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Involvement of both G protein αs and βγ subunits in β-adrenergic stimulation of vascular L-type Ca^{2+} channels

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1 Previous data have shown that activation of β_3-adrenoceptors stimulates vascular L-type Ca^{2+} channels through a Gαs-induced stimulation of the cyclic AMP/PKA pathway. The present study investigated whether β-adrenergic stimulation also uses the Gβγ/PI3K/PKC pathway to modulate L-type Ca^{2+} channels in rat portal vein myocytes.

2 Peak Ba^{2+} current (I_{Ba}) measured using the whole-cell patch clamp method was maximally increased by application of 10 μM isoprenaline after blockade of β_3-adrenoceptors by 1 μM SR59230A. Under these conditions, the isoprenaline-induced stimulation of I_{Ba} was reversed by ICI-118551 (a specific β_2-adrenoceptor antagonist) but not by atenolol (a specific β_1-adrenoceptor antagonist). The β_2-adrenoceptor agonist salbutamol increased I_{Ba}, an effect which was reversed by ICI-118551 whereas the β_1-adrenoceptor agonist dobutamine had no effect on I_{Ba}.

3 Application of PKA inhibitors (H-89 and Rp 8-Br-cyclic AMPs) or a PKC inhibitor (calphostin C) alone did not affect the β_2-adrenergic stimulation of I_{Ba} whereas simultaneous application of both PKA and PKC inhibitors completely blocked this stimulation.

4 The β_2-adrenergic stimulation of L-type Ca^{2+} channels was blocked by a pre-treatment with cholera toxin and by intracellular application of an anti-Gαs antibody (directed against the carboxyl terminus of Gαs). In the presence of H-89, intracellular infusion of an anti-$G\beta_{com}$ antibody or a βARK_1 peptide as well as a pre-treatment with wortmannin (a PI3K inhibitor) blocked the β_2-adrenergic stimulation of I_{Ba}.

5 These results suggest that the β_2-adrenergic stimulation of vascular L-type Ca^{2+} channels involves both Gαs and Gβγ subunits which exert their stimulatory effects through PKA and PI3K/PKC pathways, respectively.

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Abbreviations: I_{Ba}, Ba^{2+} current; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C

Introduction

In vascular and visceral smooth muscles, a number of studies have shown that β-adrenergic receptor stimulation leads to enhancement of L-type Ca^{2+} channel activity *via* the cyclic AMP/protein kinase A (PKA) transduction pathway (Ruiz-Velasco *et al*., 1998; Viard *et al*., 2000). After binding with agonist, the β-adrenergic receptor catalyzes the exchange of GDP for GTP on the α subunit of the heterotrimeric G proteins, resulting in dissociation of the α subunit from the βγ dimers. It is now well documented that both the Gαs subunit and the Gβγ dimers are able to transduce signals to effector molecules (Clapham & Neer, 1997). Previous studies from our laboratory have shown that muscarinic m3- and endothelin ET_A-induced Ca^{2+} release in vascular myocytes is selectively transduced by $G\alpha_q$ and $G\alpha_{11}$ subunits, respectively (Morel *et al*., 1997; Macrez *et al*., 1999). In contrast, angiotensin AT_1 receptors transduce the signal to L-type Ca^{2+} channels by Gβγ dimer (Macrez-Leprêtre *et al*., 1997). In the absence of receptor activation, infusion of Gβγ subunits in the myocytes leads to stimulation of L-type Ca^{2+} channels (Viard *et al*., 1999; Zhong *et al*., 1999).

On the basis of RT–PCR experiments, three β-adrenoceptor subtypes may be expressed in rat portal vein myocytes (Viard *et al*., 2000). The transduction pathway activated by β_3-adrenoceptors is selectively mediated through a Gαs-induced stimulation of the cyclic AMP/PKA pathway (Viard *et al*., 2000). However, the possible role of endogenous Gβγ subunits to the β-adrenergic stimulation of L-type Ca^{2+} channels is still unknown. No informations are available concerning the potential role of Gβγ subunits as possible contributors to stimulation of L-type Ca^{2+} channels in response to activation of β-adrenoceptor subtypes.

In the present study, we investigated the transduction pathways activated by non β_3-adrenoceptors (i.e. β_1- and/or β_2-adrenoceptors) in the modulation of L-type Ca^{2+} channels in rat portal vein myocytes. We combined the use of inhibitors of PKA and PKC to determine whether one or both of these kinases contribute to the response and specific β-adrenoceptor agonists and antagonists to characterize the receptor subtypes involved. We show that Gαs and Gβγ subunits participate in the β_2-adrenergic stimulation of L-

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type Ca^{2+} channels through activation of PKA and PKC, respectively.

Methods

The investigation conforms with the European Community guiding principles in the care and use of animals (86/609/CEE, CE Off J no. L358, 18 December 1986) and the French decree no. 87/748 of October 19, 1987 (J Off République Française, 20 October 1987, pp. 12245–12248). Authorizations to perform animal experiments according to this decree were obtained from the French Ministère de l'Agriculture et de la Pêche.

Cell preparation

Isolated myocytes from rat portal vein were obtained by enzymatic dispersion, as described previously (Leprêtre *et al*., 1994). Cells were seeded at density of $\sim 10^3$ cells mm^{-2} on glass slides and maintained in short-term primary culture (2–36 h) in M199 containing 5% foetal calf serum, 2 mM glutamine, 1 mM pyruvate 20 U ml^{-1} penicillin and 20 μg ml^{-1} streptomycin.

Membrane current measurement

Voltage-clamp and membrane current recordings were performed with a standard patch-clamp technique using an EPC-7 amplifier (List, Darmstadt-Eberstadt, Germany). Whole-cell recordings were performed with patch pipettes having resistances of 2–4 MΩ. Membrane potential and membrane currents were stored and analysed using a PC computer (P-clamp system, Axon Instruments, Foster City, CA, U.S.A.). Ba^{2+} currents were digitally corrected for leakage currents. In some experiments, Ba^{2+} current density is expressed as peak current amplitude per capacitance unit (in pA/pF). All experiments were performed at 30 ± 1°C. Results are expressed as means ± s.e. mean. Significance was tested by Student's *t*-test. *P* values < 0.05 were considered as significant.

Solutions

The physiological solution used to record Ba^{2+} currents contained (in mM): NaCl 130, KCl 5.6, $MgCl_2$ 1, $BaCl_2$ 5, glucose 11, HEPES 10, pH 7.4 with NaOH. The basic pipette solution contained (in mM): CsCl 130, EGTA 10, $ATPNa_2$ 5, GTP 0.1, $MgCl_2$ 2, HEPES, 10 pH 7.3, with CsOH. Gβγ proteins were stored in a solution containing 20 mM Tris, 1 mM EDTA, 11 mM CHAPS, and 20 mM β-mercaptoethanol. At the concentration of Gβγ used in the experiments, the final concentration of detergent was 100 μM CHAPS, which alone had no effects on Ba^{2+} current density (Viard *et al*., 1999). β-adrenergic agonists, phorbol ester and 8-Br-cAMP were extracellularly applied to the recorded cell by pressure ejection from a glass pipette.

Chemicals and drugs

Isoprenaline, salbutamol, propranolol, prazosin, rauwolscine, dobutamine and wortmannin were from Sigma (St Quentin Fallavier, France). 8-Br-cAMP, Rp 8-Br-cAMPs, H-89, calphostin C and cholera toxin (CTX) were from Calbiochem (Meudon, France). Atenolol, ICI-118551, phorbol 12-myristate 13-acetate and 4α-phorbol 12-myristate 13-acetate were from RBI (Natick, MA, U.S.A.). SR59230A (3-(2-ethylphenoxy)-1[(1S)-1,2,3,4-tetrahydronapth-1-ylaminol]-(2S)-propranolol-oxalate) was from Sanofi (Milano, Italy). M199 medium was from Flow Laboratories (Puteaux, France). Streptomycin, penicillin, glutamine and pyruvate were from Life Technology (Paisley, U.K.). Rabbit anti-$G\alpha_s$ subunit antibody (371732) raised to the carboxyl-terminal amino acids, RMHLRQYELL, of $G\alpha_s$ was from Calbiochem. Rabbit anti-$G\beta_{com}$ antibody (SC-378) raised to the carboxyl-terminal amino acids, TDDGMAVATG SWDSFLKIWN, of $G\beta_1$ subunit was from Santa-Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Peptides corresponding to the Gβγ binding domain of β-adrenergic receptor kinase-1 (WKKELRDAYREAQQLVQRVPKMKNKPRS) or to a region outside the Gβγ binding site (AETDRLEARKKTKNKQLGHEEDY) were synthetized by Genosys (Cambridge, U.K.). Gβγ subunits purified from bovine brain Gi/Go proteins were a gift from B. Nürnberg (University of Berlin).

Results

β-adrenergic stimulation of L-type Ca^{2+} channels in vascular myocytes

We have previously shown that a combination of propranolol (a non-selective β_1- and β_2-adrenoceptor antagonist) and SR59230A (a β_3-adrenoceptor antagonist) was required to inhibit the isoprenaline-induced maximal stimulation of L-type Ca^{2+} channels. Studied in isolation, the β_3-adrenoceptor-induced transduction pathway involves the Gαs-induced stimulation of the cyclic AMP/PKA (Viard *et al*., 2000). Here, we combined the application of both inhibitors of PKA and PKC and antagonists of β-adrenoceptor subtypes to further investigate the transduction pathways activated by β_1- and/or β_2-adrenoceptors leading to stimulation of Ca^{2+} channels. Ba^{2+} currents through L-type Ca^{2+} channels were elicited every 20 s in single vascular myocytes bathed in 5 mM $BaCl_2$ solution by 200 ms depolarizations to +10 mV from a holding potential of −40 mV. As previously reported (Viard *et al*., 1999), inward current progressively increased in amplitude over 2–3 min in almost all cells due to the diffusion of the pipette solution into the cytoplasm (Figure 1A). After steady-state was reached, application of isoprenaline (10 μM) resulted in an increase in Ba^{2+} current which reached a peak within 2–3 min (Figure 1B) without any variation in holding current. Removal of isoprenaline resulted in a progressive and slow return to basal Ba^{2+} current within 5–7 min. All the experiments reported in the present paper were carried out in the continuous presence of 1 μM SR59230A to inhibit β_3-adrenoceptors and stimulation of Ba^{2+} current was measured a time to peak effect during 3 min-lasting applications of stimulating substances. Neither time to peak current nor inactivation kinetics were modified in the presence of isoprenaline (Figure 1B).

To identify the β-adrenergic subtypes responsible for the non-β_3-adrenoceptor-induced stimulation of L-type Ca^{2+}

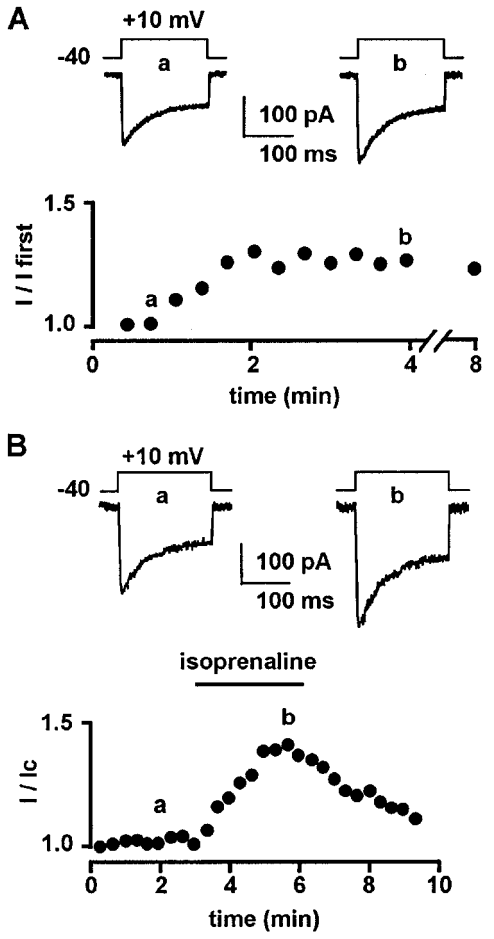

Figure 1 Effects of isoprenaline on L-type Ca^{2+} channels in rat portal myocytes. (A) Time course of peak Ba^{2+} current (expressed as a fraction of the first current obtained just after breakthrough into the whole-cell recording mode) in cells dialyzed with a control pipette solution. Inset: typical Ba^{2+} currents (a,b) evoked by a depolarization to +10 mV from a holding potential of −40 mV corresponding to lowercase letters on the curve. (B) Time course of peak Ba^{2+} current during the application of 10 μM isoprenaline, 3 min after reaching steady-state current. Inset: typical Ba^{2+} currents (a,b) corresponding to lowercase letters on the curve. External solution contained 5 mM Ba^{2+}, 10 nM prazosin and 10 nM rauwolscine (to inhibit α_1- and α_2-adrenoceptors) and 1 μM SR59230A (to inhibit β_3-adrenoceptors).

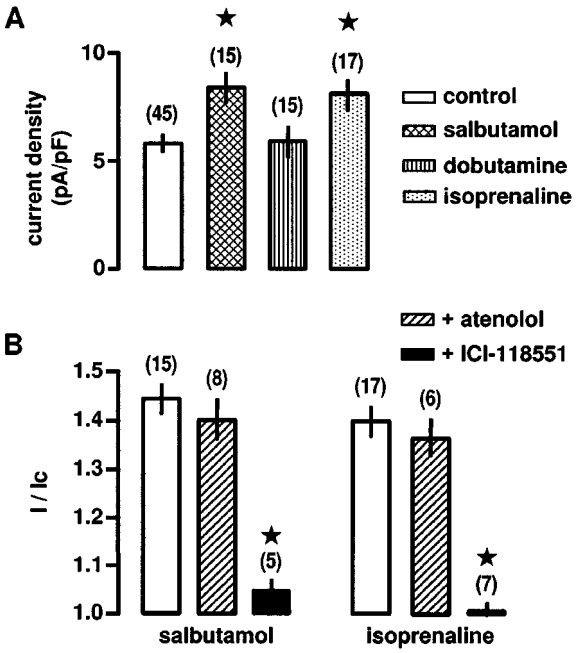

Figure 2 Effects of β-adrenoceptor agonists and antagonists on L-type Ca^{2+} channels. (A) Ba^{2+} current densities in control conditions and in the presence of 10 μM salbutamol (a β_2-adrenoceptor agonist), 10 μM dobutamine (a β_1-adrenoceptor agonist) or 10 μM isoprenaline. Current density was calculated at the time of peak effect. *Values significantly different from control ($P<0.05$). (B) Compiled data showing the effects of 1 μM ICI-118551 (a β_2-adrenoceptor antagonist) and 1 μM atenolol (a β_1-adrenoceptor antagonist) on the increase in peak Ba^{2+} current evoked by 10 μM salbutamol or 10 μM isoprenaline. Currents are expressed as a fraction of their values in the absence of agonists (I/Ic). *Values significantly different from those obtained in the presence of agonists ($P<0.05$). Data are means±s.e.mean with the number of cells tested indicated in parentheses. External solution contained 5 mM Ba^{2+}, 10 nM prazosin, 10 nM rauwolscine and 1 μM SR59230A.

channels, we tested the effects of isoprenaline and of selective β_1-and β_2-adrenergic agonists and antagonists. In control conditions, the mean Ba^{2+} current density was 5.8±0.4 pA/pF ($n=45$). Salbutamol (10 μM), a potent β_2-adrenergic agonist (Skeberdis *et al*., 1997), increased the Ba^{2+} current density by 45±7% (Figure 2A) whereas dobutamine (10 μM), a potent β_1-adrenergic agonist (Doggrell, 1990), had no detectable effect on the Ba^{2+} current density (Figure 2A). Isoprenaline (10 μM) increased the Ba^{2+} current density by 41±5% (Figure 2A), a value not significantly different from that obtained with salbutamol. The agonist-induced increases in Ba^{2+} current density were not different from the increase in peak current expressed as a fraction of control current (Figure 2B), indicating that this method was adequate for measuring the stimulatory effect of externally-applied agonists. The stimulatory effect of salbutamol was abolished by application of 1 μM ICI-118551, a selective antagonist of β_2-adrenoceptor (Bilski *et al*., 1983) or 1 μM propranolol (not shown), whereas application of 1 μM atenolol, a selective antagonist of β_1-adrenoceptors (Satake *et al*., 1996) was ineffective (Figure 2B). As the stimulatory effect of isoprenaline was abolished by application of 1 μM ICI-118551 (Figure 2B) or 1 μM propranolol (not shown) but not significantly affected by application of 1 μM atenolol (Figure 2B), it is likely that the β_2-adrenoceptors are the predominant receptor subtype underlying non β_3-adrenoceptor-induced stimulation of Ba^{2+} current.

To evaluate whether PKA is involved in the intracellular pathway activated by β_2-adrenoceptors, we tested the effects of PKA inhibitors (H-89 and Rp 8-Br-cyclic AMPs) on the stimulation of Ba^{2+} current evoked by isoprenaline (in the continuous presence of SR59203A). External application of H-89 (0.1 μM) or Rp 8-Br-cyclic AMPs (0.3 μM), applied 15 min before and throughout the current recording period did not change the peak Ba^{2+} current in control cells (data not shown) nor affect the stimulatory response of 0.1 μM PMA, an activator of PKC (Figure 3A,B). Increase in peak Ba^{2+} current evoked by 0.3 mM 8-Br-AMP was inhibited by H-89 and Rp 8-Br-cyclic AMPs, applied 15 min before and

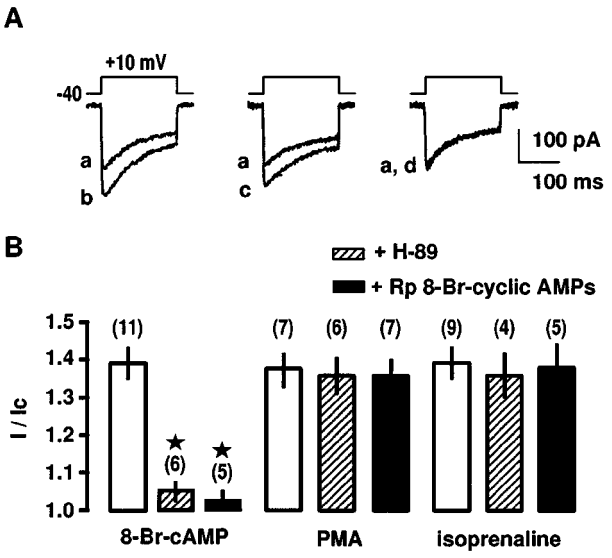

Figure 3 Effects of PKA inhibitors on β_2-adrenergic stimulation of L-type Ca^{2+} channels. (A) Ba^{2+} currents evoked by a depolarization to +10 mV from a holding potential of −40 mV before (a) and during the application of 10 μM isoprenaline in control conditions (b), in the presence of 0.1 μM H-89 for 15 min (c) or 1 μM propranolol for 15 min (d). (B) Compiled data showing the effect of 0.1 μM H-89 and 3 μM Rp 8-Br-cyclic AMPs on the increase in Ba^{2+} current evoked by 0.3 mM 8-Br-cAMP, 0.1 μM phorbol 12-myristate 13-acetate (PMA) or 10 μM isoprenaline. Currents are expressed as a fraction of their values in the absence of the stimulating substances (I/Ic). Data are means±s.e.mean with the number of cells tested indicated in parentheses. *Values significantly different from those obtained in the presence of the stimulating substances ($P<0.05$). External solution contained 5 mM Ba^{2+}, 10 nM prazosin, 10 nM rauwolscine and 1 μM SR59230A.

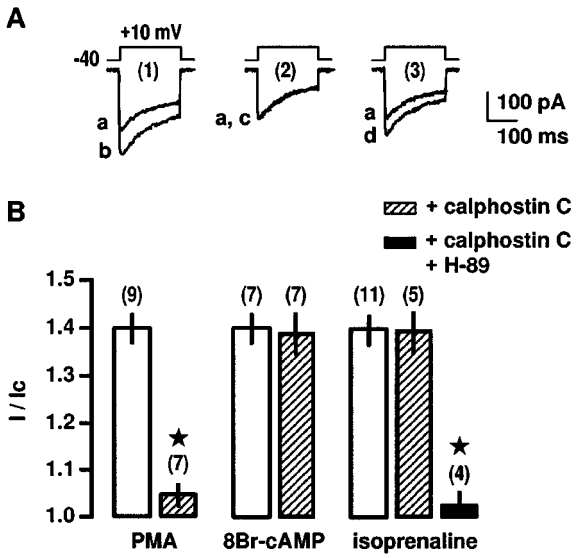

Figure 4 Effects of PKC and PKA inhibitors on β_2-adrenergic stimulation of L-type Ca^{2+} channels. (A) Ba^{2+} currents evoked by a depolarization to +10 mV from a holding potential of −40 mV before (a) and during the application of 0.1 μM PMA for 5 min (b) in control conditions (1). In the presence of 0.2 μM calphostin C for 10 min (2 and 3), Ba^{2+} currents obtained before (a) and during the application of 0.1 μM PMA for 5 min (c) or 0.3 mM 8-Br-cAMP for 8 min (d). (B) Compiled data showing the effect of 0.2 μM calphostin C alone or associated with 0.1 μM H-89 on the increase in Ba^{2+} current evoked by 0.1 μM PMA, 0.3 mM 8-Br-cAMP and 10 μM isoprenaline. Currents are expressed as a fraction of their control values in the absence of stimulating substances (I/Ic). Data are means±s.e.mean with the number of cells tested indicated in parentheses. *Values significantly different from those obtained in the presence of stimulating substances ($P<0.05$). External solution contained 5 mM Ba^{2+}, 10 nM prazosin, 10 nM rauwolscine and 1 μM SR59230A.

throughout the current recording period (Figure 3B). In contrast, application of H-89 or Rp 8-Br-cyclic AMPs alone had not significant effect on the isoprenaline-induced stimulation of peak Ba^{2+} current (Figure 3B). A similar absence of effect of PKA inhibitors was also observed on the salbutamol-induced stimulation of Ba^{2+} current ($n=9$). Because it has been recently suggested that PKC inhibitors (19–31 peptide or GF109203X) may act more selectively to some PKC isoforms (Wang *et al*., 1999), we tested calphostin C, a general inhibitor of PKCs, on the stimulation of Ba^{2+} currents involving activation of PKC or PKA. Superfusion of cells with 0.2 μM calphostin C for 10–15 min reduced the peak Ba^{2+} current in control cells by 25±5% ($n=9$). However, calphostin C-pretreatment did not affect the 8-Br-AMP-induced stimulation of L-type Ca^{2+} channels (Figure 4). Stimulation of peak Ba^{2+} current evoked by 0.1 μM PMA was blocked by 0.2 μM calphostin C (Figure 4A,B). In contrast, application of 0.2 μM calphostin C alone did not affect the isoprenaline-induced stimulation of peak Ba^{2+} current (Figure 4B). However, simultaneous applications of 0.2 μM calphostin C plus 0.1 μM H-89 completely inhibited the stimulatory effect of isoprenaline (Figure 4B). A similar inhibition was obtained when salbutamol was used to increase peak Ba^{2+} current ($n=12$). These results suggest that the β_2-adrenergic stimulation of L-type Ca^{2+} channels is likely to involve both PKA and PKC.

Simultaneous applications of 8-Br-AMP and PMA at concentrations producing a maximal stimulation of the Ba^{2+} current induced a slight further enhancement in Ba^{2+} current when compared to the application of one or the other activator alone. Thus, coapplication of both 8-Br-AMP and PMA did not result in a clear additive effect on L-type Ca^{2+} channel activity. However, when 8-Br-AMP and PMA were co-applied at submaximal concentrations, an additive stimulation of Ba^{2+} current was obtained which reached 49±5% ($n=5$); this stimulation was not different of that obtained with a maximal concentration of PKA or PKC activators.

Gαs- and Gβγ subunit-dependent pathways activated by β_2-adrenergic stimulation

We have previously shown that intracellular application of purified Gβγ subunits from bovine brain to vascular myocytes stimulates L-type Ca^{2+} channels through a PI3K/PKC pathway (Viard *et al*., 1999). However, it is unclear whether a similar coupling is involved in the response to β-adrenergic stimulation. To identify the G protein involved in the β_2-adrenergic stimulation, cells were infused with antibodies directed against the Gα-subunits. Antibodies directed against the carboxyl terminus of Gαs inhibit the interactions between the activated receptors and the Gs protein and therefore prevent the dissociation of the Gα- and Gβγ-subunits. Intracellular application of anti-Gαs antibody

through the patch-clamp pipette for 5 min before application of isoprenaline inhibited in a concentration-dependent manner the isoprenaline-induced increase in Ba^{2+} current density (Figure 5A,B). Complete inhibition was obtained at 10 μg ml^{-1} anti-Gαs antibody. In contrast, intracellular application of 10 μg ml^{-1} anti-$G\alpha_{q/11}$ antibody had no effect on the stimulation of the Ba^{2+} current evoked by isoprenaline (not shown). Furthermore, inactivated antibody obtained by heating at 95°C for 30 min had no effect on the β_2-adrenergic stimulation (Figure 5A,B). Pre-treatment with 150 ng ml^{-1} CTX for 24 h increased slightly the Ba^{2+} current density by about 10% (from 6.3±0.3 pA/pF in control cells to 7.0±0.3 pA/pF in CTX-pretreated cells, $n=24$), suggesting that the permanent activation of Gs induced a desensitization of the transduction pathway. Under these conditions, β-agonists did not change significantly the Ba^{2+} current density (7.1±0.5 pA/pF, $n=12$). These results indicate that the Gs protein is involved in the transduction pathways leading to stimulation of L-type Ca^{2+} channels in response to stimulation of β_2-adrenoceptors.

To demonstrate that the β_2-adrenergic stimulation of L-type Ca^{2+} channels involves the Gβγ subunits, intracellular infusion of either an anti-$G\beta_{com}$ antibody or a peptide corresponding to the fragment of βARK_1 was used to bind Gβγ subunits released after receptor activation and therefore to block activation of effectors. The following experiments were performed in the continuous presence of 0.1 μM H-89 to remove the PKA-activated pathway. Applications of βARK_1 peptide and anti-$G\beta_{com}$ antibody had no significant effects on the Ba^{2+} current density in non-stimulated cells. The mean Ba^{2+} current density was 6.4±0.4 pA/pF in control conditions ($n=11$), 6.0±0.5 pA/pF in the presence of 10 μM βARK_1 ($n=10$) and 6.1±0.5 pA/pF in the presence of 10 μg ml^{-1} anti-β_{com} antibody ($n=9$). As shown in Figure 6, anti-β_{com} antibody and βARK_1 peptide (corresponding to the Gβγ binding domain of βARK_1; Nair *et al*., 1995) inhibited in a concentration-dependent manner the β_2-adrenergic increase in Ba^{2+} current density. In contrast, inactive βARK_1 peptide and inactivated anti-β_{com} antibody (by heating at 95°C for 30 min) had no effect on the β_2-adrenergic stimulation (not shown). It is noteworthy that in the absence of PKA inhibitors, maximal concentrations of anti-β_{com} antibody and βARK_1 peptide alone were unable to inhibit the isoprenaline-induced stimulation of Ba^{2+} current ($n=16$). Taken together, these results suggest that both αs and βγ subunits of Gs proteins are involved in the β_2-adrenergic stimulation of L-type Ca^{2+} channels.

Finally, we pre-treated the cells with the covalent membrane permeant-PI3K inhibitor wortmannin (Yano *et al*., 1993) to reveal the presence of PI3K in the transduction pathway activated by β_2-adrenoceptors. As shown in Figure 7A,B, a pre-treatment for 30 min with increasing concentrations of wortmannin inhibited the Gβγ-induced increase in Ba^{2+} current density. Similarly, the isoprenaline-induced

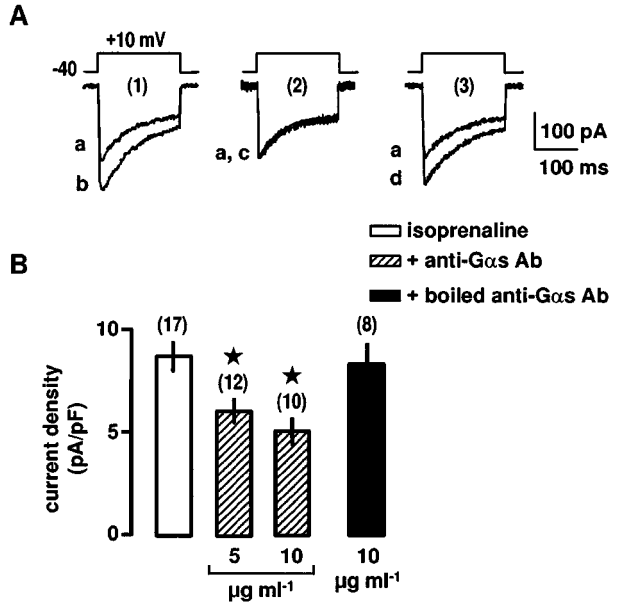

Figure 5 Effects of anti-Gαs antibody on the β_2-adrenergic stimulation of L-type Ca^{2+} channels. (A) Ba^{2+} currents evoked by a depolarization to +10 mV from a holding potential of −40 mV before (a) and during the application of 10 μM isoprenaline for 5 min in control conditions (1b), in the intracellular presence of 10 μg ml^{-1} anti-Gαs antibody (2c) or 10 μg ml^{-1} boiled anti-Gαs antibody for 5 min (3d). (B) Compiled data showing the effects of anti-Gαs antibody on the increase in Ba^{2+} current density evoked by 10 μM isoprenaline. Data are means±s.e.mean with the number of cells tested indicated in parentheses. *Values significantly different from those obtained in the presence of isoprenaline ($P<0.05$). External solution contained 5 mM Ba^{2+}, 10 nM prazosin, 10 nM rauwolscine and 1 μM SR59230A.

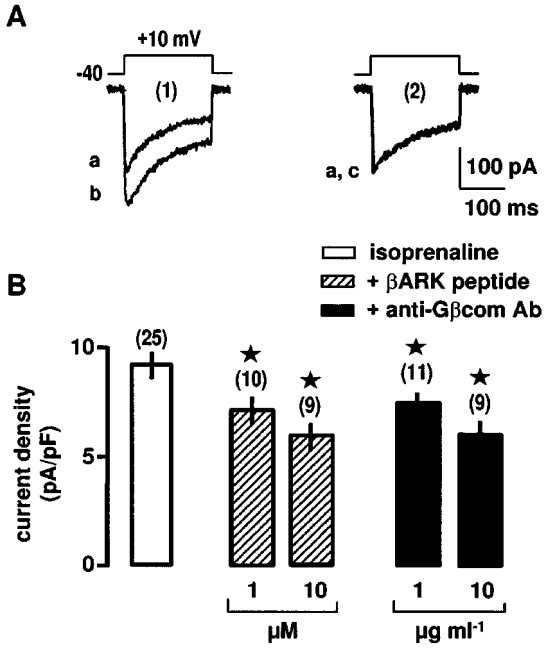

Figure 6 Effects of βARK_1 peptide and anti-$G\beta_{com}$ antibody on the β_2-adrenergic stimulation of L-type Ca^{2+} channels. (A) Ba^{2+} currents evoked by a membrane depolarization to +10 mV from a holding potential of −40 mV before (a) and during the application of 10 μM isoprenaline (b) in control conditions (1). In the intracellular presence of 10 μM βARK_1 peptide (2), Ba^{2+} currents before (a) and during the application of 10 μM isoprenaline (c). (B) Compiled data showing the effects of βARK_1 peptide and anti-$G\beta_{com}$ antibody on the increase in Ba^{2+} current density evoked by 10 μM isoprenaline. *Values significantly different from those obtained in the presence of isoprenaline alone ($P<0.05$). External solution contained 5 mM Ba^{2+}, 10 nM prazosin, 10 nM rauwolscine, 1 μM SR59230A and 0.1 μM H-89.

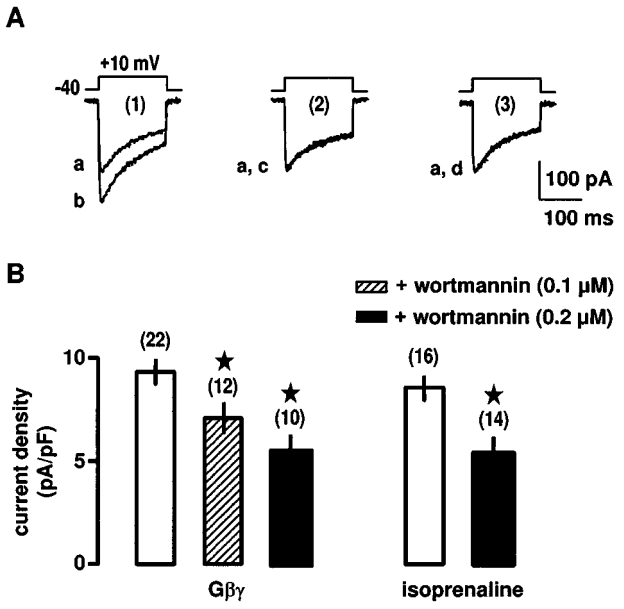

Figure 7 Effects of PI3K inhibitor on the β_2-adrenergic stimulation of L-type Ca^{2+} channels. (A) Ba^{2+} currents evoked by a membrane depolarization to +10 mV from a holding potential of −40 mV before (a) and during the intracellular application of 0.2 μM Gβγ for 5 min (b) in control conditions (1). In the presence of 0.2 μM wortmannin for 30 min (2 and 3), Ba^{2+} currents before (a) and during the application of 0.2 μM Gβγ (c) or 10 μM isoprenaline for 5 min (d). (B) Compiled data showing the effects of wortmannin on the increase in Ba^{2+} current density evoked by 0.2 μM Gβγ and 10 μM isoprenaline. *Values significantly different from those obtained in the presence of Gβγ alone ($P<0.05$). External solution contained 5 mM Ba^{2+}, 10 nM prazosin, 10 nM rauwolscine, 1 μM SR59230A and 0.1 μM H-89.

increase in Ba^{2+} current density was also completely blocked by a pre-treatment with 0.2 μM wortmannin (Figure 7A,B) in PKA inhibitor-containing solution to remove the PKA-activated pathway. No unspecific effect of wortmannin was detected and the same cells pre-treated with wortmannin were still responding to PMA by an increase in Ba^{2+} current (not shown). Taken together, these data suggest that after activation of β_2-adrenoceptors, the Gβγ subunits released from the Gs proteins may stimulate the PI3K activity, leading to activation of PKC and stimulation of L-type Ca^{2+} channels.

Discussion

The results of the present study indicate that in vascular myocytes the β-adrenergic stimulation of L-type Ca^{2+} channels depends on both β_2- and β_3-adrenoceptor-activated pathways. The β_2-adrenergic pathway involves both the Gαs and the Gβγ subunits leading to activation of different downstream effectors, PKA and PI3K/PKC, respectively.

Although it is generally accepted that the α subunits of Gs proteins play an important role in regulation of L-type Ca^{2+} channels in cardiac and smooth muscles during β-adrenergic stimulation, the role of both endogenous Gαs and Gβγ subunits is not completely clear. Gαs is generally associated with activation of the adenylyl cyclase/PKA pathway and the subsequent phosphorylation of L-type Ca^{2+} channels. In addition, PKA phosphorylation sites have been identified on both the α and β subunits of L-type Ca^{2+} channels (Hofmann *et al*., 1994). Vascular L-type Ca^{2+} channels have been shown to be stimulated by isoprenaline, 8-Br-cAMP and forskolin (Loirand *et al*., 1992; Viard *et al*., 2000), as well as by intracellular applications of exogenous Gαs and the catalytic subunit of PKA (Ruiz-Velasco *et al*., 1998). The present study provides additional evidence that β-adrenergic stimulation *via* β_2-adrenoceptors activates the Gαs/PKA pathway for regulation of vascular L-type Ca^{2+} channels. When the Gβγ pathway was eliminated by application of calphostin C or infusion with anti-$G\beta_{com}$ antibody or βARK_1 peptide, the isoprenaline-induced stimulation of L-type Ca^{2+} channel was entirely abolished by PKA inhibitors (H-89 and Rp 8-Br-cyclic AMPs). A similar transduction pathway involving Gαs and PKA has been identified in response to activation of β_3-adrenoceptors in the same myocytes (Viard *et al*., 2000). Although both PKA and PKC are involved in L-type Ca^{2+} channels stimulation in response to β_2-adrenoceptor activation, removal of one transduction coupling does not affect the stimulation of Ca^{2+} channels by the other one, suggesting that there is no additivity and that each one of the PKA- and PKC-mediated mechanisms is able to produce the maximal stimulation of L-type Ca^{2+} channels. However, it has to be noted that concentrations producing maximal effects on Ca^{2+} channel activity have been used in order to identify the transduction couplings. Therefore, this does not exclude the additivity of these couplings on the same effector, i.e. the L-type Ca^{2+} channel if PKA and PKC are activated at lower levels during physiological stimulations.

Recently, modulation of Ca^{2+} channels by Gβγ subunits has received much more attention. In neurons, presynaptic Ca^{2+} channels have been reported to be inhibited by neuromediators *via* direct interaction between the Ca^{2+} channel complex and the Gβγ subunits released from the activated G protein heterotrimer (Ikeda, 1996; De Waard *et al*., 1997; Zamponi *et al*., 1997). In contrast, we have reported that the $\beta_1\gamma_3$ dimer of G_{13} transduces the angiotensin II-induced stimulation of L-type Ca^{2+} channels in rat portal vein myocytes (Macrez-Leprêtre *et al*., 1997) and that direct infusion of purified Gβγ subunits also increases the Ca^{2+} channel current (Viard *et al*., 1999). A similar stimulatory effect of purified Gβγ subunits has been reported in rabbit portal vein myocytes (Zhong *et al*., 1999). The present data provides evidence that the Gβγ pathway also contributes to β-adrenergic stimulation by showing that the PKA inhibitors-resistant stimulation of L-type Ca^{2+} channels induced by β_2-adrenoceptors was inhibited by intracellular application of anti-$G\beta_{com}$ antibody and βARK_1 peptide.

Previously, we have proposed that the action of Gβγ subunits on L-type Ca^{2+} channels was due to activation of PKC (Viard *et al*., 1999). Several pathways may lead to stimulation of PKC, including Gβγ-activated PLC, PLD and PLA_2 (Clapham & Neer, 1997). In addition, we have shown recently that Gβγ may also activate PKC *via* PI3K; accordingly, infusion of cells with purified PI3Kγ also stimulates L-type Ca^{2+} channels (Viard *et al*., 1999). It is not yet clear what specific βγ combination of G protein is coupled to Gαs since the subunit composition of the Gs proteins that interact with the β_2- and β_3-adrenoceptors has not been identified. Different combinations of β and γ subunits (except $\beta_1\gamma_1$) have been reported to have similar

actions on various effectors (Dolphin, 1998). However, recent data have shown that activation of mitogen-activated protein kinase/extracellular signal-regulated kinase and inhibition of adenylyl cyclases V and VI appear to be Gβ isoform specific (Gβ_1 being more efficient than Gβ_5; Zhang *et al*., 1996; Bayewitch *et al*., 1998). Recombinant mammalian G$\beta_{1-3\gamma 2}$ complexes stimulate PI3Kγ with similar potency and efficacy whereas G$\beta_{5\gamma 2}$ is not effective and appears to be unable to stimulate L-type Ca^{2+} channels in vascular myocytes, suggesting that signalling specificity may be encoded in the direct protein–protein interaction between G$\beta\gamma$ and PI3K (Maier *et al*., 2000). Obviously, selective protein–protein interactions represent the first step in signalling specificity and may be a possible explanation for the absence of G$\beta\gamma$-activated pathway during β_3-adrenoceptors activation (Viard *et al*., 1999). It can be postulated that the G$\beta\gamma$ dimers coupled to Gαs may be different in relation to the existence of two forms of Gαs, as previously suggested (Chaudhry & Granneman, 1991; Chaudhry *et al*., 1994). Additional factors such as cell compartmentation, spatial and temporal expression of transduction components may be also involved in signalling specificity, but they remain to be studied in more detail.

Although three β_{1-3}-adrenergic subtypes have been identified in portal vein myocytes by RT–PCR, we found that only β_2- and β_3-adrenoceptors stimulated L-type Ca^{2+} channels. Mixed populations of β-adrenergic receptor subtypes have been previously reported in vascular and visceral smooth muscles, but predominant roles for β_2- and β_3-adrenoceptors in inducing relaxation are generally demonstrated (De Boer *et al*., 1993; Satake *et al*., 1996; Yamazaki *et al*., 1998; Roberts *et al*., 1999). Existence of several β-adrenoceptor subtypes producing the same physiological effect raises the question of the role of these receptors. Although a redundant function of these receptors cannot be discarded, specific modulations of β-adrenoceptor subtype have been previously described. For example, β_3-adrenoceptors, unlike β_1- and β_2-subtypes, lack regulatory phosphorylation sites for G protein receptor kinases (Liggett *et al*., 1993) and could be relatively resistant to agonist-induced desensitization. Thus, the function of β_3-adrenoceptors may become predominant after desensitization of the β_2-adrenoceptors when the sympathetic nervous system is highly stimulated.

The mechanisms by which activation of β-adrenoceptors induce relaxation in smooth muscles are not fully understood although several hypotheses have been proposed including Ca^{2+}-dependent and Ca^{2+}-independent mechanisms.

The Ca^{2+}-independent mechanism is mainly related to stimulation of voltage-dependent K^+ channels (Aiello *et al*. 1998) and/or ATP-sensitive K^+ channels (Randall & McCulloch, 1995) by isoprenaline and PKA which causes hyperpolarization of the membrane potential. Two Ca^{2+}-dependent mechanisms have been suggested to underlie the isoprenaline-induced relaxation in vascular myocytes: (1) Full activation of Ca^{2+}-sensitive large-conductance K^+ channels by isoprenaline and PKA requires an increase in $[Ca^{2+}]_i$ (Song & Simard, 1995) which can result from PKA- and PKC-mediated stimulation on L-type Ca^{2+} channels (Tewari & Simard, 1994; Viard *et al*., 1999 and the present study); (2) Alternately, PKA-induced stimulation of sarcoplasmic reticulum Ca^{2+}-ATPases may increase the luminal Ca^{2+} concentration and consequently stimulate the frequency of spontaneous Ca^{2+} sparks (due to the opening of clustered ryanodine-sensitive Ca^{2+} release channels; Porter *et al*., 1998). Since Ca^{2+} sparks have been reported to activate Ca^{2+}-sensitive K^+ channels (Mironneau *et al*., 1996; Perez *et al*., 1999), their stimulation by β-agonists could also lead to membrane potential hyperpolarization, closing of voltage-dependent Ca^{2+} channels and relaxation.

In conclusion, our results suggest that the β-adrenergic stimulation of vascular L-type Ca^{2+} channels involves both β_2- and β_3-adrenoceptors and that these receptors exert their effects through two different pathways involving only PKA in the case of β_3-adrenoceptors or both PKA and PKC for β_2-adrenoceptors. The reasons for this overlapping of couplings to L-type Ca^{2+} channels are not known but strengthen the role of β-adrenergic agonists in the modulation of vascular relaxation.

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