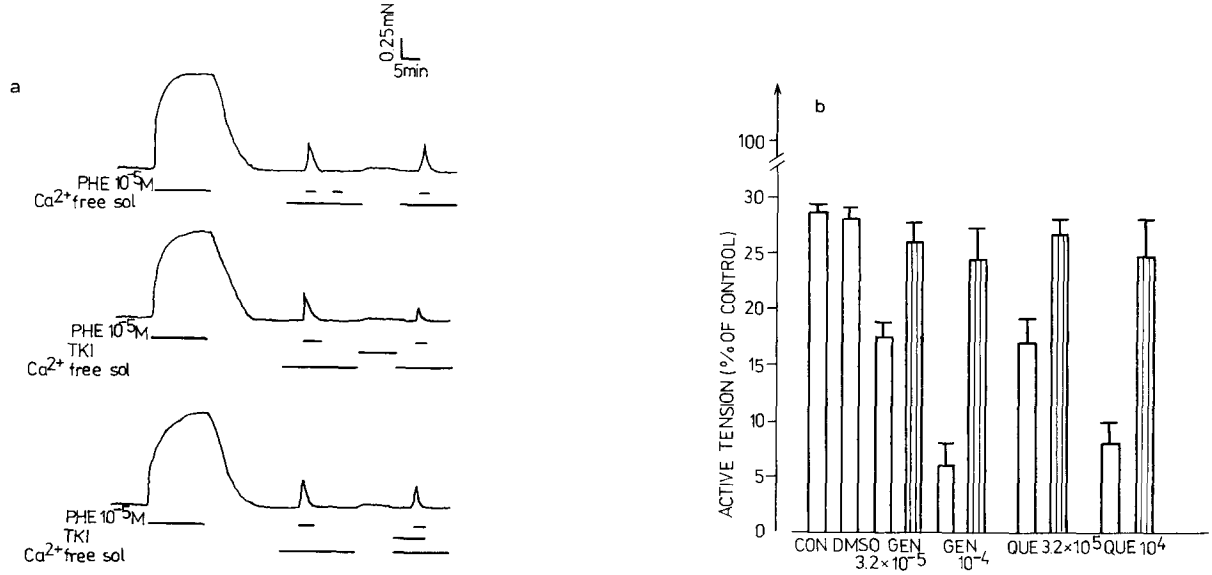

Fig. 3. (a) The protocol used to study internal Ca^{2+} store refilling. The upper trace indicates complete refilling of stores after 10 min in Ca^{2+}-containing external medium. The middle trace shows that the presence of tyrosine kinase inhibitors (TKI) largely blocked the refilling process. In the lower trace, the presence of tyrosine kinase inhibitors only in Ca^{2+}-free medium resulted in a lower degree of inhibition. Additions of different drugs are indicated below the traces (horizontal bars). (b) The statistics of the results obtained in the experiments conducted according to the protocols shown in (a). The ordinate indicates the control response to phenylephrine in Ca^{2+}-containing medium (100%), CON shows the response to phenylephrine in Ca^{2+}-free medium (2 mM EGTA added) and DMSO indicates the effect of 100 μl DMSO on Ca^{2+} store refilling. Two different concentrations of genistein (GEN) and quercetin (QUE) were tested as shown below the graph. Open columns show the effect of these inhibitors on refilling of internal Ca^{2+} stores (middle trace from (a)), whereas hatched columns show the effects of genistein or quercetin addition only in Ca^{2+}-free medium (lower trace from (a)) ($n = 4$–6 in each case).

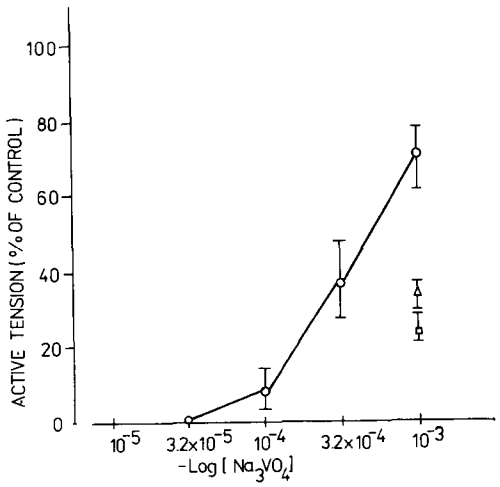

Fig. 4. Sodium orthovanadate effects on rat aortic smooth muscle tone. The ordinate axis shows the control response to phenylephrine 10^{-5} M, a square indicates the vanadate-induced response in Ca^{2+}-free medium (2 mM EGTA) and a triangle the remaining tension level after 100 μM genistein ($n = 4$–6 in each case).

3.3. Effects of sodium orthovanadate

Sodium orthovanadate in Ca^{2+}-containing medium elicits rapid contractions, with a threshold value of 50 μM and an IC_{50} value of 0.66 mM. Concentrations of sodium orthovanadate higher than 2 mM seem to be toxic (relaxation occurs). In Ca^{2+}-containing medium sodium orthovanadate (1 mM) induces for more than 15 min a stable contraction tension by 71 ± 11% ($n = 6$) of that elicited by 10^{-5} M phenylephrine. Genistein 100 μM inhibits this contraction by only 48 ± 6% ($n = 4$) (Fig. 4).

In Ca^{2+}-free medium, 1 mM sodium orthovanadate induced a transient contraction with an amplitude similar to that elicited by 10^{-5} M phenylephrine (23 ± 4%, $n = 5$). The time pattern of sodium orthovanadate- and phenylephrine-induced contractions was almost the same in Ca^{2+}-free medium. Phenylephrine and sodium orthovanadate possibly mobilise the same Ca^{2+} stores, since treatment with one of them completely abolished the subsequent response to the other ($n = 4$ in each case). The effects of sodium orthovanadate were irreversible even after 60 min washing. However, if the response to sodium orthovanadate was obtained in Ca^{2+}-free medium and thereafter this compound was washed out with Ca^{2+}-containing medium, one strong transient contraction was constantly observed (84 ± 6.7% from control, $n = 5$).

The presence of sodium orthovanadate (1 mM) during the refilling period completely abolished the subsequent response to phenylephrine in Ca^{2+}-free medium ($n = 5$, data not shown).

4. Discussion

This study produced evidence that tyrosine kinase inhibitors inhibit the contraction of rat aortic smooth muscle in Ca^{2+}-containing medium. This was also shown for guinea pig gastric smooth muscle (Yang et al., 1993), guinea pig small mesenteric arteries and taenia coli or canine arteries (Di Salvo et al., 1993a). Even if the specificity of commercially available tyrosine kinase inhibitors could be questioned, this inhibition is very probably related to a block of protein tyrosine phosphorylation.

The phenylephrine-induced contraction of smooth muscle involves pharmaco-mechanical coupling while the K^+-induced contraction is a result of electro-mechanical coupling (Somlyo, 1985). In our model, genistein and quercetin (the latter only at small concentrations) showed a preferential inhibition of phenylephrine-induced contractions over K^+-induced contractions, supporting the view of Di Salvo et al. (1993a) that protein tyrosine phosphorylation is predominantly activated during pharmaco-mechanical coupling. The lack of specificity of quercetin at high doses could be related to the inhibition of protein kinase C at these doses (Sristava, 1985). This inhibition of the maintenance of sustained contractions by tyrosine kinase inhibitors suggests a direct effect on Ca^{2+} entry. No differences between the effects of tyrosine kinase inhibitors in pre-treatment or post-treatment protocols were found. This suggests that their effects upon Ca^{2+} entry do not depend on previous activation of the process. Also, the effects of tyrosine kinase inhibitors did not interfere with those of D600, a well-known VOC blocker (Weiss, 1982) (data not shown).

The experiments aimed to investigate internal Ca^{2+} store refilling demonstrated that tyrosine kinase inhibitors also inhibit the refilling of phenylephrine-sensitive Ca^{2+} stores. Our experimental design may not have been the best choice, because smooth muscle may show a sensitizing phenomenon in response to agonists (Himpens et al., 1990). However, we used only one agonist under similar conditions and most probably the amplitude of the phenylephrine-induced contraction in Ca^{2+}-free medium reflects the degree of phenylephrine-sensitive Ca^{2+} store filling. Unfortunately, in our experimental conditions one cannot distinguish between the direct effect of tyrosine kinase inhibitors on contractile filaments (Di Salvo et al., 1993a) and the Ca^{2+} release. However, the greater extent of inhibition in the presence of tyrosine kinase inhibitors during the refilling period as compared to that observed when tyrosine kinase inhibitors were present only in the Ca^{2+}-free medium, could be considered a good argument in favor of the effects of tyrosine kinase inhibitors upon internal Ca^{2+} store refilling. Beside a direct effect upon the contractile filaments,

tyrosine kinase inhibitors could directly inhibit the Ca^{2+} release, as shown once (Ozaki et al., 1993), but not confirmed by others (Lee et al., 1993; Sargeant et al., 1993). Such an action could be an additional explanation of the effects of tyrosine kinase inhibitor pretreatment in Ca^{2+}-free solution.

The possibility that tyrosine kinase-induced phosphorylation could be involved in the control of stored Ca^{2+} in rat aortic smooth muscle is further supported by the effects of vanadate. This drug, beside its effects on Ca^{2+} ATP-ases (Carafoli, 1992), also phosphorylates phospholipase C-γ, evoking the formation of inositol 1,4,5-trisphosphate (Bianchini et al., 1993) and enhances protein tyrosine phosphorylation in guinea pig taenia coli smooth muscle (Di Salvo et al., 1993b). Under our experimental conditions only 50% of the vanadate-induced contraction is genistein sensitive. This fact clearly demonstrates that, in rat aorta, vanadate contractile effects are not exclusively a consequence of tyrosine phosphatase inhibition. In Ca^{2+}-free solution it evokes a contraction similar (time and amplitude) to that evoked with phenylephrine. This effect is less probably to be the predominant consequence of a block of sarcoplasmic reticulum Ca^{2+} ATP-ase since thapsigargin, a specific SERCA ATP-ase blocker (Lytton et al., 1992), in Ca^{2+}-free medium at 1 μM concentration develops a much slower contraction with a significantly reduced amplitude (Filipeanu et al., unpublished results). One possible explanation for the vanadate-induced transient contraction in Ca^{2+}-free medium could be enhancement of protein tyrosine phosphorylation. In favor of this is the strong phasic contraction seen after the return to Ca^{2+}-containing solution. In the study of Di Salvo et al. (1993a,1993b a similar pattern was reported for guinea pig smooth muscle, together with a marked enhancement of protein tyrosine phosphorylation. However, the effects of vanadate on Ca^{2+} ATP-ases were clearly visible in our preparation because its presence during the refilling period totally suppressed the subsequent response to phenylephrine in Ca^{2+}-free medium.

Taking into account the data obtained in the present study, one can conclude that the protein tyrosine phosphorylation/dephosphorylation process seems to be a key step of signal transduction in vascular smooth muscle. These data are consistent with the results obtained by others (Duff et al., 1993; Granat et al., 1993), showing that in vascular smooth muscle cells the action of agonists is associated with an increase in protein tyrosine phosphorylation. Apart from other consequences, this process seems to participate in the control of normal Ca^{2+} homeostasis as shown by our results as well as by those obtained in other studies (Di Salvo et al., 1993a,b; Sargeant et al., 1993; Lee et al., 1993; Ozaki et al., 1993).

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