British Journal of Pharmacology (2000) 129, 1497–1505

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Beta-3 adrenergic stimulation of L-Type Ca^{2+} channels in rat portal vein myocytes

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1 The effects of β_3-adrenergic stimulation were studied on the L-type Ca^{2+} channel in single myocytes from rat portal vein using the whole-cell mode of the patch-clamp technique.

2 Reverse transcription-polymerase chain reaction showed that β_1-, β_2- and β_3-adrenoceptor subtypes were expressed in rat portal vein myocytes. Application of both propranolol (a non-selective β_1- and β_2-adrenoceptor antagonist) and SR59230A (a β_3-adrenoceptor antagonist) were needed to inhibit the isoprenaline-induced increase in L-type Ca^{2+} channel current.

3 L-type Ca^{2+} channels were stimulated by CGP12177A (a β_3-adrenoceptor agonist with potent β_1- and β_2-adrenoceptor antagonist property) in a manner similar to that of isoprenaline. The CGP12177A-induced stimulation of Ca^{2+} channel current was blocked by SR59230A, cyclic AMP-dependent protein kinase inhibitors, H-89 and Rp 8-Br-cyclic AMPs, but was unaffected by protein kinase C inhibitors, GF109203X and 19-31 peptide. This stimulation was mimicked by forskolin and 8-Br-cyclic AMP. In the presence of okadaic acid (a phosphatase inhibitor), the β_3-adrenoceptor-induced stimulation was maintained after withdrawal of the agonist.

4 The β_3-adrenoceptor stimulation of L-type Ca^{2+} channels was blocked by a pretreatment with cholera toxin and by the intracellular application of an anti-$G\alpha_s$ antibody. This stimulation was unaffected by intracellular infusion of an anti-$G\beta_{com}$ antibody and a βARK_1 peptide.

5 These results show that activation of β_3-adrenoceptors stimulates L-type Ca^{2+} channels in vascular myocytes through a $G\alpha_s$-induced stimulation of the cyclic AMP/protein kinase A pathway and the subsequent phosphorylation of the channels.

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Keywords: β_3-adrenoceptor; isoprenaline; Ca^{2+} channels; G protein; protein kinase A; smooth muscle

Abbreviations: βARK, β-adrenergic receptor kinase; cyclic AMP, cyclic adenosine monophosphate; CTX, cholera toxin; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PCR, polymerase chain reaction; PI3K, phosphatidylinositol-3-kinase; PKA, protein kinase A; PKC, protein kinase C

Introduction

Previous data have reported that β-adrenergic activation stimulates L-type Ca^{2+} channels in vascular and visceral smooth muscles (Fukumitsu *et al*., 1990; Loirand *et al*., 1992, Muraki *et al*., 1993). Several reports support a stimulatory effect of the cyclic AMP/protein kinase A (PKA) pathway on L-type Ca^{2+} channels in vascular myocytes (Shi & Cox, 1995; Tewari & Simard, 1994; Ruiz-Velasco *et al*., 1998). These results are in contrast with other data indicating that isoprenaline and cyclic AMP exert a transient increase followed by a decrease in L-type Ca^{2+} channel activity (Xiong *et al*., 1994; Xiong & Sperelakis, 1995). The stimulatory effect of β-adrenergic receptor activation has been proposed to depend on a direct modulation of L-type Ca^{2+} channels by the activated G_s protein whereas the inhibitory effect has been attributed to G_s activation of adenylyl cyclase and the subsequent phosphorylation of the Ca^{2+} channel by PKA (Xiong & Sperelakis, 1995). However, there are increasing evidence that three or four β-adrenoceptor subtypes may exist in different cell types including heart and visceral smooth muscle (Kaumann, 1997), so that β-adrenergic stimulation cannot be considered as a single mechanism as long as β-adrenoceptors have not been identified in the studied cells. β_3-adrenoceptors have been identified in adipocytes in which they are predominantly expressed (Emorine *et al*., 1989) but also in cardiac and smooth muscles (Kaumann, 1997; Anthony *et al*., 1998). Activation of β_3-adrenoceptors has been shown to induce negative inotropic effect in human heart (Gauthier *et al*., 1996) and relaxation in visceral smooth muscle (Fujimura *et al*., 1999), but the cellular mechanisms activated by these receptors in vascular myocytes have not been elucidated.

In the present study, we identified by RT–PCR the mRNAs corresponding to β_1-, β_2- and β_3-adrenoceptor subtypes and demonstrated the existence of a β_3-adrenoceptor activated-stimulation of L-type Ca^{2+} channels in rat portal vein myocytes. We show that the transduction pathway activated by β_3-adrenoceptors is mediated through a $G\alpha_s$-induced stimulation of the cyclic AMP/PKA pathway and the subsequent phosphorylation of L-type Ca^{2+} channels.

Methods

The investigation conforms with the European Community guiding principles in the care and use of animals (86/609/CEE, CE Off J n°L358, 18 December 1986) and the French decree n°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.

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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+} 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 $\pm$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. Isoprenaline and CGP12177A were extracellularly applied to the recorded cell by pressure ejection from a glass pipette.

RNA purification and reverse transcription-polymerase chain reaction (PCR)

Total RNA was extracted from about 500 cells dissociated from rat portal vein and detrusor muscles by using RNeasy mini kit (Qiagen, Hilden, Germany) and following the instructions of the supplier. The reverse transcription reaction was performed using Sensiscript RT kit (Qiagen, Hilden, Germany). Briefly, total RNA was first incubated with random primers (Promega, Charbonnières, France) at 65°C for 5 min and cooled down 60 min at 37°C. The resulting cDNA was stored at −20°C. PCR was performed with 1 μl of cDNA, 1.25 units of HotStartTaq DNA polymerase (Qiagen), 0.5 μM of each primer and 200 μM of each deoxynucleotide triphosphate, in a final volume of 50 μl. The PCR conditions were 95°C for 15 min for HotStartTaq activation, then 35 cycles were performed as follows: 94°C for 1 min, 55°C (β_1- and β_2-adrenoceptors) or 62°C (β_3-adrenoceptor) for 1.5 min and 72°C for 1 min. At the end of PCR, samples were kept at 72°C for 10 min for final extension before being stored at 4°C. Reverse transcription and PCR were performed with a thermal cycler (Techne, Cambridge, U.K.). Amplification products were separated by electrophoresis (2% agarose gel) and visualized by ethidium bromide staining. Gels were photographed with EDAS 120 and analysed with KDS1D 2.0 software (Kodak Digital Science, Paris, France). Sense (s) and antisense (as) primer pairs specific for β_1-, β_2 and β_3-adrenoceptors were designed on the known cloned rat receptor sequences deposited in GenBank (accession numbers D00634, X17607 and S73473 for β_1-, β_2- and β_3-adrenoceptors, respectively) with Lasergene software (DNASTAR, Madison, WI, U.S.A.). The nucleotide sequences and the length of the expected PCR products (in parentheses) for each primer pair were respectively: β_1-adrenoceptor (s) TC GT G T GC A C CG T G TG G G CC, (as) AG GA AA CG GC GC TC GC AG CT (264 bp); β_2-adrenoceptor (s) GC CT GC TG AC CA AG AA TA AG, (as) CC CA TC CT GC TC CA CC TG G (328 bp); β_3-adrenoceptor (s) AC CT TG GC GC TG AC TG G, (as) AT GG GC GC AA AC GA CA C (229 bp).

Chemicals and drugs

Isoprenaline, propranolol, prazosin and rauwolscine were from Sigma (Saint Quentin Fallavier, France). Forskolin, 8-Br-cyclic AMP, Rp-8-Br-cyclic AMPs, H-89, 19-31 peptide and cholera toxin (CTX) were from Calbiochem (Meudon, France). Phorbol ester 12,13-dibutyrate and 4α-phorbol 12,13-dibutyrate were from LC Laboratories (Woburn, MA, U.S.A.). The protein kinase C (PKC) inhibitor, GF109203X, was a gift from Glaxo (Les Ulis, France). CGP12177A was from RBI (Natick, MA, U.S.A.). SR59230A (3-(2-ethylphenoxy)-1[(1S)-1,2,3,4-tetrahydronapth-1-ylaminol] - (2S)-propanolol-oxalate) was from Sanofi (Milano, Italy). M199 medium was from Flow Laboratories (Puteaux, France). Streptomycin, penicillin, glutamine and pyruvate were from Gibco (Paisley, U.K.). Rabbit anti-$G\alpha_s$ subunit antibody (371732) raised to the carboxyl-terminal animo acids, RMHLRQYELL, of $G\alpha_s$ was from Calbiochem. Rabbit anti-$G\beta_{com}$ antibody (SC 378) raised to the carboxyl-terminal amino acids, TDDGMAVATGSWDSFLKIWN, of $G\beta_1$ subunit was from Santa-Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Peptides corresponding to the $G\beta\gamma$ binding domain of β-adrenergic receptor kinase-1 or to a region outside the $G\beta\gamma$ binding site (Viard *et al*., 1999) were synthetized by Genosys (Cambridge, U.K.). PCR primers were synthetized by Eurogentec (Seraing, Belgium). Molecular-weight marker, *HinfI*, was from Promega (Charbonnières, France).

Results

Effects of isoprenaline on voltage-dependent Ba^{2+} current

Inward currents were elicited every 20 s in single myocytes bathed in 5 mM $BaCl_2$ solution by 200 ms depolarization to +10 mV from a holding potential of −40 mV and they gradually increased in size over 2–3 min in almost all cells, as previously reported (Viard *et al*., 1999). After the amplitude of inward current became steady application of 10 μM isoprenaline (in the continuous presence of 10 nM prazosin and 10 nM rauwolscine to inhibit α_1- and α_2-adrenoceptors, Leprêtre *et al*., 1994) resulted in an increase in Ba^{2+} current reaching $39 \pm 8\%$ ($n=5$) within 3–4 min (Figure 1). Under both conditions (with or without isoprenaline), the recorded Ba^{2+} currents were completely blocked by 1 μM oxodipine or isradipine (data not shown). After a 10 min pre-treatment with either 1 μM propranolol (a non-selective β_1- and β_2-adrenoceptor antagonist) or 0.1 μM SR59230A alone (a β_3-adrenoceptor antagonist, Manara *et al*., 1996), the isoprenaline-induced stimulation of Ba^{2+} current was not significantly affected (Figure 1B). In contrast, application of both compounds inhibited the stimulation of the Ba^{2+} current evoked by 10 μM isoprenaline (Figure 1A,B). Figure 1C illustrates the inhibitory effect of increasing concentrations of SR59230A in the absence or in the

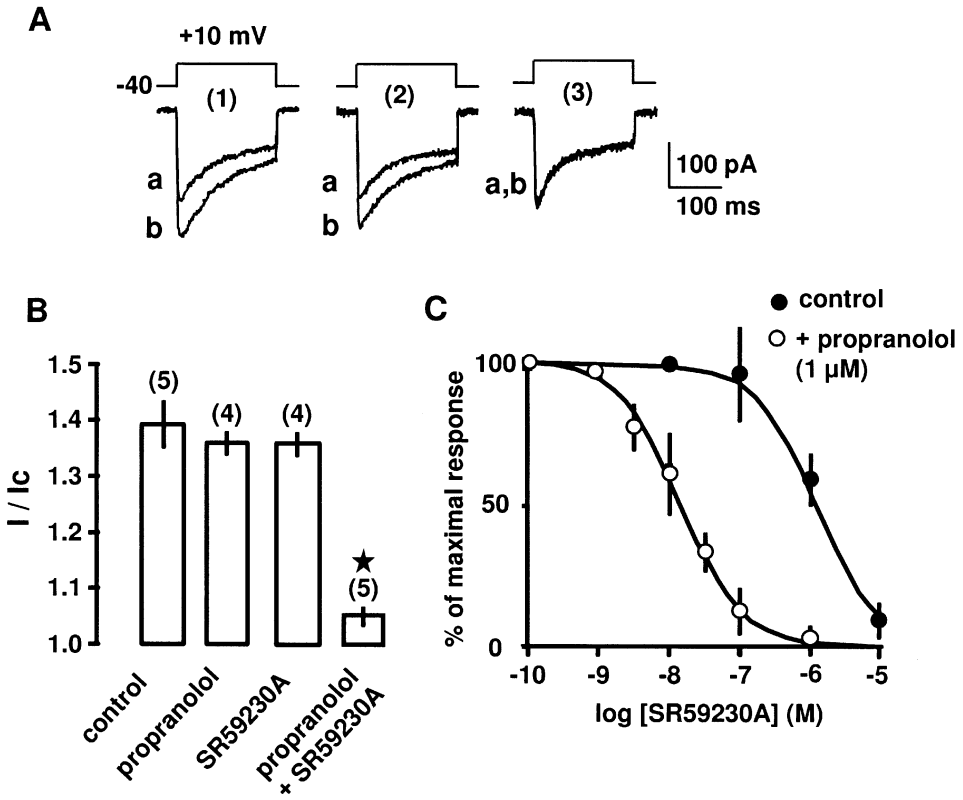

Figure 1 Effects of isoprenaline and β-adrenoceptor antagonists on L-type Ca^{2+} channels in rat portal vein myocytes. (A) Ba^{2+} currents evoked by a depolarization to +10 mV from a holding potential of −40 mV before (a) and during the application of 10 μM isoprenaline for 3 min (b) in control conditions (1), in the presence of 1 μM propranolol for 10 min (2) or in the presence of both 1 μM propranolol and 0.1 μM SR59230A for 10 min (3). (B) Compiled data showing the effects of β-adrenoceptor antagonists on the isoprenaline-induced increase in L-type Ba^{2+} current. Currents are expressed as a fraction of their control values (I/Ic). Results are means ± s.e.mean, with the number of cells tested indicated in parentheses. ★, Values significantly different from control values ($P<0.05$). (C) Concentration-response curves showing the inhibition of isoprenaline-induced increase in L-type Ba^{2+} current by SR59230A in the absence or in the presence of 1 μM propranolol. External solution contained 5 mM Ba^{2+}, 10 mM prazosin and 10 nM rauwolscine.

presence of 1 μM propranolol. It appears that the inhibitory concentration-response curve for SR59230A was shifted to lower concentrations in the presence of propranolol, as the SR59230A concentration corresponding to half-maximal inhibition decreased from 1.2 μM to 14 nM.

In order to identify the β-adrenoceptors potentially involved in the effects of isoprenaline, mRNA purified from rat portal vein myocytes was reversibly transcribed into cyclic DNA, and a fragment of cyclic DNA of each receptor subtype was amplified using subtype-specific primers for the PCR. As illustrated in Figure 2, products of expected sizes corresponding to β_1-, β_2- and β_3-adrenoceptor mRNAs were detected in rat portal vein and detrusor muscle (used as a positive control, Seguchi *et al*., 1998). These results suggest that the three β-adrenoceptor subtypes are involved in the stimulation of Ba^{2+} current through independent transduction pathways which, however, present a common step since their effects are not additive. Furthermore, it appears that SR59230A is a specific antagonist of β_3-adrenoceptors at concentrations lower than 100 nM.

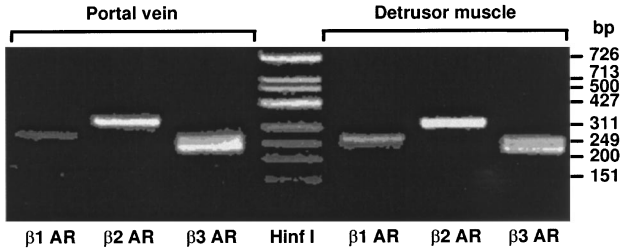

Figure 2 Expression of β-adrenoceptor mRNA in rat portal vein and detrusor smooth muscles. Amplified cDNA fragments corresponding to β_1-, β_2- and β_3-adrenoceptors (AR) were separated on a 2% agarose gel and visualized by staining with ethidium bromide. *Hinf*I: molecular size standards in base pairs (bp). For RNA purification and PCR conditions, see Methods.

Stimulation of Ba^{2+} current by the selective β_3 agonist CGP12177A

In the following experiments, CGP12177A was used as a β_3-adrenergic agonist and 1 μM propranolol, 10 nM prazosin and 10 nM rauwolscine were continuously added to the perfusion solution. Stimulation of Ba^{2+} current was detectable with 10 nM CGP12177A and increased with the concentration of CGP12177A to reach a maximum with 10 μM CGP12177A (Figure 3A,B). The maximal stimulation of Ba^{2+} current in the presence of 10 μM CGP12177A was 43 ± 4% ($n=25$), not significantly different from that obtained with 10 μM isoprenaline (Figure 3B). The concentration of CGP12177A and isoprenaline producing a half-maximal stimulation of Ba^{2+} current were 0.14 and 1.35 μM, respectively (Figure 3B). CGP12177A-induced stimulation of Ba^{2+} current was concentration-dependently inhibited by SR59230A with a half-maximal inhibition obtained at 18 nM (Figure 3C). As illustrated by the current-voltage relationship (Figure 4A), the

maximal Ba^{2+} current was increased by 10 μM CGP12177A without any change in the voltage threshold, the potential for the maximal current, and the extrapolated reversal potential. Steady-state inactivation of the Ba^{2+} current was examined with a two-pulse protocol (Figure 4B). A test pulse to +10 mV (V_2) from a holding potential of −40 mV was preceded by a prepulse (V_1) of 20 s duration and of variable amplitude (−60 to −20 mV). For each prepulse, the amplitude of the test current was taken as an index of the remaining activatable channels. Relative availability was expressed by plotting the test current against the prepulse potential value. The amplitude of the test current was expressed as a fraction of the current obtained at the most negative prepulse. As shown in Figure 4B, the curves obtained in the absence and presence of 10 μM CGP12177A were superimposed. Since the Ba^{2+} currents in cells stimulated by CGP12177A appeared to be slightly more enhanced at negative potentials than at positive potentials, we calculated the conductance (G) by dividing the peak Ba^{2+}

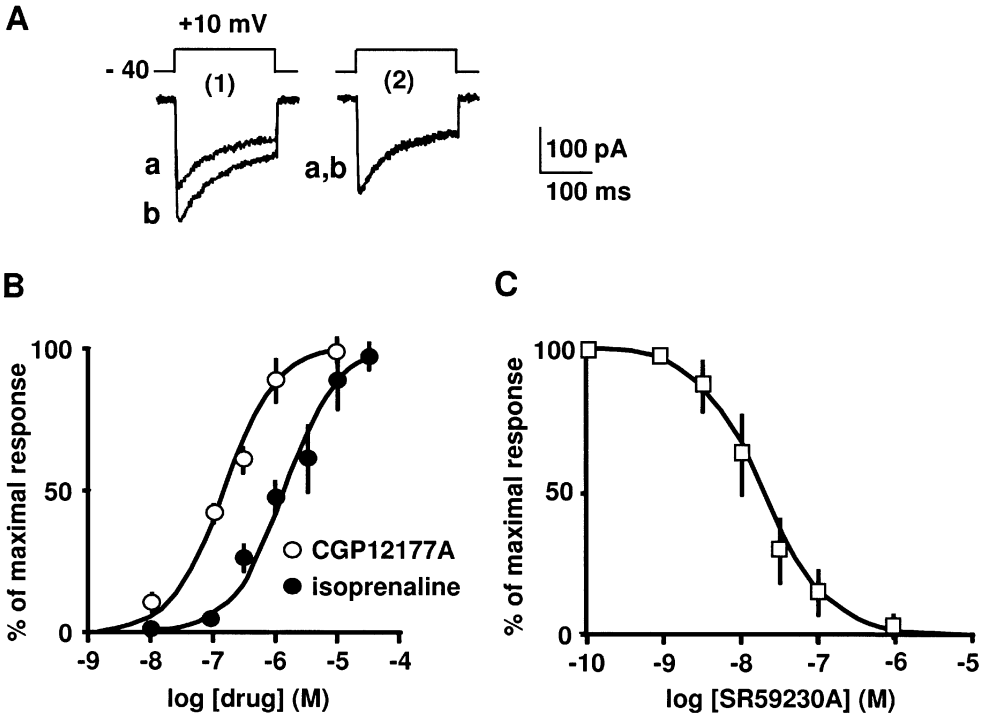

Figure 3 Effects of CGP12177A, a β_3-adrenoceptor selective agonist, on L-type Ca^{2+} channels. (A) Ba^{2+} currents evoked by a depolarization to +10 mV from a holding potential of −40 mV before (a) and during the application of 10 μM CGP12177A for 3 min (b) in control conditions (1) or in the presence of 0.1 μM SR59230A for 10 min (2). (B) Concentration-response curves showing the stimulatory effect of CGP12177A and isoprenaline. Ba^{2+} currents are expressed as a percentage of the maximal response obtained with 10 μM CGP12177A, on each cell tested. Results are means±s.e.mean for 5–10 cells. (C) Inhibition curve for CGP12177A-induced increase in L-type Ba^{2+} current by SR59230A. Values are expressed as a percentage of the maximal response obtained with 10 μM CGP12177A. Results are means±s.e.mean for 4–8 cells. External solution contained 5 mM Ba^{2+}, 1 μM propranolol, 10 nM prazosin and 10 nM rauwolscine.

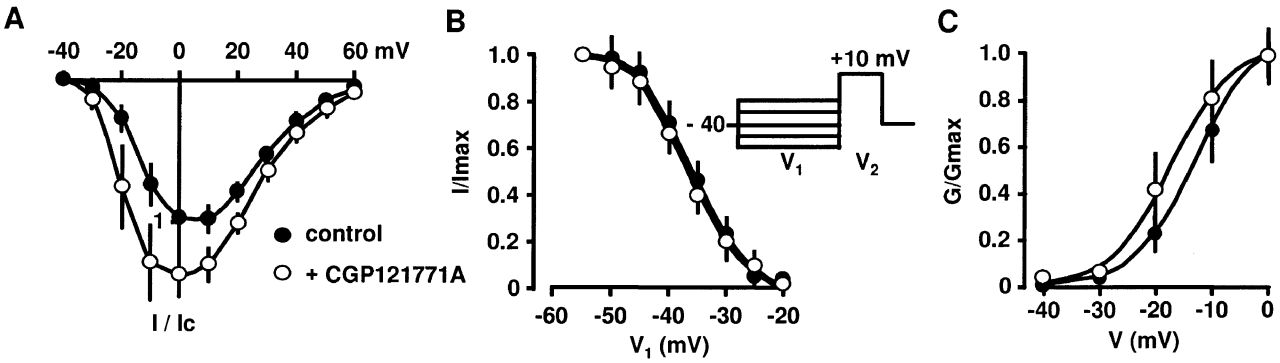

Figure 4 Effect of CGP12177A on the current-voltage relationship and the activation and inactivation curves of L-type Ca^{2+} channels. (A) Current-voltage relationships obtained from a holding potential of −40 mV in control conditions and during the application of 10 μM CGP12177A for 3 min. Currents are expressed as a fraction of the maximal current obtained in control conditions (I/Ic). Data are means±s.e.mean for 4–7 cells. (B) Steady-state inactivation curve obtained with the two-pulse protocol (inset). Currents are expressed as a fraction of maximal current (I/I_{max}) obtained in control conditions or during application of 10 μM CGP12177A. (C) Activation curve expressed as a fraction of G/G_{max} in control conditions or during application of 10 μM CGP12177A. Data are fitted by curves of form 1/1 [1+exp (Vm−Vh)/k], in which Vh is the potential at which half of the current is inactivated, Vm is the membrane potential, and k is the slope factor. Data are given as means±s.e.mean for 5–7 cells. External solution contained 5 mM Ba^{2+}, 1 μM propranolol, 10 nM prazosin and 10 nM rauwolscine.

current at each negative potential tested by the driving force ($V-V_{rev}$). The normalized conductance (G/G_{max}) is plotted as a function of membrane potential in Figure 4C. In control conditions we found a half-activation potential of -14.0 ± 2.0 mV ($n=4$). In the presence of 10 μM CGP12177A, the half-activation potential was not significantly modified (-16.5 ± 2.5 mV, $n=4$, $P>0.05$). Taken together, these results indicate that CGP12177A stimulates L-type Ca^{2+} channels without affecting the gating properties of these channels.

Transduction pathway activated by β_3-adrenoceptors

Modulation of L-type Ca^{2+} channels in vascular myocytes by β-adrenoceptor activation is believed to involve the cyclic AMP/PKA pathway through phosphorylation of channel subunits by PKA (Ishikawa *et al*., 1993) and/or a possible direct modulation of the channel activity by α subunits of Gs protein (Xiong *et al*., 1995).

To elucidate the mechanism of stimulation of Ba^{2+} current by CGP12177A, we applied forskolin, a direct activator of adenylyl cyclase. Forskolin (5 μM) increased the Ba^{2+} current by $40\pm6\%$ ($n=10$) within 4–5 min (Figure 5A,B). Similarly, 8-Br-AMPc (0.3 mM), applied extracellularly, stimulated the Ba^{2+} current by $35\pm6\%$ ($n=5$; Figure 5A) indicating that activation of PKA may stimulate L-type Ca^{2+} channels in vascular myocytes. To evaluate whether PKA is involved in the intracellular pathway activated by β_3-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 CGP12177A. Superfusion of cells with H-89 (0.1 μM) or Rp 8-Br-cyclic AMPs (10 μM) for 10–15 min did not change the peak Ba^{2+} current in control cells (data not shown) nor affect the stimulatory response of 1 μM phorbol dibutyrate, an activator of PKC (control: $37\pm4\%$, $n=4$; in the presence of H-89: $42\pm4\%$, $n=4$; Figure 5B). In contrast, the stimulation of Ba^{2+} current evoked by forskolin, 8-Br-AMPc (not shown) or CGP12177A was inhibited by H-89 (Figure 5B) and Rp 8-Br-cyclic AMPs (not shown), suggesting that PKA is involved in the stimulation of Ca^{2+} channel activity by β_3-adrenergic agonist. To ensure that the stimulation of Ba^{2+} current was mediated by a phosphorylation of L-type Ca^{2+} channels, we applied a protein phosphatase inhibitor, okadaic acid, before stimulation with CGP12177A or forskolin. Okadaic acid (5 μM) by itself had no significant effect on the Ba^{2+} current density when applied for 10–15 min (control: 8.1 ± 0.6 pA/pF; in the presence of okadaic acid: 8.9 ± 0.7 pA/pF, $n=5$) in agreement with previous data on L-type Ca^{2+} channels (Chahine *et al*., 1996). In control conditions, stimulation of the Ba^{2+} current by 10 μM CGP12177A or 5 μM forskolin was maximal within 3–4 min. After withdrawal of the stimulating substances, the Ba^{2+} current returned to its control value within 4–5 min (Figure 6A–C). In the presence of 5 μM okadaic acid, the CGP12177A-induced increase in Ba^{2+} current was similar to that obtained in control conditions but persisted after wash-out of CGP12177A (Figure 6B,C). Similar results were obtained with forskolin (not shown). These results support the idea that a phosphorylation mechanism is involved in the effects of CGP12177A and forskolin on L-type Ca^{2+} channels.

To identify the G protein coupling the β_3-adrenoceptors to adenylyl cyclase we used cholera toxin (CTX) which ADP ribosylates G_s proteins, leading to their permanent activity. Cells were incubated in a culture medium containing 160 ng ml^{-1} CTX for 20 h. This pretreatment suppressed the CGP12177A-induced stimulation of Ba^{2+} current (Figure 7A) whereas it did not affect the clonidine-induced stimulation of

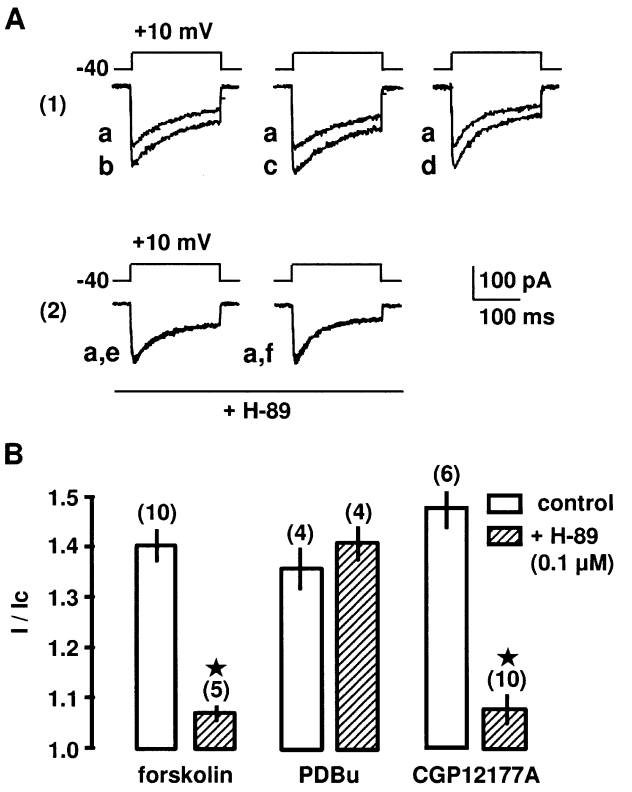

Figure 5 Effects of activators and inhibitors of PKA on L-type Ca^{2+} channels. (A) Ba^{2+} currents evoked by a depolarization to +10 mV from a holding potential of −40 mV before (a) and during the external application of 0.3 mM 8-Br-AMPc (b), 5 μM forskolin (c) or 10 μM CGP12177A (d), in control conditions (1). In the presence of 0.1 μM H-89 for 10 min (2), currents before (a) and during application of 5 μM forskolin (e) or 10 μM CGP12177A (f). (B) Compiled data showing the effect of 0.1 μM H-89 on the increase in L-type Ba^{2+} current evoked by 5 μM forskolin, 0.1 μM phorbol dibutyrate (PDBu) and 10 μM CGP12177A. Currents are expressed as a fraction of their control values (I/Ic). Data are means±s.e.mean with the number of cells tested indicated in parentheses. ★, Values significantly different from control values ($P<0.05$). External solution contained 5 mM Ba^{2+}, 1 μM propranolol, 10 nM prazosin and 10 nM rauwolscine.

Ba^{2+} current (control: $41\pm5\%$, $n=5$; CTX-pre-treated cells: $40\pm6\%$, $n=5$) which has been demonstrated to depend on activation of a G_i protein (Leprêtre *et al*., 1995). Antibodies directed against the carboxyl terminus of the α subunits of G proteins have been shown to be useful tools for identifying transduction couplings. When a carboxyl-terminal anti-Gα_s antibody was added to the basic pipette solution for 4 min, the CGP12177A-induced stimulation of Ba^{2+} current was concentration-dependently inhibited, with maximal inhibition obtained at 10 μg ml^{-1} anti-Gα_s antibody (Figure 7B,C). Intracellular application of the anti-Gα_s antibody inactivated by heating at 95°C for 30 min did not significantly affect the CGP12177A-induced stimulation of Ba^{2+} current (Figure 7B). These results indicate that the G_s protein transduces the signal for stimulation of Ca^{2+} channels in response to activation of β_3-adrenoceptors.

The anti-Gα_s antibody, however, cannot distinguish whether α or $\beta\gamma$ subunits interact with adenylyl cyclase. Therefore, intracellular infusion of either an anti-Gβ_{com} antibody or a peptide corresponding to a fragment of βARK$_1$ was used to bind G$\beta\gamma$ subunits and to block activation of effectors. Applications of βARK$_1$ peptide and anti-Gβ_{com} antibody had no significant effects on the Ba^{2+} current density

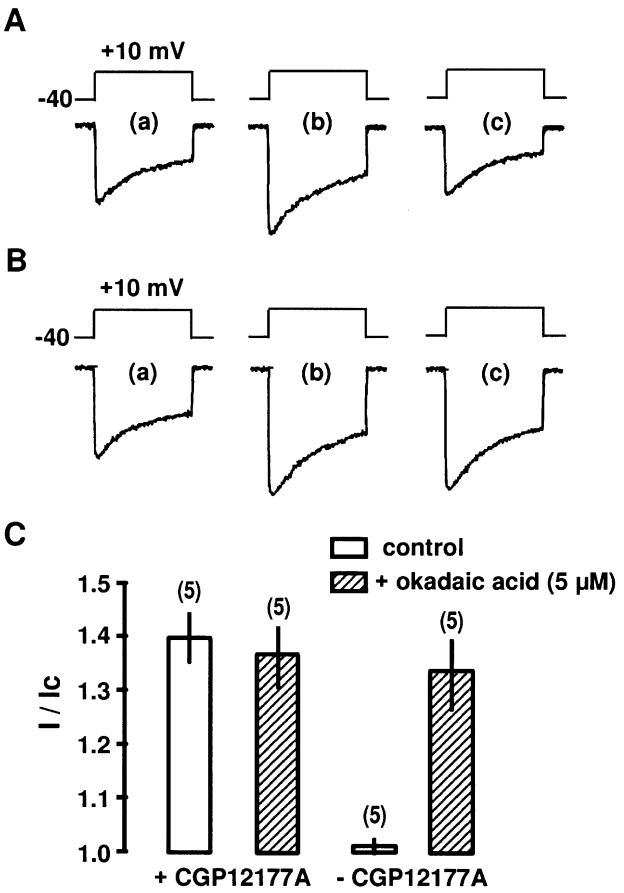

Figure 6 Effects of okadaic acid on CGP12177A-induced stimulation of L-type Ca^{2+} channels. (A) Ba^{2+} currents on control conditions (a), in the presence of 10 μM CGP12177A for 4 min (b), and after withdrawal of the agonist for 5 min (c). (B) Ba^{2+} currents in the presence of 5 μM okadaic acid before (a) and during the applications of 10 μM CGP12177A (b), and after withdrawal of the agonist for 5 min (c). (C) Compiled data showing the stimulatory effects of 10 μM CGP12177A and the recovery after withdrawal of the agonist for 5 min in control conditions and in the presence of 5 μM okadaic acid. Currents are expressed as a fraction of their control values (I/Ic). Data are means$\pm$s.e.mean with the number of cells tested indicated in parentheses. External solution contained 5 mM Ba^{2+}, 1 μM propranolol, 10 nM prazosin and 10 nM rauwolscine.

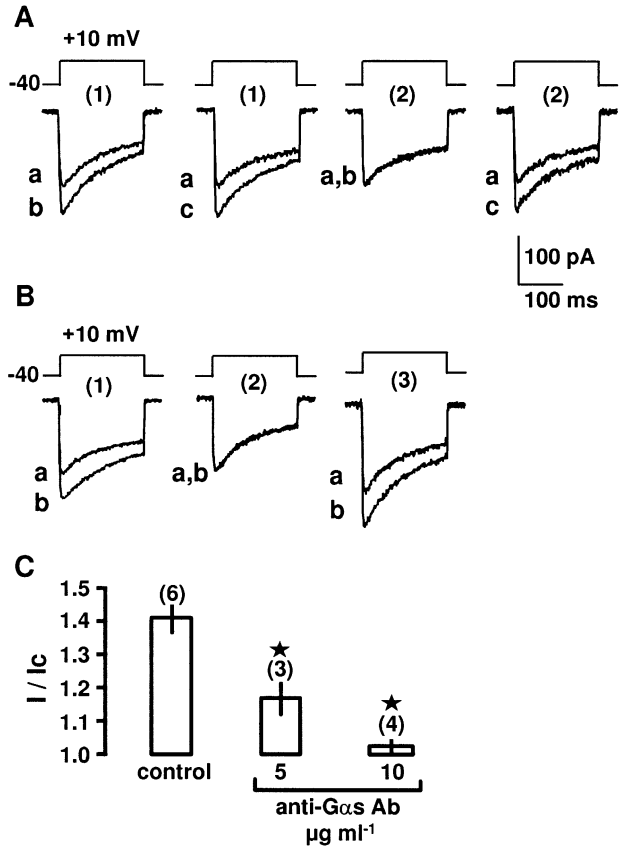

Figure 7 Effects of cholera toxin and anti-$G\alpha_s$ antibody on the CGP12177A-induced stimulation of L-type Ca^{2+} channels. (A) Ba^{2+} currents evoked by a membrane depolarization to +10 mV from a holding potential of -40 mV before (a) and during application of 10 μM CGP12177A (b) or 10 μM clonidine (c) in control conditions (1) or after a pretreatment with 160 μg ml^{-1} CTX for 20 h (2). (B) Ba^{2+} currents before (a) and during application of 10 μM CGP12177A in control conditions (1), in cells dialyzed with 10 μg ml^{-1} anti-$G\alpha_s$ antibody for 4 min (2) or boiled anti-$G\alpha_s$ antibody (3). (C) Compiled data showing the inhibition of CGP12177A-induced increase in L-type Ba^{2+} current by increasing concentrations of anti-$G\alpha_s$ antibody. Currents are expressed as a fraction of their control values (I/Ic). Data are means$\pm$s.e.mean, with the number of cells tested indicated in parentheses. ★, Values significantly different from control values ($P<0.05$). External solution contained 5 mM Ba^{2+}, 1 μM propranolol, 10 nM prazosin and 10 nM rauwolscine.

in non-stimulated myocytes. The mean Ba^{2+} current density was 8.5 ± 0.5 pA/pF in control conditions ($n=21$), 7.8 ± 0.7 pA/pF in the presence of 10 μM βARK$_1$ ($n=11$) and 7.6 ± 0.6 pA/pF in the presence of 10 μg ml^{-1} anti-β_{com} antibody for 5–7 min ($n=9$). As shown in Figure 8A, 10 μg ml^{-1} anti-Gβ_{com} antibody or 10 μM βARK$_1$ peptide (corresponding to the G$\beta\gamma$ binding region of βARK$_1$, Nair *et al*., 1995; Stehno-Bittel *et al*., 1995) had no significant effects on CGP12177A-induced stimulation of Ba^{2+} current (control: $40\pm3\%$, $n=8$; in the presence of βARK$_1$: $43\pm4\%$, $n=5$, or anti-Gβ_{com} antibody: $41\pm4\%$, $n=5$). In contrast, in the same cell batches, the angiotensin II-induced stimulation of Ba^{2+} current was selectively inhibited by both the anti-Gβ_{com} antibody and the βARK$_1$ peptide (Figure 8B; control: $44\pm4\%$, $n=10$; in the presence of βARK$_1$: $2\pm1\%$, $n=5$, or anti-Gβ_{com} antibody: $3\pm2\%$, $n=5$, $P<0.05$). Taken together, these results suggest that G$\beta\gamma$ subunits are not involved in the β_3-adrenergic stimulation of adenylyl cyclase in vascular myocytes.

We have previously shown that the vascular L-type Ca^{2+} channels may be stimulated by PKC-dependent mechanisms (Leprêtre *et al*., 1994; Viard *et al*., 1999). Stimulation of Ba^{2+} current evoked by 0.1 μM PDBu was completely blocked by PKC inhibitors, i.e. external application of 1 μM GF109203X (Figure 9A,B) or infusion of 1 μM 19–31 peptide. Both GF109203X and 19–31 peptide had no direct effects on the control Ba^{2+} current activated by membrane depolarization (data not shown) as well as on the stimulation of Ba^{2+} current evoked by forskolin and CGP12177A (Figure 9A,B). In addition, simultaneous applications of both 0.1 μM PDBu and 5 μM forskolin resulted in a further increase in Ba^{2+} current. This stimulatory effect is higher than that obtained with PDBu or CGP12177A alone (Figure 9B) and suggests an additive effect of PKC and PKA on L-type Ca^{2+} channel activity. These results confirm that a PKC-dependent pathway does not mediate the β_3-adrenergic-induced stimulation of Ca^{2+} channels.

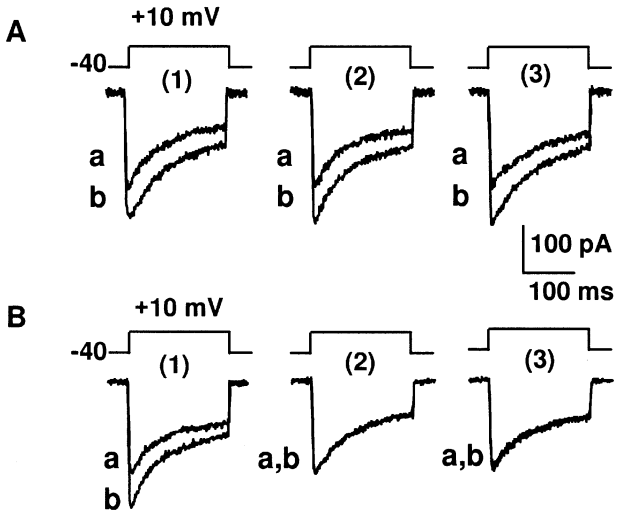

Figure 8 Effects of anti-Gβ_{com} antibody and βARK$_1$ peptide on CGP12177A- and angiotensin II-induced stimulation of L-type Ca^{2+} channels. (A) Typical Ba^{2+} currents evoked by a depolarization to +10 mV from a holding potential of −40 mV before (a) and during application of 10 μM CGP12177A (b) in control conditions (1), in cells dialyzed with 10 μM βARK$_1$ peptide (2) or 10 μg ml^{-1} anti-Gβ_{com} antibody for 4 min (3). (B) Typical Ba^{2+} currents before (a) and during applications of 10 nM angiotensin II (b) in control conditions (1), in cells dialyzed with 10 μM βARK$_1$ peptide (2) or 10 μg ml^{-1} anti-Gβ_{com} antibody for 4 min (3). External solution contained 5 mM Ba^{2+}, 1 μM propranolol, 10 nM prazosin and 10 nM rauwolscine.

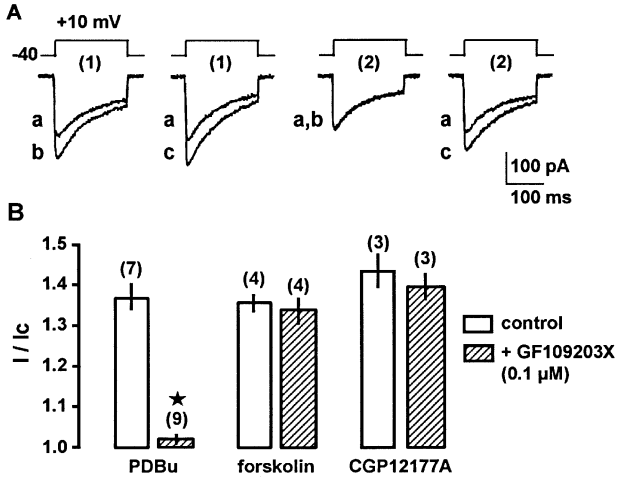

Figure 9 Effects of PKC inhibitor on the PDBu-, forskolin- and CGP12177A-induced stimulation of L-type Ca^{2+} channels. (A) Ba^{2+} currents evoked by a depolarization to +10 mV from a holding potential of −40 mV before (a) and during application of 10 μM PDBu (b) or 5 μM forskolin (c) in control conditions (1) and in the presence of 0.1 μM GF109203X for 10 min (2). (B) Compiled data showing the effects of 0.1 μM GF109203X on PDBu-, forskolin- and CGP12177A-induced increase of L-type Ba^{2+} current. Currents are expressed as a fraction of their control values (I/Ic). Data are means±s.e.mean with the number of cells tested indicated in parentheses. ★, Values significantly different of those obtained in the absence of GF109203X. External solution contained 5 mM Ba^{2+}, 1 μM propranolol, 10 nM prazosin and 10 nM rauwolscine.

Discussion

The results of the present study indicate that in vascular myocytes: (1) β_1-, β_2- and β_3-adrenoceptor mRNAs are expressed, as identified by RT–PCR; (2) the stimulatory effect of the β_3-adrenoceptor agonist CGP12177A on L-type Ca^{2+} channels is selectively prevented by SR59230A (the β_3-adrenoceptor antagonist); (3) the CGP12177A-induced stimulation of Ca^{2+} channels is blocked by cyclic AMP dependent protein kinase inhibitors, H-89 and Rp 8-Br-cyclic AMPs, but not by PKC inhibitors, GF109203X and 19–31 peptide. This stimulation was mimicked by forskolin and 8-Br-AMPc. In the presence of okadaic acid, inhibition of recovery after withdrawal of β_3-adrenoceptor agonist and forskolin suggests that phosphorylation plays a major role on L-type Ca^{2+} channel modulation; (4) the β_3-adrenoceptor stimulation of L-type Ca^{2+} channels was removed by a pretreatment with CTX and by the intracellular application of an anti-Gα_s antibody but was unaffected by intracellular infusion of an anti-Gβ_{com} antibody and a βARK$_1$ peptide. These observations indicate that the transduction coupling activated by β_3-adrenoceptors involves a Gα_s-induced stimulation of the cyclic AMP/PKA pathway leading to phosphorylation and subsequent activation of L-type Ca^{2+} channels.

The β_3-adrenoceptors have been cloned and sequenced in several species including rat and human (Strosberg, 1997). Recent studies have suggested the existence of putative β_4-adrenoceptors which have not been cloned and sequenced up to now (Kaumann *et al*., 1998). However, since these putative β_4-adrenoceptors have been shown to be insensitive to SR59230A (Galitzky *et al*., 1997), our results showing a complete inhibition with SR59230A support the idea that only β_3-adrenoceptors mediate the CGP12177A-induced stimulation of L-type Ca^{2+} channels in vascular myocytes. Activation of the G_s/PKA pathway by β_3-adrenoceptors has been previously reported in rodent adipocytes (Strosberg, 1997). Although it is generally accepted that α subunits of G_s protein play an important role in regulation of L-type Ca^{2+} channels in cardiac and smooth muscles during β-adrenergic stimulation, the signalling pathways underlying the modulation of L-type Ca^{2+} channels by activated G_s proteins remain controversial. In cardiac myocytes, the β-adrenergic-induced stimulation of Ca^{2+} channels is essentially *via* activation of adenylyl cyclase and subsequent phosphorylation of the channel (McDonald *et al*., 1994). In addition, a direct G protein activation of Ca^{2+} channels has been also proposed in the heart in response to β-adrenergic stimulation (Yatani *et al*., 1987). In rabbit portal vein myocytes, intracellular application of activated Gα_s subunits mimics the stimulatory effect of isoprenaline, and this effect has been interpreted as the result of a direct action of G_s protein on L-type Ca^{2+} channels (Xiong & Sperelakis, 1995). However, recent reports have shown that 8-Br-cyclic AMP and the catalytic subunit of PKA significantly increased peak Ba^{2+} currents, and their effects could be blocked by PKA inhibitors (Ruiz-Velasco *et al*., 1998). In the present study, the β_3-adrenoceptor-induced stimulation of Ba^{2+} current was entirely blocked by H-89 and Rp 8-Br-cyclic AMPs, inhibitors of PKA, and by intracellular infusion of the anti-Gα_s antibody. In addition, application of okadaic acid, a protein phosphatase inhibitor, suppressed the CGP12177A- and forskolin-induced recovery of Ba^{2+} current after withdrawal of the stimulating substances. These results support the idea that the activated α_s subunits of G_s proteins elicit their stimulatory effect through the cyclic AMP/PKA pathway and the subsequent phosphorylation of L-type Ca^{2+} channels.

In addition to G_s protein, β_3-adrenoceptors have been shown to interact with $G_{i/o}$ proteins in adipocytes (Chaudhry *et al*., 1994) and human cardiac cells (Gauthier *et al*., 1996). Recently, it has been demonstrated that the human β_3-adrenoceptor may activate the mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK) 1 and 2 through $G_{i/o}$ protein and phosphatidylinositol-3 kinase

(PI3K) (Gerhardt *et al*., 1999). Moreover, the isoprenaline-induced activation of MAPK has been reported to involve the $G\beta\gamma$ dimers derived from $G_{i/o}$ protein (Crespo *et al*., 1995). Using an anti-$G\beta_{com}$ antibody and a βARK_1-derived $G\beta\gamma$ binding peptide, we have reported that the $\beta\gamma$ dimer from G_{13} is responsible for the transduction pathway during angiotensin II-induced stimulation of L-type Ca^{2+} channels (Macrez *et al*., 1997). Intracellular infusion of purified $G\beta\gamma$ proteins has been shown to stimulate the activity of L-type Ca^{2+} channels; this effect is largely inhibited by PKC inhibitors but remains insensitive to PKA inhibitors (Viard *et al*., 1999). In contrast, we show here that the β_3-adrenoceptor-induced stimulation of Ba^{2+} current is removed by H-89 but not affected by PKC inhibitors and after intracellular infusion of an anti-$G\beta_{com}$ antibody or a βARK_1 peptide, which act as $G\beta\gamma$ scavengers. Thus, our results suggest that in vascular myocytes only the α subunits of G_s may play a role in the regulation of L-type Ca^{2+} channels during β_3-adrenoceptor activation. Although different combinations of β and γ subunits of G proteins may have similar actions on Ca^{2+} channels (Dolphin, 1998), recent data have shown that activation of MAPK/ERK and inhibition of adenylyl cyclases V and VI appear to be $G\beta$ isoform specific ($G\beta_1$ being more efficient than $G\beta_5$) (Zhang *et al*., 1996; Bayewitch *et al*., 1998). As the subunit composition of the G_s protein that interacts with the β_3-adrenoceptor of vascular myocytes has not been identified, it can be postulated that this $\beta\gamma$ dimer may be unable to stimulate the $G\beta\gamma$-sensitive pathway leading to stimulation of L-type Ca^{2+} channels.

Activation of β-adrenoceptors is known to induce a relaxation in smooth muscle cells and several hypotheses have been proposed including Ca^{2+}-dependent and Ca^{2+}-independent mechanism. Although global increases in $[Ca^{2+}]_i$ regulate contraction, local Ca^{2+} transients (Ca^{2+} sparks) caused by opening of ryanodine-sensitive Ca^{2+} release channels in the sarcoplasmic reticulum may activate K_{Ca} channels in the surface membrane (Mironneau *et al*., 1996; Perez *et al*., 1999) leading to hyperpolarization and closing of voltage-dependent Ca^{2+} channels (Knot & Nelson, 1998). We have shown that Ca^{2+} sparks can be triggered by activation of L-type Ca^{2+} currents, particularly in the voltage range between -20 and 0 mV (Arnaudeau *et al*., 1997). In contrast, propagated Ca^{2+} waves are obtained only when large ($+10$ mV) and durable (>200 ms) depolarizations are applied (Arnaudeau *et al*., 1997). Preliminary results indicate that 1 μM CGP12177A and 5 μM forskolin increase the frequency of Ca^{2+} sparks in portal vein myocytes held at -50 mV (F. Coussin and J. Mironneau, unpublished data), then promoting increased activation of K_{Ca} channels, hyperpolarization and, ultimately, vasodilation. Therefore, it is proposed that the β_3-adrenoceptor-mediated modulation of L-type Ca^{2+} channels may be important in the regulation of smooth muscle tension.

In conclusion, the present study shows that in vascular myocytes activation of β_3-adrenoceptors stimulates the L-type Ca^{2+} channels though a $G\alpha_s$-induced activation of the cyclic AMP/PKA pathway and the subsequent phosphorylation of the channels.

This work was supported by grants from Centre National de la Recherche Scientifique and Fondation pour la Recherche Médicale (to P. Viard), France. We thank N. Biendon for secretarial assistance and C. Le Sénéchal for participating to PCR experiments.

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(Received July 19, 1999
Revised November 5, 1999
Accepted January 6, 2000)