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Ras proteins increase Ca^{2+}-responsiveness of smooth muscle contraction

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G-proteins may be involved in receptor-mediated Ca^{2+}-sensitization of smooth muscle contraction, but the responsible G-proteins are not yet known. Here we show that in β-escin skinned mesenteric microarteries, H-ras p21 proteins, preactivated with GTP or GTPγS, increase force at constant submaximal Ca^{2+} (pCa 6.3) concentration dependently. The GTP-bound form of the wild-type H-ras p21 and the oncogenic mutant (p21[G12V]) were equally effective. The nucleotide-free and the inactive GDP-bound form of ras p21 had no effect on force. The tyrosine kinase inhibitor, tyrphostin, partially reversed the effect of the ras proteins in the GTP-bound form on force. Thus, ras proteins mimic the Ca^{2+}-sensitizing effect of GTPγS and vasoconstrictors in mesenteric microarteries which may involve tyrosine phosphorylation.

Ras p21; Permeabilized microartery; Ca^{2+}-sensitivity; Smooth muscle; Tyrosine phosphorylation; G protein

1. INTRODUCTION

α-Adrenergic activation of vascular smooth muscle may occur with little if any change in membrane potential ([1], pharmacomechanical coupling). Pharmacomechanical coupling involves at least two intracellular signalling cascades leading to the IP_3 mediated release of Ca^{2+} from intracellular stores [2] and sensitization of the myofilaments to Ca^{2+} [3,4]. The mechanism of the latter effect is not understood though it had been proposed that it may involve inhibition of myosin phosphatase [5–7], a G protein coupled mechanism [4,8] possibly involving rhoA p21 [9] or activation of protein kinase C [10]. Here we demonstrate, for the first time, a possible role of H-ras p21 in the mechanism causing Ca^{2+}-sensitization of vascular smooth muscle.

The effect of low molecular mass proteins, such as ras proteins, on tension development at 'clamped' [Ca^{2+}] can be investigated in smooth muscle preparations permeabilized with β-escin. In these preparations, the receptor effector coupling is still intact [11] while at the same time the diffusion barrier for these proteins is abolished. We tested two types of H-ras p21 (ras proteins), the wild-type H-ras p21 (c-ras) and its oncogenic mutant (p21 [G12V]; v-ras). Like the α-subunits of heterotrimeric G proteins, ras p21 can switch from an active GTP-bound form (GTP-ras) to an inactive GDP-bound form (GDP-ras) [12]. While the intrinsic GTPase activity of c-ras is stimulated by a GTPase activating protein (GAP), the GTPase activity of the oncogenic mutant, v-ras, is not subject to regulation by GAP [12]. The GTP-bound form of v-ras is therefore a permanently active form of ras p21.

We have recently shown [13] that norepinephrine-induced contractions in intact mesenteric microarteries were inhibited by tyrosine kinase inhibitors. Here we show that the Ca^{2+}-sensitizing effect of the ras proteins is partially reversed by tyrphostin, a synthetic tyrosine kinase inhibitor [14], suggesting the involvement of protein tyrosine phosphorylation in the ras p21 mediated increase in Ca^{2+}-responsiveness.

2. MATERIALS AND METHODS

Female guinea pigs (200–250 g body weight) were stunned and bled. Mesenteric microarteries (outer diameter in situ; 118 ± 4 μm, n = 54) were isolated and mounted on a myograph as reported previously [15]. Following an equilibration period of about 60 min in physiological salt solution buffered with HEPES (20 mM, pH 7.4 at 25°C) during which several contractions were elicited with KCl (125 mM), tissues were permeabilized with 50 μg/ml β-escin for 30 min at 25°C in relaxing solution. The relaxing solution contained (final concentration in mM): imidazole 20, EGTA 4, ATP 7.5, potassium methanesulfonate 110, magnesium acetate 10, NaN_3 1, creatine phosphate 10, at pH 7.0 and 25°C. The contracting solutions were obtained by varying the ratio of EGTA to Ca-EGTA [16]. To allow addition of proteins and guanosine nucleotides without diluting the ionic composition of the solutions,

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Abbreviations. Gpp(NH)p, guanylyl-5′-imidodiphosphate; GAP, GTPase activating protein; G protein, GTP-binding protein; c-ras p21, wild type H-ras p21; v-ras p21, mutant H-ras p21 (p21[G12V]); HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid].

they were made up as doubly concentrated stock solutions and stored in aliquots at −20°C [16]. This concentrate was diluted twofold by the addition of (final concentrations) creatine kinase (100 U/ml), calmodulin (1 μM), leupeptin (1 μM), dithioerythritol (2 mM) as well as ras proteins and guanosine nucleotides when indicated. Solutions were contained in thermostatted 0.2- or 1-ml cups. Prior to activation, each tissue was treated with 1 μM A23187 for 20 min in the relaxing solution to deplete Ca^{2+} from the intracellular store sites.

Recombinant nucleotide-free ras proteins were prepared as reported previously [17]. Protein concentration was determined by the method of Bradford [18] using IgG as a standard To obtain the GTPγS-bound form of c-ras, 300 μM nucleotide-free c-ras was reacted with 600 μM GTPγS and 1 mM $MgCl_2$ (final volume 100 μl) for 5 min at 25°C. The excess GTPγS was removed by a commercial gel filtration column (NAP5, Pharmacia) at 4°C in 20 mM imidazole [19]. In this way, 61 μM c-ras complexed with GTPγS was obtained. The protein was either shock frozen and stored at −80°C or kept on ice to minimize the dissociation of GTPγS and was used within 6 h. To obtain the GTP- and GDP-bound form of ras proteins, the nucleotide-free protein and the nucleotide were added to the incubation solution in the indicated concentrations and allowed to react for at least 5 min at 25°C prior to incubation of the tissues. The conditions were sufficient to exchange the nucleotides completely for the bound guanosine; the dissociation of the nucleotides and the cleavage of GTP is negligeable (cf. [17]). Tyrphostin was generously provided by J. Di Salvo, Dept. of Medical and Molecular Physiology, University of Minnesota, Duluth, USA.

Statistics Values are shown as means ± S.E.M. (n = number of experiments). Difference of responsiveness among groups was tested by the one-way analysis of variance, followed by paired or unpaired Student's t-test. P values < 0.05 were considered as indicating significant differences.

3. RESULTS

Guinea pig mesenteric microarteries permeabilized with β-escin responded to activation with pCa 6.3 with a submaximal contraction which amounted to 24 ± 4% of the maximal force obtained with pCa 4.9 (n = 24). GTPγS and Gpp(NH)p, two non-hydrolyzable GTP analogues, enhanced submaximal (Figs. 1A and 2D) but not maximal tension in this preparation. The effect of the GTP analogues was mimicked by c-ras preactivated with GTPγS (Fig. 1B,C). As unbound GTPγS was removed by gel filtration, the increase in Ca^{2+}-responsiveness was unlikely due to activation of endogenous G proteins by free GTPγS. The possibility, however, remains that microarteries may contain GTP/GDP exchange factors which might release GTPγS from GTPγS-ras. GTPγS may then bind to endogenous G proteins and permanently activate these. We addressed this problem by testing the effect of ras proteins activated with GTP.

Figs. 2 and 3 show that tension at submaximal Ca^{2+} was enhanced in the presence of 3 μM v-ras which was preactivated with different concentrations of GTP (0.01 to 10 μM). Interestingly, c-ras preactivated with GTP was equally effective as v-ras in increasing Ca^{2+}-responsiveness (Fig. 3). Moreover, we did not observe a difference in the time course (up to 12 min) of the tension response to the two GTP-ras proteins. In parallel experiments, we tested whether GTP when it is not bound to ras proteins also affects submaximal force. Fig. 3 shows that GTP alone is less effective in increasing force at all concentrations tested.

We performed several control experiments to test whether the effect of the GTP-ras proteins was specific for the active form. In the nucleotide free form, neither c- nor v-ras affected force (Fig. 2B). In the inactive, GDP-bound form, the ras proteins had only a minor effect on submaximal force (Fig. 2A). Thus, force in the presence of 3 μM v-ras bound to equimolar [GDP] was 127 ± 8% of the Ca^{2+}-activated force. In the presence of GDP-v-ras, the dose–response relation to GTP appears to be suppressed somewhat (Fig. 3), but this was not statistically significant. Following permanent activation of endogenous G proteins by Gpp(NH)p (0.3 μM) which increased Ca^{2+}-responsiveness 3- to 4-fold, neither GTP nor GTP-ras proteins (Fig. 2D) produced a further increase in force.

The ras p21 induced increase in Ca^{2+}-responsiveness was partially reversed by tyrphostin, a synthetic tyrosine kinase inhibitor (Fig. 2B,C). Tyrphostin partially

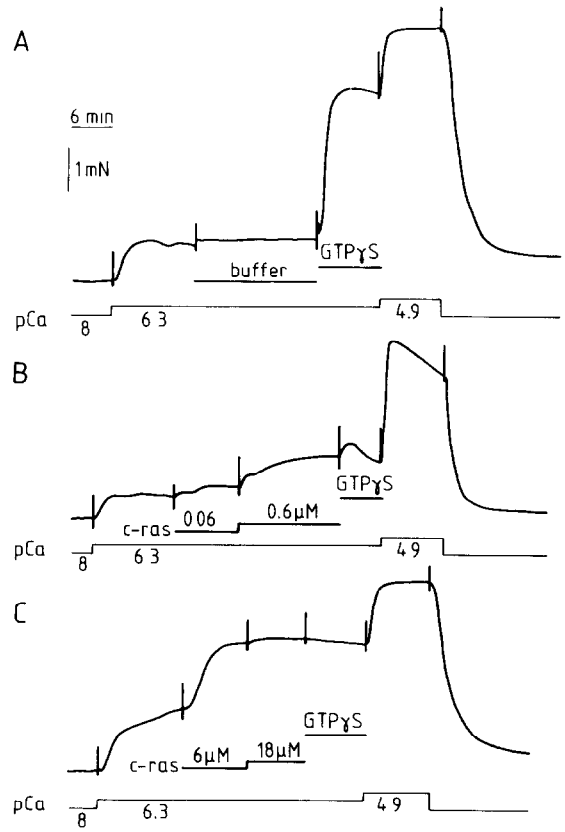

Fig. 1. Effect of c-ras preactivated with GTPγS on submaximal force in β-escin skinned mesenteric microarteries (A) Control, GTPγS (3 μM) but not the ras-buffer increased force elicited by submaximal [Ca^{2+}]. (B and C) C-ras preactivated with GTPγS (see section 2) increased submaximal force concentration dependently. Threshold concentration was < 0.6 μM. Following incubation with high concentrations of preactivated c-ras, GTPγS had no further effect on force (C).

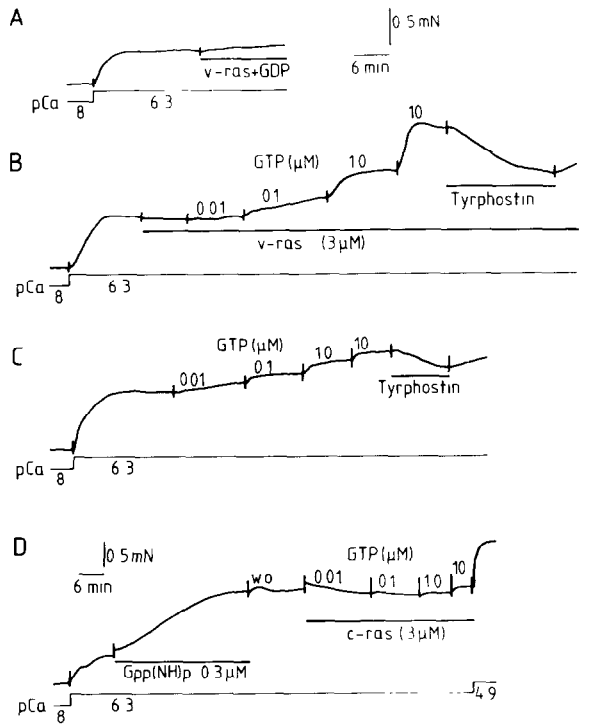

Fig. 2. Effect of ras proteins on submaximal force (elicited at pCa 6.3) in mesenteric microarteries. (A) The inactive GDP bound form of ras (3 μM v-ras bound to equimolar GDP) has only a minor contractile effect. (B) Following activation with pCa 6.3, the microartery was first incubated with nucleotide free ras and then with ras preactivated with GTP. Note that nucleotide free ras proteins (3 μM) were mixed with the indicated concentrations of GTP at least 5 min prior to incubation of the tissue; rapid (spike like) vertical tension changes indicate the solution changes to a new solution with the next higher [GTP] at constant [ras]. (C) Effect of GTP alone. (D) Gpp(NH)p enhanced submaximal force irreversibly (w.o. = washout), c-ras preactivated with GTP (cf. lane B) had no further effect on force, but force could be further enhanced by pCa 4.9. The tyrosine kinase inhibitor, tyrphostin (50 μM), partially reversed the effects of GTP-ras and GTP (B,C).

relaxed both the force enhanced by 3 μM v-ras preactivated with 10 μM GTP (GTP-ras: 250 ± 4% of Ca^{2+}-induced force, + tyrphostin: 150 ± 9%, n = 5) as well as the force enhanced by 10 μM GTP alone (GTP: 140 ± 13% of Ca^{2+}-induced force, + tyrphostin: 124 ± 12%, n = 4). Tyrphostin also partially reversed the tension increase induced by GTP bound c-ras but had no effect on Ca^{2+}-induced force [13].

4. DISCUSSION

In this study we present evidence that active H-ras p21 proteins increased force at constant submaximal [Ca^{2+}] in β-escin skinned mesenteric microarteries. Thus, ras proteins in the GTP and GTPγS bound form mimicked the Ca^{2+}-sensitizing effect of agonists such as norepinephrine [3,4] and non-hydrolyzable GTP analogues in vascular smooth muscle [3,4,6,8] while the inactive forms of ras proteins had no (nucleotide free ras) or only a minor effect (GDP-ras) on force. Following activation of endogenous G proteins with the non-hydrolyzable GTP analogue, Gpp(NH)p, exogenously added GTP-ras proteins no longer enhanced force suggesting that it may affect endogenous signalling pathways involved in Ca^{2+}-sensitization (cf. Fig. 2D). It should be noted that the recombinant ras proteins used in this study are not post-translationally modified suggesting that their Ca^{2+}-sensitizing action does not require post-translational modification.

The Ca^{2+}-sensitizing effect was concentration-dependent and occurred at submicromolar concentrations of active ras proteins (0.6 μM GTPγS-ras or 3 μM ras proteins activated with 0.1 μM GTP). Although the GTP-ras complexes were not purified by gel filtration, the free [GTP] in the incubation solution was negligeable when the ras proteins were activated with substoichiometric amounts of GTP due to the high affinity of ras proteins for GTP [17,20]. Furthermore, no significant dissociation of the nucleotides from the complexes has to be expected under our incubation conditions based on the work of Neal et al. [19] and the dissociation rate constants [17,20]. There is, however, some uncertainty concerning the stability of the GTP-ras complex within the microarteries as GTP may be released from the ras proteins by exchange factors and may then bind

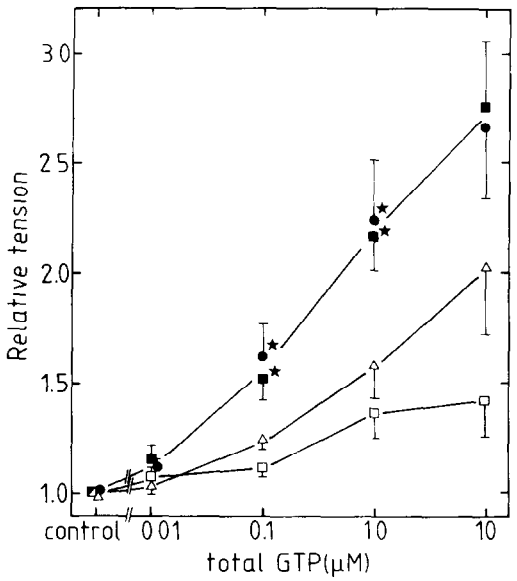

Fig. 3. Summary of the effect of ras proteins preactivated with GTP (■,●) in the concentrations given on the abscissa on submaximal force (pCa 6.3) Note that c-ras (●) and v-ras (■) were equally effective. GTP either alone (△) or in the continued presence of 3 μM GDP-v-ras (□) had a significantly (asterix) smaller effect on force. Experimental protocol as in Fig. 2B and C. Force was expressed relative to the tension elicited by pCa 6.3 (control). Total GTP (given on the abscissa) indicates either free [GTP] (experiments with GTP alone or in the presence of GDP-ras) or the concentrations of GTP (bound and free) with which the ras proteins were preactivated before incubation of the tissues. Values are mean ± S.E.M. of 4 (□) and 8 microarteries (△,■,●).

to and activate endogenous G proteins; obviously the maximal concentration of released GTP would be equal to the concentration with which the ras proteins were activated. If this were the case, then GTP alone should increase force to a similar extent as GTP-ras. However, the effect of GTP alone was significantly smaller than that of GTP-ras (Fig. 3).

In signalling cascades involving ras p21, phosphorylation of proteins on tyrosyl residues (e.g. GAP and extracellular signal regulated kinase, MAP2 kinase) has been reported [21,22]. Tyrosine phosphorylation may also be involved in the increase in Ca^{2+}-responsiveness observed in the presence of GTP-ras as it is partially inhibited by the tyrosine kinase inhibitor, tyrphostin. As the cellular and mutant GTP-bound form of ras proteins were equally effective in increasing submaximal force GAP does not appear to be functional under our experimental conditions. This suggests that the Ca^{2+}-sensitization may involve tyrosine phosphorylation downstream of ras p21, e.g. MAP2 kinase. It is interesting to note that norepinephrine induced contractions in mesenteric microarteries are also inhibited by tyrphostin [13] and in smooth muscle cells, several proteins are phosphorylated on tyrosyl residues in response to a number of vasoconstrictors [23].

Recently, it was reported that rhoA p21, another small G protein, increases Ca^{2+}-sensitivity in saponin skinned smooth muscle [9]. This protein shares 30–40% amino-acid sequence homology with ras p21 [24]. How ras p21 and rhoA p21 increase Ca^{2+}-responsiveness is presently unknown. The so far identified downstream effects of ras p21 and rho p21 are different [22,25,26]. It is therefore unlikely that they interact with the same effector associated with the myofilaments. Signalling pathways involving ras p21 and rho p21 may, however, be coupled [27] probably in a hierarchical manner as has recently been observed in swiss 3T3 cells [28]. Alternatively, they may act independently but have a common biological effect in smooth muscle cells, namely increasing Ca^{2+}-sensitivity.

In other cell systems, ras increases the arachidonic acid production [25] and activates MAP2 kinase [22]. Both effects, if operating in smooth muscle, may increase Ca^{2+}-sensitivity. Arachidonic acid increases Ca^{2+}-sensitivity of smooth muscle contraction, which is associated with an increase in myosin light chain phosphorylation and inhibition of phosphatase [7]. MAP2 kinase phosphorylates caldesmon [29,30], which results in weakening of the binding of caldesmon to actin [29]. Assuming that unphosphorylated caldesmon inhibits smooth muscle contraction [31,32], phosphorylation of caldesmon may be a mechanism to increase Ca^{2+}-sensitivity independent of an increase in myosin light chain phosphorylation [33]. It will, therefore, be interesting to see whether active H-ras p21 increases arachidonic acid production and/or stimulates MAP2 kinase activity in smooth muscle cells.

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