



HAL
open science

Regulation of the Cystic Fibrosis Transmembrane Conductance Regulator Channel by β -Adrenergic Agonists and Vasoactive Intestinal Peptide in Rat Smooth Muscle Cells and Its Role in Vasorelaxation

Renaud Robert, Vincent Thoreau, Caroline Norez, Anne Cantereau, Alain Kitzi, Yvette Mettey, Christian Rogier, Frédéric Becq

► To cite this version:

Renaud Robert, Vincent Thoreau, Caroline Norez, Anne Cantereau, Alain Kitzi, et al.. Regulation of the Cystic Fibrosis Transmembrane Conductance Regulator Channel by β -Adrenergic Agonists and Vasoactive Intestinal Peptide in Rat Smooth Muscle Cells and Its Role in Vasorelaxation. *Journal of Biological Chemistry*, 2004, 279 (20), pp.21160-21168. <10.1074/jbc.M312199200>. <hal-03120019>

HAL Id: hal-03120019

<https://hal.science/hal-03120019v1>

Submitted on 26 Jan 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons CC BY-NC-ND 4.0 - Attribution - Non-commercial use - No Derivative Works - International License

Regulation of the Cystic Fibrosis Transmembrane Conductance Regulator Channel by β -Adrenergic Agonists and Vasoactive Intestinal Peptide in Rat Smooth Muscle Cells and Its Role in Vasorelaxation*

Received for publication, November 7, 2003, and in revised form, March 8, 2004
Published, JBC Papers in Press, March 11, 2004, DOI 10.1074/jbc.M312199200

Renaud Robert[‡], Vincent Thoreau[§], Caroline Norez[‡], Anne Cantereau[‡], Alain Kitzis[§],
Yvette Mettey[¶], Christian Rogier[‡], and Frédéric Becq[‡]||

From the [‡]Laboratoire des Biomembranes et Signalisation Cellulaire CNRS Unité Mixte de Recherche 6558, Université de Poitiers, 86022 Poitiers, France, [§]Laboratoire de Génétique Cellulaire et Moléculaire, Unité Propre de Recherche de l'Enseignement Supérieur EA 2622, Centre Hospitalier Universitaire de Poitiers, 86022 Poitiers, France, and [¶]Laboratoire de Chimie Organique, Faculté de Médecine et de Pharmacie de Poitiers, BP 199, 86002 Poitiers Cedex, France

The signaling events that regulate vascular tone include voltage-dependent Ca^{2+} influx and the activities of various ionic channels; which molecular entities are involved and their role are still a matter of debate. Here we show expression of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel in rat aortic smooth muscle cells. Immunoprecipitation and *in vitro* protein kinase A phosphorylation show the appearance of mature band C of CFTR. An immunohistochemistry study shows CFTR proteins in smooth muscles of aortic rings but not in skeletal muscles. Using the iodide efflux method, a combination of agonists and pharmacological agents was used to dissect the function of CFTR. Agonists of the cAMP pathway, the β -adrenergic agonist isoproterenol, and the neuropeptide vasoactive intestinal peptide activate CFTR-dependent transport from cells maintained in a high but not low extracellular potassium-rich saline, suggesting that depolarization of smooth muscle is critical to CFTR activation. Smooth muscle CFTR possesses all of the pharmacological attributes of its epithelial homologues: stimulation by the CFTR pharmacological activators MPB-07 ($\text{EC}_{50} = 158 \mu\text{M}$) and MPB-91 ($\text{EC}_{50} = 20 \mu\text{M}$) and inhibition by glibenclamide and diphenylamine-2-carboxylic acid but not by 5,11,17,23-tetrasulfonato-25,26,27,28-tetramethoxy-calix[4]arene. Contraction measurements on isolated aortic rings were performed to study the contribution of CFTR to vascular tone. With aortic rings (without endothelium) precontracted by high K^+ saline or by the α -adrenergic agonist norepinephrine, CFTR activators produced a concentration-dependent relaxation. These results identify for the first time the expression and function of CFTR in smooth muscle where it plays an unexpected but fundamental role in the autonomic and hormonal regulation of the vascular tone.

The balance between contraction and relaxation signals determines the vascular tone, the major determinant of the re-

sistance to blood flow through the circulation. The regulation of the general vascular tone depends on the activity of plasma membrane ion channels, through which the movement of ions determines, to a large extent, the membrane potential of vascular smooth muscle cells (SMC)¹ (1, 2). Activation of SMC by contractile agents (e.g. α -adrenergic agonist) induces a cascade of events resulting in an increase in cytosolic Ca^{2+} concentration and smooth muscle contraction (1, 2). Smooth muscle contraction is also initiated by membrane depolarization (e.g. induced by elevation of extracellular K^+ concentration), which opens voltage-dependent Ca^{2+} channels, resulting in the influx of Ca^{2+} .

Relaxation of SMC, on the other hand, is dependent on at least two processes. One is the passive relaxation following the removal of the contractile agents. Second is the active relaxation supported by cyclic nucleotide-dependent pathways, in the continued presence of the contractile agent (3). Cyclic AMP- and cyclic GMP-dependent protein kinases modulate SMC relaxation by mechanisms independent of changes in intracellular Ca^{2+} concentrations or of the state of myosin light chain phosphorylation (1–3). In SMC, the neuropeptide vasoactive intestinal peptide (VIP) induces smooth muscle relaxation (4). VIP also increases the cellular cAMP in SMC (4) as well as in other cells (5), but the precise contribution of cAMP to the vasodilation effect of VIP is not known (4).

In vitro studies have provided evidence that Cl^- currents are important for equilibrium of membrane potential, intracellular pH, cell volume maintenance, and SMC contraction (1, 2, 6–8). Among the ionic channels identified in SMC are Ca^{2+} -activated Cl^- channels (I_{ClCa}) (2, 6–8). More recently, the voltage-dependent chloride channel $\text{ClC}-3$ has been identified in canine colonic smooth muscle, the activation of which depends on cellular volume (9). Here we present evidence that another chloride channel is expressed in SMC (i.e. the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel). The CF gene encodes CFTR, a protein that functions as a

* This work is in partial fulfillment of Ph.D. requirements for R. R. and was supported by a fellowship from Vaincre la Mucoviscidose (VLM). This work was also supported by grants from VLM and CNRS. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

|| To whom correspondence should be addressed. E-mail: frederic.becq@univ-poitiers.fr.

¹ The abbreviations used are: SMC, smooth muscle cells; CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; VIP, vasoactive intestinal peptide; MPB-07, 6-hydroxy-10-chlorobenzo[c]quinolinium chloride; MPB-91, 5-butyl-6-hydroxy-10-chlorobenzo[c]quinolinium chloride; MPB-80, 10-fluoro-6-hydroxybenzo[c]quinolinium chloride; IBMX, 3-isobutyl-1-methylxanthine; TS-TM calix[4]arene, 5,11,17,23-tetrasulfonato-25,26,27,28-tetramethoxy-calix[4]arene; PKA, cAMP-dependent protein kinase; DPC, diphenylamine-2-carboxylic acid; AgII, angiotensin II; cpt-cAMP, 8-chlorophenylthio-cAMP.

cAMP-dependent Cl⁻ channel in the apical membrane of secretory epithelial cells (10, 11). Whereas CFTR has been generally regarded as specifically expressed in epithelial cells, evidence for CFTR expression and/or function as a Cl⁻ conductance has been observed in cardiac muscle cells (12–14), brain (15), and endothelia (16, 17). In this study, we demonstrated expression of PKA-regulated CFTR in rat smooth muscle cells, explored its pharmacology and regulation by various physiologic and pharmacologic agonists (*i.e.* the β -adrenergic agonist isoproterenol, the neuropeptide VIP, forskolin, IBMX, and benzo[c]quinolizinium derivatives), and provided evidence for a role of CFTR in the autonomic and hormonal regulation of the vascular tone.

EXPERIMENTAL PROCEDURES

Vessel Preparation—All experiments were performed on male Wistar rats (250–300 g). The thoracic aorta of animals killed by cervical dislocation was removed and placed into Krebs solution containing 120 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 15 mM NaHCO₃, 1.2 mM KH₂PO₄, 11 mM D-glucose, 10 mM Hepes, pH 7.4. After separation of connective tissues, the thoracic segment of aorta was cut into rings of 3 mm in length. The preparation was then transferred into a 5-ml organ bath containing Krebs solution bubbled with a mixture of 95% O₂ and 5% CO₂. Each aortic ring was suspended between two stainless steel hooks. One of the hooks was mounted at the bottom of the bath, whereas the other was connected to force displacement transducer IT1–25 (Emka Technologies). All experiments were performed at 37 °C. A basal tension of 2 g was applied in all experiments. The endothelium from all arterial rings was removed by rubbing its luminal surface. Endothelium integrity or functional removal was verified by the presence or absence, respectively, of the relaxant response to 10⁻⁵ M acetylcholine.

SMC Isolation—For each culture, thoracic aortas from four rats were excised and placed in modified Krebs solution containing 120.8 mM NaCl, 5.9 mM KCl, 0.2 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 2 mM NaHCO₃, 11 mM D-glucose, 10 mM Hepes, and pH 7.4. Cleaned aortas were placed for 20 min at 37 °C in modified Krebs solution containing 10 units/ml elastase (Serva, Germany), 170 units/ml collagenase (CLS2; Worthington), 0.6 mg/ml trypsin inhibitor (Sigma), 1 mg/ml bovine albumin (Sigma). Tunica adventicia and endothelium were removed, and the remaining medial layers were minced and digested for 50 min in α -minimal essential medium (Invitrogen) containing 0.024 M Hepes, 300 units/ml collagenase, 65 units/ml elastase. The suspension was filtered, collected in α -minimal essential medium containing 0.024 M Hepes plus 10% fetal calf serum, and centrifuged at +4 °C. Cells were resuspended in α -minimal essential medium plus 10% fetal calf serum, plated onto glass coverslips or 24-well plates, and incubated at 37 °C in 5% CO₂. Characteristics of SMC were verified by positive immunostaining with the monoclonal anti- α -smooth muscle actin antibody (clone 1A4; Sigma) and by their inability to react with von Willebrand Factor VIII antibody (Sigma), a marker of endothelial cells. All solutions contained 100 units/ml penicillin and 0.1 mg/ml streptomycin.

Immunoprecipitation and Phosphorylation of CFTR—Smooth muscle and HT29 cells (a human epithelial colonic cell line) were washed three times in phosphate-buffered saline, scraped in a sufficient volume of radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 1% Triton X-100) supplemented with protease inhibitors (20 μ M leupeptin, 0.8 μ M aprotinin, 10 μ M pepstatin, and 2.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride), and homogenized by several passes through a 23-gauge syringe needle. Cell lysates were incubated on ice for 30 min and clarified by centrifugation at 15,000 \times g for 5 min at 4 °C. Cell lysates were then supplemented with 3 volumes of NET buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.05% Nonidet P-40) and incubated overnight at 4 °C with 2 μ g of CFTR mouse monoclonal antibody clone 24–1 (R & D Systems) or 2 μ g of nonimmune mouse IgG (Sigma). To precipitate immune complexes, an incubation with 3 μ g of protein G-Sepharose (Amersham Biosciences) was conducted for 1 h at 4 °C. Bead-bound complexes were washed three times with ice-cold NET buffer and resuspended in 50 μ l of phosphorylation buffer (50 mM Tris-HCl, 0.1 mg/ml bovine serum albumin, 10 mM MgCl₂, pH 7.5). CFTR was phosphorylated *in vitro* (1 h at 32 °C) using 2 units of the catalytic subunit of PKA (Sigma) and 10 μ Ci of [γ -³²P]ATP (3000 Ci/mmol; Amersham Biosciences). After several washes in ice-cold NET buffer, proteins were denatured in Laemmli buffer for 15 min at room temperature. Samples were then separated on 6% polyacrylamide SDS-PAGE, and, after drying, phosphorylated proteins were visualized by autoradiography.

Immunohistochemistry and Immunofluorescence—For immunohistochemical analysis of CFTR protein expression in intact tissue, rat aorta and rat skeletal muscle were dissected out, and rat aorta endothelium was removed. Tissues were fixed for 3 h in 0.01% phosphate buffer containing 0.01 M sodium meta-periodate, 0.075 M lysine, 3% paraformaldehyde at room temperature and stored overnight in 30% saccharose at 4 °C. Coronal sections (10 μ m thick) were cut with a cryostat and mounted on polylysine-coated glass slides. Sections were washed three times in Tris-buffered saline containing 0.4% Triton X-100, and non-specific binding sites were blocked with Tris-buffered saline containing 0.4% Triton X-100, 0.5% bovine serum albumin, and 10% goat serum for 1 h. Anti-CFTR C-terminal monoclonal antibody (1:100, Ig2a, mouse anti-human; R & D Systems) was used as primary antibody. Organ sections were incubated with primary antibody overnight at 4 °C. After three washes, organ sections were incubated with the biotinylated anti-mouse IgG secondary antibody (1:200; Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Samples were then washed three times in Tris-buffered saline and incubated with the fluorescein isothiocyanate-conjugated streptavidin for detection of biotin. Nucleic acids were stained in blue with TO-PRO-3 iodide (Molecular Probes, Inc., Eugene, OR) for 15 min at room temperature (1:200 in Tris-buffered saline), and preparations were mounted in the same antifade solution Vectashield Mounting Medium (Vector Laboratories). In control experiments, the primary antibody was omitted. Fluorescence was detected using confocal laser-scanning microscopy (Bio-Rad MRC 1024). In all experiments, CFTR appears in *green*, and nuclei appear in *blue* (see Fig. 2).

Functional Study of Chloride Channel Activity in SMC—Chloride channel activity was assayed at 37 °C by measuring the rate of iodide (¹²⁵I) efflux as previously described (18, 19) and adapted to SMC. All experiments were performed at 37 °C. Cells were placed in multiwell plates in order to perform parallel experiments and comparison analysis using a robotic system (MultiProbe II EXT®; PerkinElmer Life Sciences) adapted to the iodide efflux method. Two different efflux buffers were used. Classical Krebs solution containing 120 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 15 mM NaHCO₃, 1.2 mM KH₂PO₄, 11 mM D-glucose, 10 mM Hepes, pH 7.4, or a modified Krebs solution with high K⁺ content (hereafter denoted 80 mM K⁺). This solution consisted of Krebs solution with 80 mM KCl where Na⁺ was replaced by an equimolar concentration of K⁺ to maintain a constant ion strength. SMC were first washed with the classical Krebs solution. Cells were then incubated in Krebs solution containing 1 μ M KI and 1 μ Ci Na¹²⁵I/ml (PerkinElmer Life Sciences) during 1 h at 37 °C to permit the ¹²⁵I to reach equilibrium. Then cells were washed with the Krebs solution to remove extracellular ¹²⁵I. The loss of intracellular ¹²⁵I was determined by removing the medium with either Krebs solution or Krebs solution containing 80 mM K⁺ every 1 min for up to 9 min. The first three aliquots were used to establish a stable base line in efflux buffer alone. A medium containing the appropriate drug was used for the remaining aliquots. Residual radioactivity was extracted with 0.1 N NaOH and determined using a γ counter (Cobra II; PerkinElmer Life Sciences). The fraction of initial intracellular ¹²⁵I lost during each time point was determined, and time-dependent rates (k = peak rate, min⁻¹) of ¹²⁵I efflux were calculated from $k = \ln(^{125}I_{t_1}/^{125}I_{t_2})/(t_1 - t_2)$ where ¹²⁵I_t is the intracellular ¹²⁵I at time t , and t_1 and t_2 are successive time points (18, 19). Curves were constructed by plotting k versus time. Relative rates were calculated and correspond to $k_{\text{peak}} - k_{\text{basal}}$ (min⁻¹) (*i.e.* k representing the peak rate excluding the third point used to establish the base line). For experiments using the chloride transport inhibitors, glibenclamide, diphenylamine-2-carboxylic acid (DPC), and TS-TM calix[4]arene, they were present in the loading solution and in the efflux buffer. Results are expressed as mean \pm S.E. of n observations.

Contraction Measurement on Isolated Aortic Rings—During 1 h, tissues were rinsed three times in Krebs solution, and the basal tone was always monitored and adjusted to 2 g. High K⁺ solution (Krebs solution with 80 mM of KCl, where Na⁺ was replaced by an equimolar concentration of K⁺ to maintain a constant ionic strength) or 10⁻⁶ M norepinephrine (denoted NE) were used to evoke the sustained contractile response. Once the sustained tension was established, the tissues were allowed to equilibrate further for 30 min before cumulative addition of agonist to the bath. Cumulative concentration-response relationships for the relaxant effect of MPB compounds was determined in aortic rings following stable contraction. The relaxant effect of CFTR agonists was expressed as percentage contraction of the agonist-constricted arterial rings. IC₅₀ was calculated as the drug concentration inducing a half-maximal vasorelaxation (or inhibition of contraction). Data are presented as mean \pm S.E. of n experiments.

Chemicals—The benzo[c]quinolizinium compounds 10-chloro-6-hy-

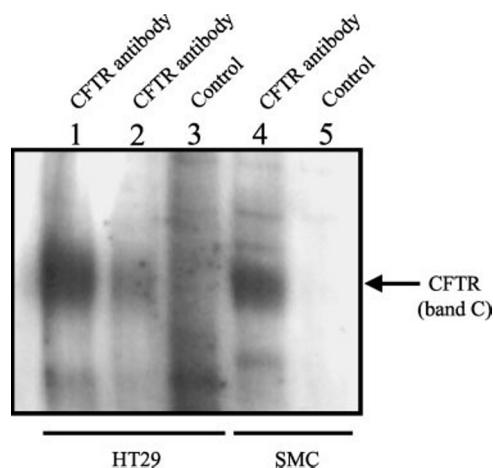


FIG. 1. Identification of CFTR in smooth muscle cells. The presence of CFTR protein was assessed by immunoprecipitation followed by *in vitro* PKA phosphorylation. CFTR is expressed in epithelial colonic HT-29 cells (lanes 1 and 2; lane 2 corresponds to a 5-fold dilution of the phosphorylation reaction) and in SMC (lane 4) as indicated. Lanes 3 (HT-29) and 5 (SMC), control immunoprecipitation with nonimmune mouse IgG.

droxybenzo[c]quinolinium chloride (MPB-07), 5-butyl-10-chloro-6-hydroxybenzo[c]quinolinium chloride (MPB-91), and 10-fluoro-6-hydroxybenzo[c]quinolinium chloride (MPB-80) were prepared as described previously (18, 19). 5,11,17,23-Tetrakisulfonato-25,26,27,28-tetramethoxy-calix[4]arene (TS-TM calix[4]arene) derivative, an inhibitor of outwardly rectifying Cl^- channels, was generously provided by Drs. Singh and Bridges (University of Pittsburgh, Pittsburgh, PA) (21). The chloride channel inhibitors DPC and glibenclamide, the α -adrenergic agonist norepinephrine, the β -adrenergic agonist isoproterenol, and the neuropeptide vasoactive intestinal peptide were from Sigma. All other products were from Sigma. All compounds were dissolved in Me_2SO (final Me_2SO concentration 0.1%) except isoproterenol, norepinephrine, VIP, MPB-07, and MPB-80, which were dissolved in water.

Statistics—All results are expressed as mean \pm S.E. of n observations. Sets of data were compared with either an analysis of variance or Student's t test. Differences were considered statistically significant when $p < 0.05$. Nonsignificant difference was as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. All statistical tests were performed using GraphPad Prism version 3.0 for Windows (Graphpad Software).

RESULTS

Identification of CFTR in SMC by Immunoprecipitation and *In Vitro* Phosphorylation Studies—In epithelia and nonepithelia, the activity of CFTR channels is under the control of cAMP-dependent process leading to the phosphorylation by protein kinases (principally PKA) of the regulatory (R) domain of CFTR (10–18). Since CFTR has been detected in ventricular heart cells (11–14), we raised the question of its expression in vascular cells. We first searched for the presence of CFTR in SMC at a protein level. CFTR protein expression was analyzed using immunoprecipitation experiments using anti-CFTR antibody followed by *in vitro* PKA phosphorylation. Results are shown in Fig. 1. We used the HT29 epithelial cell line as a positive control, because these cells express CFTR endogenously (10). The expected phosphorylated band obtained by CFTR immunoprecipitation in HT29 cells is presented Fig. 1 (lanes 1 and 2). A similar pattern was obtained with SMC as shown in lane 4 in Fig. 1. The major CFTR form corresponds to band C (10, 18) at the 175-kDa protein, as determined by molecular mass standards. Controls with nonimmune mouse IgG are also provided in Fig. 1, lanes 3 and 5, for HT29 and SMC, respectively. These results demonstrated that CFTR endogenously expressed in SMC can be phosphorylated *in vitro* by PKA as in epithelial cells (10, 11, 18).

Immunolocalization of CFTR Proteins in Smooth Muscle—We investigated the location of CFTR in aortic rings. Results from

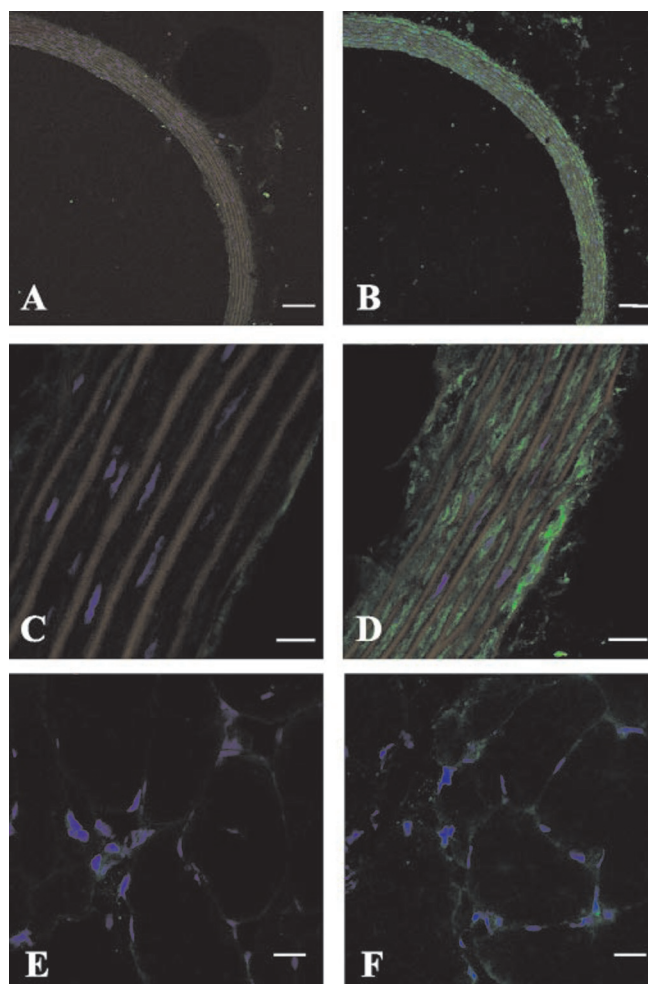


FIG. 2. Immunolocalization of CFTR in rat aorta section. A and B, localization of CFTR in rat aorta section without endothelium. C and D, high magnification of rat aorta section. A, C, and E, negative controls in which primary anti-CFTR was omitted. E and F, absence of CFTR in rat skeletal muscles. CFTR is stained in green. Nuclei are stained in blue. Scale bars, 100 μm (A and B) and 20 μm (C–F).

experiments using 10- μm -thick coronal sections of rat aortas (without endothelium) cut with a cryostat and mounted on polylysine-coated glass slides are shown in Fig. 2. Staining of rat denuded aortic rings was performed with anti-CFTR C-terminal monoclonal antibody (as primary antibody) and incubation with the biotinylated anti-mouse IgG secondary antibody. Samples were further incubated with the fluorescein isothiocyanate-conjugated streptavidin for detection of biotin. We found CFTR localization within the thickness of rat aorta in smooth muscle cells (Fig. 2B) as compared with the control in which primary anti-CFTR was omitted (Fig. 2A). High magnifications of rat aorta sections are presented in Fig. 2, C and D. With the negative control (Fig. 2, A and C), only the nuclei, stained in blue, are apparent. From Fig. 2B, it can be seen that CFTR is localized in smooth muscle cells arranged in concentric layers separated by elastic tissues. Autofluorescence in brown, which corresponds to the intact elastic lamellar structure typical of aortic tissue, is detected with (Fig. 2, B and D) or without the primary antibody (Fig. 2, A and C). Finally, using the same antibody, CFTR was found absent from rat skeletal muscle cells (Fig. 2, E and F).

Functional Analysis of CFTR in SMC: Activation by cAMP Agonists—Implicit in the successful identification of CFTR in SMC was the assumption that it would function as a cAMP-regulated Cl^- channel as in epithelial and nonepithelial cells (10–18). Therefore, several series of experiments were con-

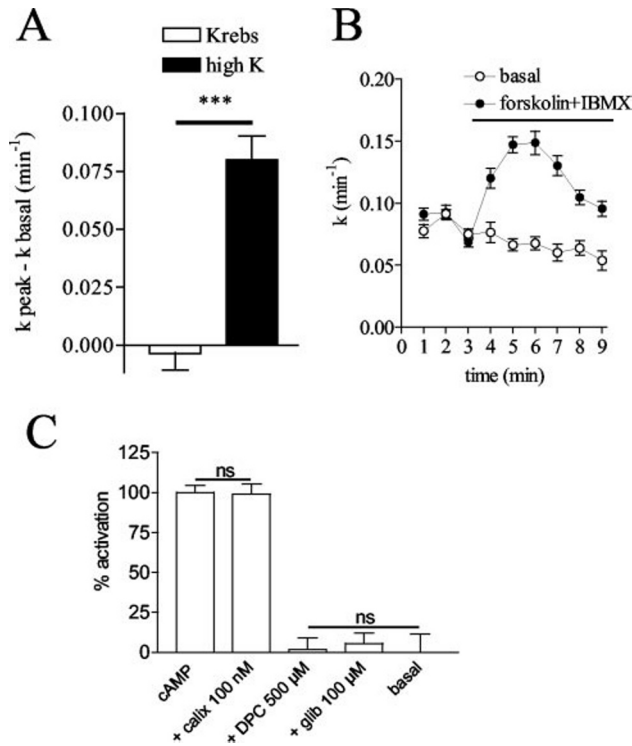


FIG. 3. cAMP-dependent CFTR Cl^- channel activity in rat smooth muscle cells. The stimulation of iodide efflux rate (k) as a function of time was evoked in a solution containing 80 mM K^+ by cAMP agents (10 μM forskolin, 500 μM IBMX, 500 μM cpt-cAMP, $n = 20$). *A*, histograms of the data obtained using Krebs medium or 80 mM K^+ solution after stimulation with the cAMP mixture. *B*, time-dependent stimulation of the efflux in resting cells (*basal*) or in the presence of cAMP agonists indicated by the horizontal bar ($n = 8$ in all experiments). *C*, histograms showing activation of efflux determined as a percentage of the maximal stimulation in the presence of cAMP agonist or cAMP agonists plus 100 μM glibenclamide, 500 μM DPC, or 100 nM TS-TM calix[4]arene ($n = 8$ for each). *basal*, vehicle alone. Data are presented as mean \pm S.E. ***, $p < 0.001$. *ns*, nonsignificant difference.

ducted to investigate first whether CFTR is functional as a cAMP- and agonist-dependent chloride channel and, second, since CFTR is present in the concentric layers of smooth muscle within the thickness of rat aorta, whether it could play a role in the vascular reactivity. We first searched for a cAMP-regulated Cl^- channel activity by measuring the rate of iodide (^{125}I) efflux (18, 19) from isolated SMC. We performed two series of experiments. First, SMC were bathed in a classical Krebs saline (containing 4.7 mM K^+) mimicking the physiological extracellular medium. Second, we used a modified Krebs solution containing 80 mM extracellular K^+ , because it is known that high K^+ depolarizes the cell membrane and promotes the contraction of SMC by activating voltage-gated Ca^{2+} channels (2, 22). A cAMP-promoting mixture containing 10 μM forskolin, 500 μM IBMX, and 500 μM cpt-cAMP was used. Two important results were obtained and presented in Fig. 3. With Krebs saline, no stimulation by cAMP agonists could be detected as compared with basal conditions (Fig. 3A, $n = 8$ each). In contrast, with SMC bathed in the high K^+ solution, a dramatic stimulation of iodide effluxes in the presence of a cAMP-promoting mixture (Fig. 3, *A* and *B*, $n = 20$) was observed with isolated rat smooth muscle cells as compared with resting cells (labeled *basal* in Fig. 3B, $p < 0.001$). The stimulation of iodide efflux results in a rapid increase of the rate of efflux (k) within the first 2 min after the addition of the agonists (Fig. 3B). The peak rate was $0.148 \pm 0.009 \text{ min}^{-1}$ ($n = 20$) for cells stimulated by cAMP agonists and $0.067 \pm 0.005 \text{ min}^{-1}$ ($n = 8$) for cells maintained in basal conditions.

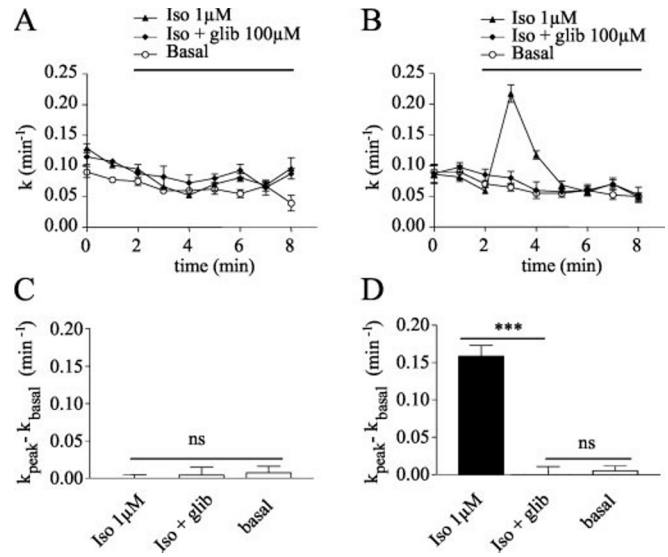


FIG. 4. Isoproterenol-activated CFTR Cl^- channels. Stimulation of iodide efflux rate (k) as a function of time evoked in Krebs medium (*A* and *C*) or solution containing 80 mM K^+ (*B* and *D*) by 1 μM isoproterenol (*Iso*) in smooth muscle cells ($n = 8$) as compared with resting cells without isoproterenol (*basal*; $n = 8$). *C* and *D*, histograms showing mean \pm S.E. of n observations using Krebs (*C*) or 80 mM K^+ solution (*D*). The inhibition of isoproterenol stimulation by 100 μM glibenclamide ($n = 8$) of efflux is shown (*A* and *B*) and quantified (*C* and *D*). *basal*, vehicle alone in Krebs (*A* and *C*) or 80 mM K^+ (*B* and *D*). ***, $p < 0.001$; *ns*, nonsignificant difference.

Three different classes of Cl^- channel inhibitors were used to establish the pharmacological signature of CFTR: glibenclamide and DPC, inhibitors of CFTR channels (23, 24), and the TS-TM calix[4]arene derivative, an inhibitor of outwardly rectifying Cl^- channels but not of CFTR (19, 21, 24). We found that the stimulation of iodide efflux by cAMP agonists with SMC bathed in high K^+ saline was fully inhibited ($p < 0.001$) by 100 μM glibenclamide and 500 μM DPC but not by 100 nM TS-TM calix[4]arene (Fig. 3C). This pharmacological profile of inhibition is in perfect agreement with that determined for the epithelial CFTR (19, 21, 24).

Activation of CFTR Channels by the β -Adrenergic Agonist Isoproterenol—To begin to understand the physiological significance of CFTR expression in SMC and since pharmacological cAMP agonists stimulate the chloride channel activity of CFTR in epithelia (10–18), we used for the following experiments more physiological modulators of SMC functions. Among the numerous physiological regulators of SMC leading to an elevation of the cellular cAMP level, we first selected the β -adrenergic agonist isoproterenol (22, 25). In Krebs saline, no significant stimulation was noted with 1 μM isoproterenol ($n = 8$; Fig. 4, *A* and *C*). However, a significant stimulation ($p < 0.001$, for stimulation compared in high and low K^+) of iodide effluxes by 1 μM isoproterenol was observed with a solution containing 80 mM K^+ as shown in Fig. 4B ($n = 8$) as compared with resting cells without isoproterenol ($n = 8$, $p < 0.001$) (Fig. 4, *B* and *D*). The typical time course for stimulation by isoproterenol of the efflux is illustrated in Fig. 4B, which shows a rapid increase of the rate of efflux (k) within the first 2 min after the addition. The corresponding calculated peak rate k was $0.217 \pm 0.001 \text{ min}^{-1}$ ($n = 8$). As for the cAMP agonists forskolin and IBMX, the isoproterenol-dependent stimulation was significantly different between high K^+ ($n = 8$; Fig. 4D) and Krebs solutions ($n = 8$; Fig. 4C, $p < 0.001$) and fully inhibited by glibenclamide (100 μM , $n = 8$; Fig. 4, *B* and *D*).

Activation of CFTR Channels by the Neuropeptide VIP—The second physiological regulator of SMC function examined here

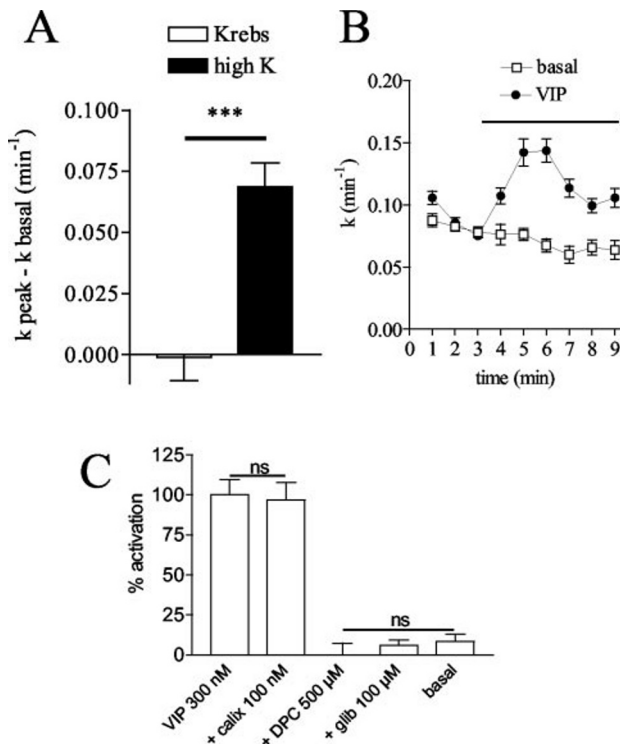


FIG. 5. VIP activated smooth muscle CFTR Cl^- channels. *A*, histograms showing stimulation of iodide efflux rate (k) by 300 nM VIP as a function of time evoked in solution containing 80 mM K^+ ($n = 14$) or in Krebs solution ($n = 8$). *B*, time-dependent activation of iodide efflux by 300 nM VIP in 80 mM K^+ . *basal*, vehicle alone in 80 mM K^+ . *C*, inhibition by 100 μM glibenclamide and 500 μM DPC but not TS-TM calix[4]arene ($n = 8$ for each) of efflux stimulated by VIP. Data are mean \pm S.E. of n observations. ***, $p < 0.001$. *ns*, nonsignificant difference.

was the neuropeptide VIP, also known to increase the cellular cAMP in SMC (4) as well as in other cells (5) and to stimulate the activity of CFTR channels in epithelial cells (26, 27). We thus explored the possibility that VIP could stimulate CFTR activity in SMC. As noted from the experiments using cAMP agonists and isoproterenol, VIP-dependent stimulation of iodide effluxes was significantly different with cells bathed in high K^+ ($n = 14$) or Krebs solutions ($n = 8$) (Fig. 5A) ($p < 0.001$). A significant iodide efflux was stimulated by 300 nM VIP with SMC bathed in a solution containing 80 mM K^+ as compared with control experiments without VIP ($n = 14$; $p < 0.001$) (Fig. 5B). The corresponding peak rate k was $0.143 \pm 0.009 \text{ min}^{-1}$ ($n = 14$). In agreement with the above data, we also found that the VIP response was fully inhibited by 100 μM glibenclamide and 500 μM DPC but not by 100 nM TS-TM calix[4]arene ($n = 8$ each; Fig. 5C).

Effect of Angiotensin II on Iodide Efflux in Isolated SMC—In rat vascular smooth muscle cells, angiotensin II (AgII) is a vasoconstrictor agent that stimulates calcium-dependent chloride channel via increases in $[\text{Ca}^{2+}]_i$ (28, 29). We therefore investigated whether calcium-dependent iodide efflux could be activated in SMC. Fig. 6 shows that a significant stimulation of iodide effluxes by 100 nM AgII was obtained independently of the concentration of extracellular K^+ . Indeed, the calculated peak rates were similar (*i.e.* $k = 0.22 \pm 0.006 \text{ min}^{-1}$ ($n = 8$) and $k = 0.21 \pm 0.004 \text{ min}^{-1}$ ($n = 8$)) with cells bathed in a solution containing either low (4.7 mM) (Fig. 6A) or high K^+ (80 mM) (Fig. 6B), respectively. The time course for stimulation of the efflux is shown in Fig. 6, A and B. Importantly, the corresponding peak rates in the presence of 100 μM glibenclamide were $0.23 \pm 0.01 \text{ min}^{-1}$ ($n = 4$) (Fig. 6A) and $0.21 \pm 0.004 \text{ min}^{-1}$

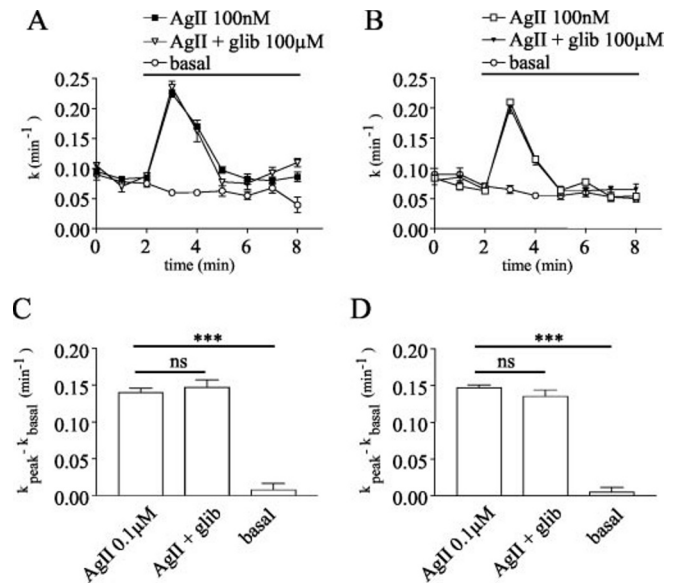


FIG. 6. Effect of angiotensin II on iodide efflux. *A* and *B*, the stimulation of iodide efflux rate (k) as a function of time was evoked in solution containing Krebs (*A* and *C*) or 80 mM K^+ (*B* and *D*) by 100 nM AgII in rat SMC ($n = 8$) as compared with basal ($n = 8$). *C* and *D*, summary of the data using Krebs (*C*) or 80 mM K^+ solution (*D*). Adding 100 μM glibenclamide ($n = 8$) has no effect on the AgII-dependent iodide efflux. *basal*, vehicle alone in Krebs (*A* and *C*) or 80 mM K^+ (*B* and *D*). Data are mean \pm S.E. of n observations. ***, $p < 0.001$. *ns*, nonsignificant difference.

($n = 4$) (Fig. 6B) were not different from those without glibenclamide (Fig. 6, C and D). Thus, angiotensin II activated a chloride transport that is not sensitive to extracellular potassium nor to glibenclamide in contrast to the response obtained with either cAMP agonists, isoproterenol or VIP.

Pharmacological Activation of CFTR Channels in SMC—If CFTR in SMC and in epithelial cells are similar, then we should observe stimulation of CFTR in SMC with activators of the epithelial homologues. Previously, we identified and synthesized, through a structure-activity relationship, pharmacological activators of the epithelial CFTR (19, 20). Two of these drugs, MPB-07 and MPB-91, are potent activators, whereas a third one, MPB-80, is not able to stimulate the epithelial CFTR (see structures in Fig. 7). With rat isolated SMC, we found stimulation of iodide efflux by MPB-07 and MPB-91 but not by MPB-80 (Fig. 7A) only with high K^+ saline and not with normal Krebs solution (Fig. 7B). The corresponding peak rates were $0.207 \pm 0.01 \text{ min}^{-1}$ (MPB-07, $n = 36$), $0.270 \pm 0.01 \text{ min}^{-1}$ (MPB-91, $n = 36$), $0.063 \pm 0.05 \text{ min}^{-1}$ (MPB-80, $n = 8$), and $0.076 \pm 0.04 \text{ min}^{-1}$ (basal, $n = 8$). We determined from eight separate experiments the half-maximal effective concentrations (EC_{50}) for stimulation of iodide efflux in SMC by MPB-07 ($\text{EC}_{50} = 158 \pm 1.3 \mu\text{M}$, $n = 4$) and MPB-91 ($\text{EC}_{50} = 20 \pm 1.6 \mu\text{M}$, $n = 4$). The corresponding curves are presented in Fig. 7C. These values are in remarkable agreement with those determined in a parallel study performed with the human airway epithelial Calu-3 cell endogenously expressing CFTR (MPB-07: $\text{EC}_{50} = 140 \pm 1.5 \mu\text{M}$, $n = 4$; MPB-91: $\text{EC}_{50} = 23 \pm 1.4 \mu\text{M}$, $n = 4$, not shown). Again, we found similar pharmacological profiles for inhibition by glibenclamide and DPC but not by TS-TM calix[4]arene for MPB-07 (Fig. 7D) and MPB-91 (Fig. 7E). These results demonstrated that specific CFTR activators like physiological regulators have similar effect with comparable pharmacology on the CFTR-dependent chloride transport in SMC.

Role of CFTR in Agonist-dependent Vasorelaxation of SMC—Since the activity of CFTR in SMC was only apparent in high

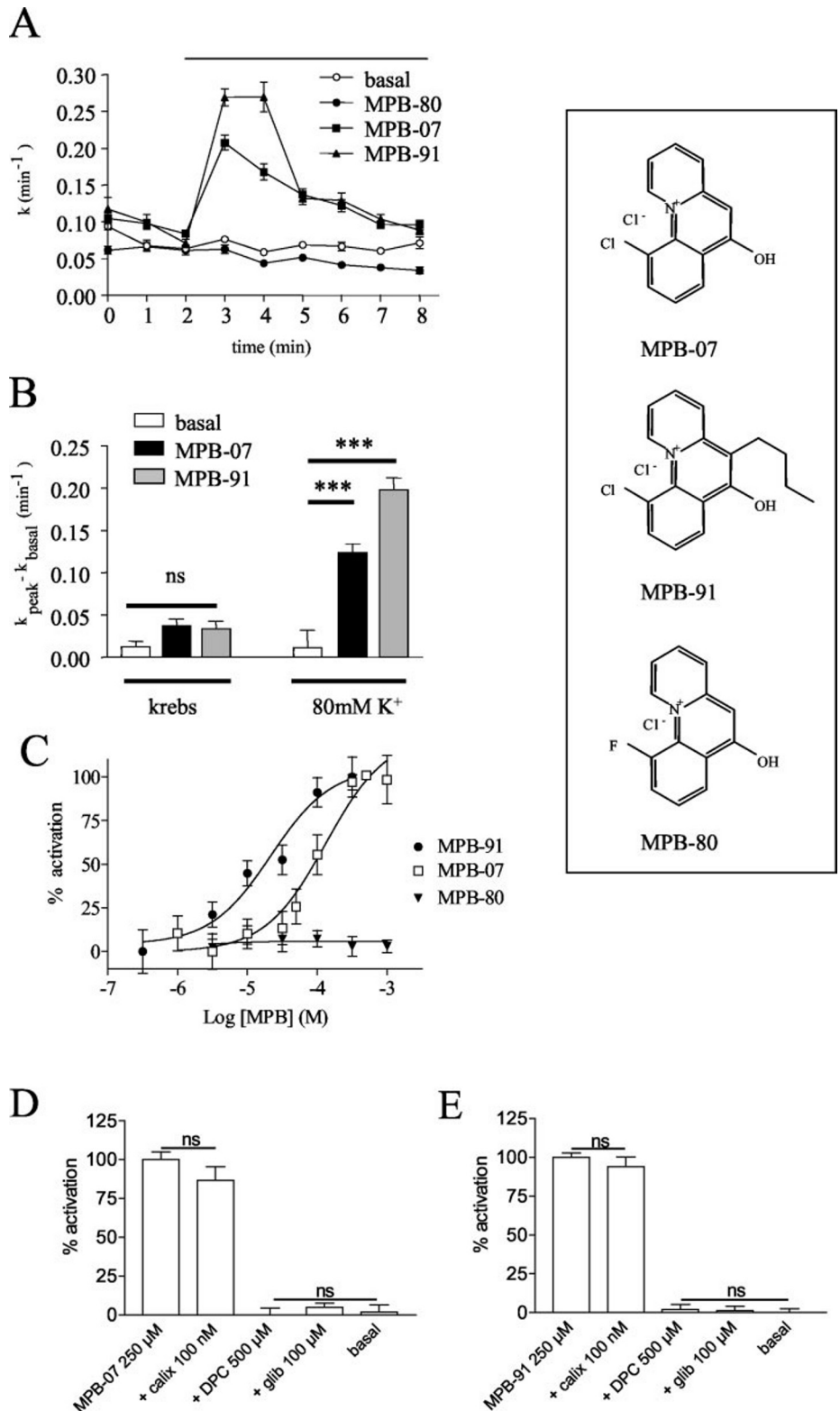
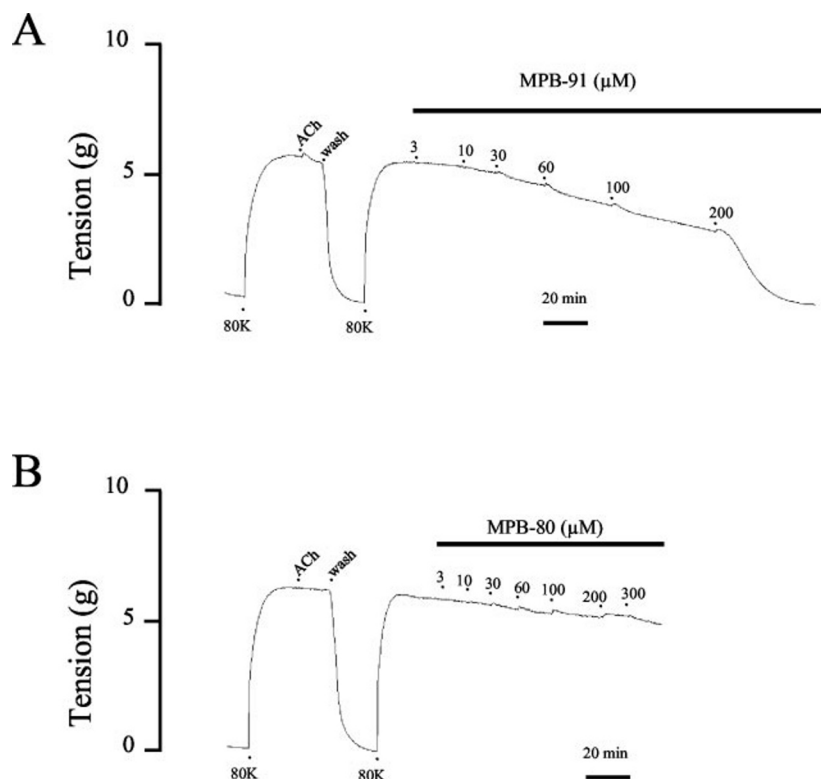


FIG. 7. Pharmacological activation of CFTR Cl⁻ channel activity in rat smooth muscle. *A*, effect of a 250 μM concentration of the CFTR activators MPB-07 (*n* = 36) and MPB-91 (*n* = 36) on the iodide efflux in 80 mM K⁺ solution in SMC. Note that 250 μM MPB-80 has no effect (*n* = 8). *B*, summary of the data for each experimental condition in normal Krebs or 80 mM K⁺ solution. *C*, half-maximal effective concentrations (EC₅₀) for stimulation of iodide efflux by MPB-07 (EC₅₀ = 158 ± 1.3 μM, *n* = 4) and MPB-91 (EC₅₀ = 20 ± 1.6 μM, *n* = 4). The stimulation of iodide efflux was evoked in saline containing 80 mM K⁺. Note that MPB-80 has no effect. *D* and *E*, inhibition by 100 μM glibenclamide and 500 μM DPC but not TS-TM calix[4]arene (*n* = 8 for each) of efflux stimulated by MPB-07 (*D*) or MPB-91 (*E*). Data are mean ± S.E. ***, *p* < 0.001. *ns*, nonsignificant difference. *basal*, vehicle alone in Krebs (*B*) or 80 mM K⁺ (*A*, *D*, and *E*). *Inset*, the chemical structures of benzo[*c*]quinolizinium derivatives.

extracellular K⁺ saline, which normally leads to the contraction of muscle via depolarization-induced activation of Ca²⁺ channels, we made the assumption that CFTR could be activated during vasoconstriction to promote vasorelaxation. To address this, we performed experiments on rat aortic rings mounted in an organ bath apparatus and measured their muscular activity. To avoid interaction with the endothelium, aortic rings were denuded, and the integrity of the preparation was subjected to acetylcholine as indicated under “Experimental

Procedures.” Reversible constriction was obtained after perfusion of the medium used for iodide efflux experiments containing 80 mM K⁺. Fig. 8*A* shows as a typical experiment in which increasing the concentrations of MPB-91 from 3 to 200 μM (*n* = 8 for each concentration) but not of MPB-80 (Fig. 8*B*, *n* = 8 for each concentration) induced the complete relaxation of rat denuded aortic rings precontracted by high K⁺ solution. The two CFTR activators MPB-07 and MPB-91 relaxed precontracted aortic rings with half-maximal relaxation value IC₅₀ of 166 ± 1.05 μM

FIG. 8. Effect of CFTR activators on the wall tension of rat aorta. Continuous traces from experiments performed with denuded aortic rings reversibly precontracted with 80 mM K^+ solution (80K at the bottoms of the traces). The absence of endothelium was verified by the addition of acetylcholine (ACh) (10^{-5} M). After washing the 80 mM K^+ solution, the tension returned to the control level before a second constriction was obtained by perfusion of the 80 mM K^+ solution again. Then the effect on tension of various concentrations of MPB-91 (A) and MPB-80 (B) was examined.



($n = 16$) and $70 \pm 1.12 \mu\text{M}$ ($n = 8$), respectively (Fig. 9) (note that these concentrations are in good agreement with our efflux data). MPB-80, which had no relaxation property (Figs. 8B and 9) also failed to stimulate CFTR-mediated iodide efflux. Finally, to verify that the vasorelaxant action of CFTR activators was not solely observed with aortic rings precontracted by high K^+ saline, we also used the α -adrenergic agonist norepinephrine. Fig. 10 presents a typical experiment representative of six different experiments showing that the CFTR activator MPB-07 produced concentration-dependent relaxation with $IC_{50} = 36 \pm 1.07 \mu\text{M}$ ($n = 6$). We concluded from these series of experiments that the activation of CFTR channels leads to vasorelaxation of rat aortic rings constricted by either high KCl or α -adrenergic receptor stimulation.

DISCUSSION

This report provides the first description of CFTR chloride channels expression in smooth muscle cells. Three salient features of this report are as follows. 1) Activation of β -adrenergic or VIP receptors and subsequently of the adenylyl cyclase-cAMP-PKA phosphorylation pathway leads to activation of CFTR in SMC. 2) Pharmacological activation/inhibition of CFTR in SMC is similar to that of the epithelial CFTR. 3) CFTR activation in SMC leads to vasorelaxation. Taken together, these observations suggest a novel mechanism for the autonomic and hormonal control of the vascular tone and are discussed below.

cAMP-dependent Regulation of CFTR in Smooth Muscle—We showed that vascular CFTR proteins can be phosphorylated *in vitro* by PKA and that agonists of the cAMP pathway, like isoproterenol, VIP, forskolin, and IBMX, stimulated CFTR chloride channel activity in rat SMC. The pharmacology of the vascular CFTR channel activity is remarkably similar to that of the epithelial CFTR, both for activation (MPB derivatives) and for inhibition (glibenclamide and DPC). For example, we found that the benzo[c]quinolizinium drugs originally demonstrated as epithelial CFTR activators (20) activated the vascular CFTR with similar EC_{50} values. Moreover, the structural

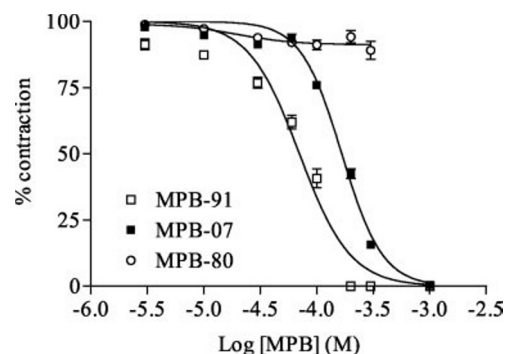


FIG. 9. Vasorelaxant effect of CFTR activators on rat aorta. Concentration-dependent curves are displayed, showing the vasorelaxation of aortic rings precontracted by 80 mM K^+ for MPB-07 ($IC_{50} = 166 \pm 1.05 \mu\text{M}$, $n = 16$) and MPB-91 ($IC_{50} = 70 \pm 1.12 \mu\text{M}$, $n = 8$) but not by MPB-80 ($n = 8$). Some error bars are smaller than the symbols.

and pharmacological specificity of benzo[c]quinolizinium (*i.e.* the different activity of MPB-80, MPB-07, and MPB-91) are conserved for CFTR in epithelia and SMC. Based on these findings, CFTR appears to be the major chloride channel activated by cAMP-dependent agonists, most probably via phosphorylation of the R domain of CFTR by PKA (14). The cAMP pathway is an important regulator of the vascular tone. In this regard, β -adrenergic receptors are located on vascular smooth muscle cells and mediate vasodilating effects of endogenous catecholamines (22, 25). The action of β -adrenergic receptors involves stimulation of adenylyl cyclase via interaction of the agonist-receptor complex with G_s and subsequent intracellular cAMP concentration increase (25). In addition, relaxation could be elicited by direct activation of adenylyl cyclase (22). This signal transduction pathway has also been described for CFTR-dependent secretion in epithelia (14). Furthermore, we also showed that the neuropeptide VIP, another potent endogenous vasodilator (4), activated CFTR in SMC. In SMC, VIP induces an increase in cAMP concentration and activates PKA with multiple effects on the sarcolemmal Ca^{2+} pump ATPase and on

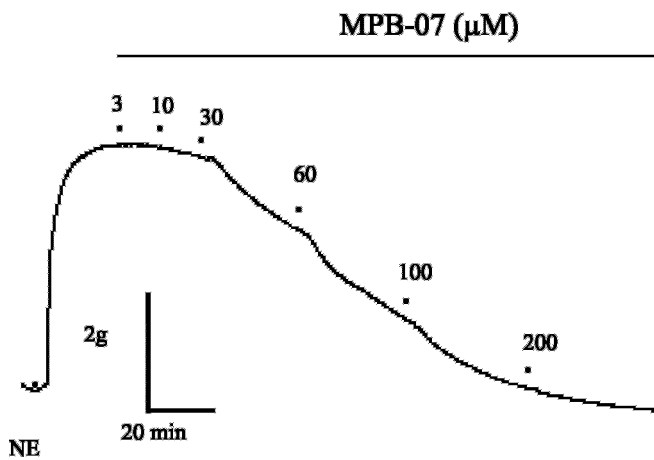


FIG. 10. Effect of MPB-07 on the tension of rat aorta precontracted by the α -adrenergic agonist norepinephrine. Shown is the original trace of an experiment performed with a denuded aortic ring precontracted by norepinephrine (NE at the bottom of the trace). The effect on tension of various concentrations of MPB-07 was examined. The corresponding IC_{50} value is $36 \pm 1.07 \mu M$ ($n = 6$).

the affinity of myosin light chain kinase for the Ca^{2+} -calmodulin complex (30). These effects produce smooth muscle relaxation and vasodilation. Therefore, CFTR activation might constitute at least in part a molecular entity by which VIP and β -adrenergic receptors relax smooth muscles (4).

The Activation of CFTR Occurs during Contraction of Smooth Muscle—We observed the stimulation of CFTR chloride channel activity by cAMP agonists, isoproterenol, or VIP when smooth muscle cells were in a depolarizing high potassium solution. Indeed, with SMC bathed in normal potassium solution, no evidence for active CFTR was observed. Because the Cl^- equilibrium potential in SMC is thought to be positive to the resting potential, between -30 and -40 mV (7, 8), activation of calcium-dependent chloride channels (or I_{ClCa}) during agonist stimulation would produce membrane depolarization, Ca^{2+} entry through voltage-dependent Ca^{2+} channels, and contraction. During contraction and upon stimulation of the intracellular cAMP pathway, since CFTR channel gating is voltage-independent (14), a substantial CFTR-dependent outward current (corresponding to an hyperpolarizing inward flow of anions) activated by isoproterenol, VIP, and other CFTR agonists would partly counter the depolarizing influence of the Ca^{2+} current, leading to attenuated contractile machinery and to relaxation. In support for that finding, cellular hyperpolarization is one major process that leads to muscle relaxation (1, 6, 7), and β -adrenergic stimulation by isoproterenol hyperpolarized and relaxed smooth muscle (25). We propose that CFTR in SMC controls the vascular tone by modulating the membrane potential in the activation window of voltage-dependent Ca^{2+} channels.

Cardiac Function and Hypertension in Cystic Fibrosis—Cystic fibrosis causes dramatic damage to the digestive system and lungs, but constant progress in medical therapy has resulted in improved survival, such that many CF patients are living well into adulthood. However, as the disease progresses, these patients develop disabling respiratory and nonrespiratory diseases and eventually respiratory failure, portal and pulmonary hypertension, and cor pulmonare. Cor pulmonare is defined as a hypertrophy of the right ventricle resulting from diseases affecting the function and/or structure of the lung (31). The pathophysiology of pulmonary hypertension in CF is thought to be related to progressive destruction of the lung parenchyma and pulmonary vasculature and to pulmonary vasoconstriction

secondary to hypoxemia (32–34). Vasoconstriction of the small- and medium-sized pulmonary arteries play an important role in the pathogenesis of pulmonary hypertension during the early stages of the disease (32). Although the underlying pathogenic mechanisms of pulmonary hypertension remain poorly understood, several hypothesis exist (32). Among them are pulmonary artery endothelial cell dysfunction, abnormal smooth muscle phenotype, abnormal expressions of ion channels, increased levels of vasoconstrictor mediators, and reduced production of vasodilators. Interestingly, early observations showed a decreased β -adrenergic responsiveness in patients with CF, indicating a defect in the cAMP-dependent pathway in vascular smooth muscle cells (35). After cardiorespiratory problems and transplantation complications, liver disease is the commonest cause of death, accounting for 2.3% of overall mortality (36). The incidence of CF-associated liver cirrhosis varies from 1.5 to 25% of adolescent and young adults, the most serious complication being portal hypertension, with the subsequent occurrence of variceal bleeding (36). The role of CFTR in these different processes is unknown. For example, although the cardiac variant of CFTR is one of the most studied cardiac chloride channel (11–14), its contribution to human cardiac electrophysiology is still debated and controversial (reviewed in Ref. 37). Cardiac abnormalities are infrequently associated with CF, and the cardiac manifestations are generally considered to occur secondary to pulmonary pathology.

The demonstration that CFTR is present in endothelium (15, 16) together with our present observations that CFTR is expressed in SMC may therefore help to explain the role of this ion channel in cardiovascular physiology. Further investigation will be required to understand the role of CFTR in cardiorespiratory complications such as pulmonary hypertension in CF. Our observations have therefore several important implications related to understanding how the vascular tone is physiologically regulated and what is the role of CFTR Cl^- channels in these processes.

Acknowledgments—We thank A. Gaillard for advice on immunostaining; C. Jouglu, N. Bizard, and B. Moreau for technical assistance; and P. Bois, J. Lenfant, and M. Joffre for advice and discussions.

REFERENCES

- Jackson, W. F. (2000) *Hypertension* **35**, 173–178
- Carl, A., Lee, H. K. & Sanders, K. M. (1996) *Am. J. Physiol.* **271**, C9–C34
- Woodrum, D. A. & Brophy, C. M. (2001) *Mol. Cell. Endocrinol.* **177**, 135–143
- Henning, R. J. & Sawmiller, D. R. (2001) *Cardiovasc Res.* **49**, 27–37
- Laburthe, M. & Couvineau, A. (2002) *Regul. Pept.* **108**, 165–173
- Chipperfield, A. R. & Harper, A. A. (2000) *Prog. Biophys. Mol. Biol.* **74**, 175–221
- Kitamura, K. & Yamazaki, J. (2001) *Jpn. J. Pharmacol.* **85**, 351–357
- Large, W. A. & Wang, Q. (1996) *Am. J. Physiol.* **271**, C435–C454
- Dick, G. M., Bradley, K. K., Horowitz, B., Hume, J. R. & Sanders, K. M. (1998) *Am. J. Physiol.* **275**, C940–C950
- Riordan, J. R., Rommens, J. M., Kerem, B.-S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsky, N., Chou, J.-L., Drumm, M. L., Iannuzzi, M. C., Collins F. S. & Tsui, L.-C. (1989) *Science* **245**, 1066–1073
- Gadsby, D. C. & Nairn, A. C. (1999) *Physiol. Rev.* **79**, S77–S107
- Levesque, P. C., Hart, P. J., Hume, J. R., Kenyon, J. L. & Horowitz, B. (1992) *Circ. Res.* **71**, 1002–1007
- Horowitz, B., Tsung, S. S., Hart, P., Levesque, P. C. & Hume, J. R. (1993) *Am. J. Physiol.* **264**, H2214–H2220
- Gadsby, D. C., Nagel, G. & Hwang, T. C. (1995) *Annu. Rev. Physiol.* **57**, 387–416
- Weyler, R. T., Yurko-Mauro, K. A., Rubenstein, R., Kollen, W. J., Reenstra, W., Altschuler, S. M., Egan, M. & Mulberg, A. E. (1999) *Am. J. Physiol.* **277**, C563–C571
- Tousson, A., Van Tine, B. A., Naren, A. P., Shaw, G. M. & Schwiebert, L. M. (1998) *Am. J. Physiol.* **275**, C1555–C1564
- Wei, L., Freichel, M., Jaspers, M., Cuppens, H., Cassiman, J. J., Droogmans, G., Flockerzi, V. & Nilius, B. (2001) *BMC Physiol.* **1**, 3
- Dormer, R. L., Dérand, R., McNeilly, C. M., Mettey, Y., Bulteau-Pignoux, L., Métayé, T., Vierfond, J. M., Gray, M. A., Galiotta, L. J., Morris, M. R., Pereira, M. M., Doull, I. J., Becq, F. & McPherson, M. A. (2001) *J. Cell Sci.* **114**, 4073–4081
- Dérand, R., Bulteau-Pignoux, L., Mettey, Y., Zegarra-Moran, O., Howell, L. D., Randak, C., Galiotta, L. J., Cohn, J. A., Norez, C., Romio, L., Vierfond, J. M., Joffre, M. & Becq, F. (2001) *Am. J. Physiol.* **281**, C1657–C1666
- Becq, F., Mettey, Y., Gray, M. A., Galiotta, L. J., Dormer, R. L., Merten, M.,

- Métayé, T., Chappe, V., Marvingt-Mounir, C., Zegarra-Moran, O., Tarran, R., Bulteau, L., Dérand, R., Pereira, M. M., McPherson, M. A., Rogier, C., Joffre, M., Argent, B. E., Sarrouilhe, D., Kammouni, W., Figarella, C., Verrier, B., Gola, M. & Vierfond, J. M. (1999) *J. Biol. Chem.* **274**, 27415–27425
21. Singh, A. K., Venglarik, C. J. & Bridges, R. J. (1995) *Kidney Int.* **48**, 985–993
22. Chruscinski, A., Brede, M. E., Meinel, L., Lohse, M. J., Kobilka, B. K. & Hein, L. (2001) *Mol. Pharmacol.* **60**, 955–962
23. Sheppard, D. N. & Welsh, M. J. (1993) *Ann. N. Y. Acad. Sci.* **707**, 275–284
24. Schultz, B. D., Singh, A. K., Devor, D. C. & Bridges, R. J. (1999) *Physiol. Rev.* **79**, 109–144
25. Kotlikoff, M. I. & Kamm, K. E. (1996) *Annu. Rev. Physiol.* **58**, 115–141
26. Becq, F., Hollande, E. & Gola, M. (1993) *Pflügers Arch.* **425**, 1–8
27. Ito, O., Naruse, S., Kitagawa, M., Ishiguro, H., Ko, S., Nakajima, M. & Hayakawa, T. (1998) *Regul. Pept.* **78**, 105–112
28. White, C. R., Elton, T. S., Shoemaker, R. L. & Brock, T. A. (1995) *Proc. Soc. Exp. Biol. Med.* **208**, 255–262
29. Marrero, M. B., Schieffer, B., Berstein, K. E. & Ling, B. N. (1996) *Clin. Exp. Pharmacol. Physiol.* **23**, 83–88
30. Murray, K. J. (1990) *Pharmacol. Ther.* **47**, 329–345
31. Bright-Thomas, R. J. & Webb, A. K. (2002) *J. R. Soc. Med.* **95**, 2–10
32. Salvi, S. (1999) *Chest* **115**, 1708–1719
33. Fraser, K. L., Tullis, D. E., Sasson, Z., Hyland, R. H., Thornley, K. S. & Hanly, P. J. (1999) *Chest* **115**, 1321–1328
34. Vizza, C. D., Lynch, J. P., Ochoa, L. L., Richardson, G. & Trulock, E. P. (1998) *Chest* **113**, 576–583
35. Eichler, H. G., Eichler, I., Lewiston, N., Blaschke, T. F. & Hoffman, B. B. (1989) *Clin. Sci. (Lond.)* **76**, 283–287
36. Diwakar, V., Pearson, L. & Beath, S. (2001) *Paediatr. Resp. Rev.* **2**, 340–349
37. Sorota, S. (1999) *Cardiovasc. Res.* **42**, 361–376