M2 Muscarinic Receptors Induce Airway Smooth Muscle Activation via a Dual, Gβγ-mediated Inhibition of Large Conductance Ca2+-activated K+ Channel Activity*

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Airway smooth muscle is richly endowed with muscarinic receptors of the M_2 and M_3 subtype. Stimulation of these receptors inhibits large conductance calcium-activated K+ (BK) channels, a negative feed back regulator, in a pertussis toxin-sensitive manner and thus facilitates contraction. The underlying mechanism, however, is unknown. We therefore studied the activity of bovine trachea BK channels in HEK293 cells expressing the M_2 or M_3 receptor (M_2R or M_3R). In M_2R- but not M_3R-expressing cells, maximal effective concentrations of carbamoylcholine (CCh) inhibited whole cell BK currents by 53%. This M_2R-induced inhibition was abolished by pertussis toxin treatment or overexpression of the Gβγ scavenger transducin-α. In inside-out patches, direct application of 300 nM purified Gβγ decreased channel open probability by 55%. The physical interaction of Gβγ with BK channels was confirmed by co-immunoprecipitation. Interestingly, inhibition of phospholipase C as well as protein kinase C activities also reversed the CCh effect but to a smaller (~20%) extent. Mouse tracheal cells responded similarly to CCh, purified Gβγ and phospholipase C/protein kinase C inhibition as M_2R-expressing HEK293 cells. Our results demonstrate that airway M_2Rs inhibit BK channels by a dual, Gβγ-mediated mechanism, a direct membrane-delimited interaction, and the activation of the phospholipase C/protein kinase C pathway.

The parasympathetic nerves provide the dominant autonomic control of airway smooth muscle (ASM).[3] The neurotransmitter acetylcholine exerts its effects by binding to muscarinic receptors of which five subtypes (M_1-M_5) have been identified. All are members of the heptahelical family of G protein-coupled receptors (1–3). On mammalian ASM, the M_2 (M_2R) and M_3 (M_3R) receptor subtypes are expressed with M_2R representing the larger population (4, 5). Additionally, M_4 receptors (M_4R) are present in rabbit small airways and parenchyma (4, 6), but their function remains unknown. M_3Rs in ASM are coupled to phospholipase C (PLC)/protein kinase C (PKC) pathway via pertussis toxin (PTX)-insensitive G proteins of the $G_{q/11}$ family. The contractile response evoked by M_3R stimulation is attributed to the formation of inositol trisphosphate (IP_3), the subsequent release of Ca^{2+} from intracellular stores, the additional influx of extracellular calcium, and the Ca^{2+}-sensitizing effect of PKC (7–11). Stimulation of M_2 muscarinic receptors (M_2Rs) in ASM inhibits adenylyl cyclase via activation of PTX-sensitive G proteins of the $G_{i/o}$ family (12, 13), and therefore M_2Rs are thought to counteract relaxation that requires activation of adenylyl cyclase, as for example the $β_2$-adrenoreceptor agonist-induced ASM relaxation (14). Recently, however, evidence has been provided that M_2Rs participate directly in ASM contraction. In mice with a targeted deletion of the M_2R, the muscarinic receptor agonist carbamoylcholine (CCh) induced less ASM contraction than in the wild-type littermates (15). In addition, in mice deleted of the M_3R contractile responses in ASM to CCh still occur (16). The mechanism by which the M_2Rs in ASM induce contraction is, however, still unknown. In isolated guinea pig tracheal strips, methacholine-induced contractions involved activation of PTX-sensitive $G_{i/o}$ proteins and required the activity of large conductance, Ca^{2+}-activated potassium channels (BK channels) (17). Because BK channels function as a rheostat, limiting Ca^{2+} influx through voltage-dependent Ca^{2+} channels by shifting the membrane potential to more inside-negative values (18, 19), it is conceivable that inhibition of BK channel activity favors contraction. By using the heterologous expression system of HEK293 cells and native tracheal cells, we show herein that Gβγ subunits released from $G_{i/o}$ proteins upon stimulation of M_2 muscarinic receptors inhibit BK channels by a dual mechanism. First, they directly bind to the BK channel pore-forming α subunit or an associated protein and inhibit the open probability. Second, they activate the PLC/PKC pathway, thereby contributing to PKC-dependent BK channel inhibition.

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[3] The abbreviations used are: ASM, airway smooth muscle; BK channel, large conductance Ca^{2+}-activated K+ channel; BKα, pore-forming α subunit of the BK channel; TSMC, tracheal smooth muscle cell; HEK, human embryonic kidney; TD, transducin; GIRK, G protein-gated inwardly rectifying K+ channel; CCh, carbamoylcholine; NA, noradrenaline; PTX, pertussis toxin; M_2R, M_2 receptor; M_3R, M_3 receptor; M_4R, M_4 receptor; PLC, phospholipase C; PKC, protein kinase C; IP_3, inositol trisphosphate; PMA, phorbol 12-myristate 13-acetate; PSS, physiological saline solution; AR, adrenoceptor.

EXPERIMENTAL PROCEDURES

Materials—Carbamoylcholine chloride and *dl*-noradrenaline hydrochloride were obtained from Sigma, and Ro 31-8220, Gö 6976, and staurosporine were from Merck. U-73122, U-73343, and PMA were purchased from Biomol (Hamburg, Germany); iberiotoxin was from Alomone Laboratories (Jerusalem, Israel); and PTX was from List Biological Laboratories (Campbell, CA). The drugs were either dissolved in physiological saline solution (PSS; see solutions) or in Me_2SO. The maximum 0.1% final concentration of Me_2SO in the bath solution did not affect BK currents. Solutions with iberiotoxin contained 0.1% bovine albumin fraction V (Sigma). Collagenase type H (lot 014K8605), hyaluronidase type I-S, papain, and 1,4-dithio-D,L-threitol were purchased from Sigma.

Purification of Transducin α Subunits and βγ Dimers—Bleached bovine rod outer segment membranes were prepared from bovine retinae as described previously (20). The bovine retinae were obtained from a local abattoir. Transducin (TD) was eluted from the membranes by hypotonic elution in the presence of 100 μM GTP, and the subunits were separated by affinity chromatography on Blue Sepharose (Bio-Rad). The buffers in which the proteins were eluted from the columns were changed to the intracellular pipette solution (see below) by repeated extensive dialysis, and the proteins were stored in aliquots at −80 °C. The protein concentration was determined according to Bradford with IgG as standard.

Cell Lines and Transfection Procedure—HEK293 cells stably transfected with the human M₂R, M₃R, and M₄R (Ref. 21; M₂R, 4.6; M₃R, 7.8; and M₄R, 2.1 pmol receptor/mg of membrane protein; kindly provided by Dr. D. Meyer zu Heringdorf, Essen, Germany) or the murine α_{2A}-adrenoreceptor (α_{2A}-AR) (Ref. 22; 23.1 pmol of receptor/mg of membrane protein; kindly provided by Dr. L. Hein, Freiburg, Germany) were transiently transfected with the BK channel α subunit (GenBank™ accession number AY033472) in pcDNA3 (23). Some α_{2A}-AR-expressing cells, and for comparison cells expressing the M₂R and M₄R, were transiently transfected with a bicistronic expression vector encoding for the G protein regulated inward rectifying potassium channel (GIRK) subunits GIRK1 and GIRK4 (Ref. 22; kindly provided by Dr. M. Bünemann, Würzburg, Germany). To evaluate G$\beta\gamma$-mediated effects by CCh, M₂R-expressing cells were transiently co-transfected with the BK channel α subunit and with bovine transducin α (TDα) subcloned into the pAdTrack-CMV vector. In some experiments, HEK293 cells were transiently co-transfected with the BK channel α subunit and either with the human M₃R in pcDNA3.1 (UMR cDNA Resource Center) or with the β_1 subunit of the BK channel cloned from bovine trachea in pcDNA3 (kindly provided by Dr. Maria Garcia, Department of Ion Channels, Merck Research Laboratories, Rahway, NJ). Stably transfected HEK293 cells were cultured in Dulbecco's modified Eagle's medium/Ham's medium (Biochrom, Berlin, Germany) containing 10% fetal calf serum, 100 units ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, and 500 μg ml⁻¹ G418. All other HEK293 cells were cultured in minimum essential medium supplemented with Earle's salts medium (Biochrom, Berlin, Germany) containing 10% fetal calf serum, 100 units ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomy-

cin. HEK293 cells were cultured for 24 h and then transiently transfected with the DNA plasmids by calcium phosphate precipitation for 18 h at 35 °C and 3% CO_2. After washing, the cells were cultivated for another 48–72 h at 37 °C and 6% CO_2. After the medium was exchanged several times with PSS (see below), the cells were transferred in a 35-mm dish to the stage of an inverted microscope (Zeiss Axiovert 200) for electrophysiological measurements. The transfection efficiency varied between 40 and 70%.

Animals and Cell Preparation—All of the experimental procedures were carried out according to the animal welfare guidelines of the University Medical Center Hamburg-Eppendorf. C57BL/6 mice of either sex were obtained from a colony bred and maintained at the animal house of the University Medical Center Hamburg-Eppendorf. The tracheae were dissected from mice killed by CO_2. After the connective tissue was removed, the tissue was cut with a sharp blade into pieces of ~1 mm side length and incubated under gentle agitation at 37 °C in Ca^{2+}-free PSS including 0.7 mg ml⁻¹ papain, 1 mg ml⁻¹ 1,4-dithio-D,L-threitol, and 1 mg ml⁻¹ fat-free bovine serum albumin. The tissue pieces were transferred 30 min later into PSS containing 50 μM Ca^{2+}, 1 mg ml⁻¹ collagenase, 1 mg ml⁻¹ hyaluronidase, and 1 mg ml⁻¹ albumin and digested for another 8–10 min at 37 °C. Single cells were released by gentle trituration of the digested tissue using a narrow-bore, fire-polished Pasteur pipette and stored in PSS at room temperature. After isolation, >50% of the cells were relaxed, and only these cells were used for the electrophysiological studies. The experiments were conducted within 6 h of cell isolation. A small aliquot of the solution containing the isolated cells was placed in an open perfusion chamber (1 ml) mounted on the stage of an inverted microscope (Zeiss Axiovert 200). The myocytes were allowed to adhere to the bottom of the chamber for 5–10 min and were then superfused at 2–3 ml min⁻¹ with PSS at room temperature.

Recording Techniques—Standard patch clamp recording techniques were used to measure currents in the cell-attached, inside-out, or whole cell patch clamp configuration (24). Patch electrodes were fabricated from borosilicate glass capillaries (MTW 150F; World Precision Instruments, Inc., Sarasota, FL) and filled with prefiltered solutions of different composition (see below). The currents were recorded at room temperature with an EPC-7 amplifier (HEKA Elektronik, Lambrecht, Germany), connected via a 16-bit A/D interface to a pentium IBM clone computer. The signals were low pass filtered (1 kHz) before 5 kHz digitization. Data acquisition and analysis were performed with an ISO-3 multitasking patch clamp program (MFK M. Friedrich, Niedernhausen, Germany). Pipette resistance ranged from 2 to 3 MΩ in whole cell experiments and from 8 to 9 MΩ in the excised patch experiments. The amplitude of single-channel currents was derived from an amplitude distribution histogram. Determination of the average channel open probability (NP_o) in patches has been described elsewhere (25). After the patch had been equilibrated for at least 5 min, the drugs were superfused for another 3–5 min before the mean NP_o was determined. A multi-barreled perfusion pipette placed 200 μm away from the patch was used to switch the superfusion

solution. The holding potential in the whole cell patch clamp experiments was -10 mV.

Solutions—The bath was superfused with PSS containing 127 mM NaCl, 5.9 mM KCl, 2.4 mM CaCl$_2$, 1.2 mM MgCl$_2$, 11 mM glucose, and 10 mM HEPES adjusted to pH 7.4 with NaOH. For whole cell experiments the pipette solution contained 126 mM KCl, 6 mM NaCl, 1.2 mM MgCl$_2$, 5 mM EGTA, 11 mM glucose, 3 mM dipotassium ATP, 0.1 mM Na$_3$GTP, and 10 mM HEPES adjusted to pH 7.4 with KOH. The free Ca^{2+} concentration was adjusted to 0.3 μM by adding the appropriate amount of CaCl$_2$ as described earlier (25). In some experiments the Ca^{2+} concentration was increased to 3 μM. Free Ca^{2+} was checked by fura 2-fluorescence. For inside-out experiments, the extracellular (pipette) solution contained 132 mM KCl, 6 mM NaCl, 1.2 mM MgCl$_2$, 5 mM EGTA, 11 mM glucose, 10 mM HEPES, pH 7.4, adjusted with KOH, and the bath solution (cytosolic surface of the patch) contained 126 mM KCl, 6 mM NaCl, 1.2 mM MgCl$_2$, 5 mM EGTA, 11 mM glucose, 3 mM dipotassium ATP, 0.1 mM Na$_3$GTP, and 10 mM HEPES, pH 7.4, adjusted with KOH. The free Ca^{2+} concentration was 0.3 μM, and in some experiments it was 1 μM. For the measurement of GIRK currents, whole cell recordings were used with a bath solution containing 120 mM NaCl, 20 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, pH 7.3, adjusted with NaOH. The internal (pipette) solution contained 100 mM potassium aspartate, 40 mM KCl, 5 mM NaCl, 2 mM MgCl$_2$, 5 mM MgATP, 0.1 mM Na$_3$GTP, 2 mM EGTA, and 10 mM HEPES adjusted to pH 7.3 with KOH.

Co-immunoprecipitation—HEK293 cells were seeded in 10-cm culture dishes and transfected with 4 μg of pcDNA3.2-BK channel α subunit, 4 μg of pcDNA6/His-Gβ_1IRESγ_2 (kind gift of Dr. H. J. Hippe, Heidelberg, Germany), or a combination of both and up to 8 μg of an empty pcDNA control vector. Forty-eight hours after transfection, the cells were lysed with 1000 μl of immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride) and homogenized for 30 s. After centrifugation (26,000 $\times$ g for 10 min at 4 °C), 40 μl of a 1:1 slurry of protein A-Sepharose (GE Healthcare) was added for preclearing the lysates. The lysates were separated from protein A-Sepharose, and then 4 μg of anti-His$_6$-antibody (Qiagen) was added to each sample and incubated for 30 min on ice. After the addition of 40 μl equilibrated protein A-Sepharose, the samples were gently shaken for 4 h at 4 °C. Then the Sepharose beads were washed three times with immunoprecipitation buffer, and the bound proteins were eluted with 25 μl of sample buffer for 5 min at 95 °C. The precipitated proteins were analyzed by immunoblot with an anti-Gβ_{com} (1:1000 dilution; Santa Cruz Biotechnology) or an anti-BK channel α subunit antibody (26) (1:500 dilution).

Data Analysis—SigmaPlot for windows (Jandel Scientific, version 8) was used for statistical analyses. Significance was determined by paired or unpaired t test or by one-way analysis of variance. When a significant effect was detected with analysis of variance, Student's t test was used for pairwise comparisons. The effects were deemed significant when a $p < 0.05$ was obtained. The results are expressed as the means $\pm$ S.E. where applicable.
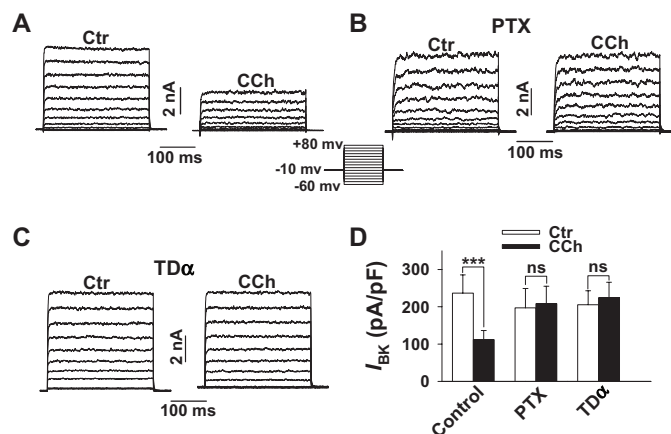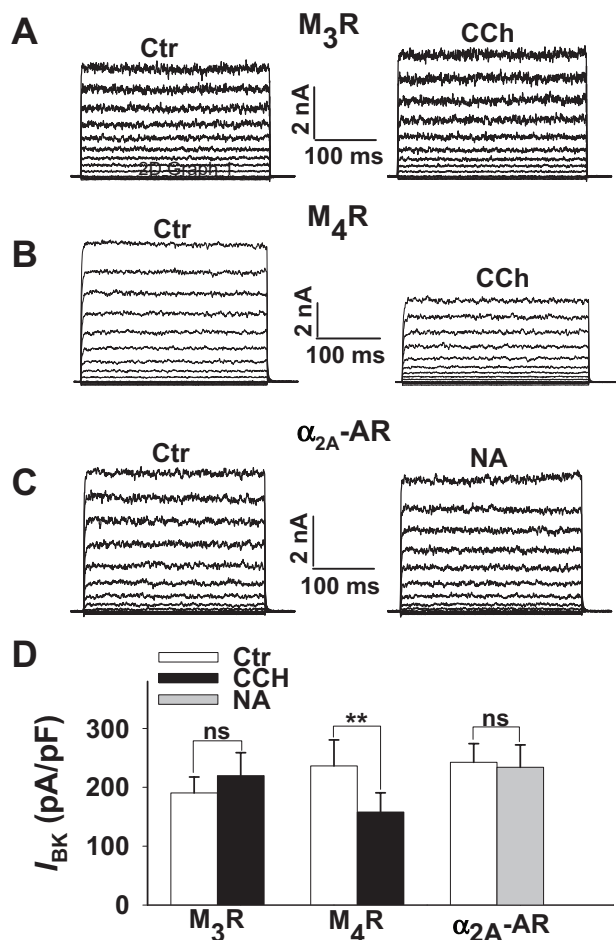

FIGURE 1. **Inhibition of BK channel currents by CCh in HEK293 cells stably expressing the M$_2$R and transiently transfected with the pore-forming BK channel α subunit.** Whole cell BK outward currents (I_{BK}) were elicited from a holding potential of -10 mV by depolarizing the cells every 5 s for 300 ms from -60 to $+80$ mV in 10-mV increments. *A*, original current traces before and after application of 10 μM CCh. *B* and *C*, abolition of CCh-induced inhibition of I_{BK} after pretreatment of the cells with PTX (*B*) or after co-transfection with TDα (*C*). *D*, summary of the effects of CCh on current densities of I_{BK} at a membrane potential of $+80$ mV. The *bars* represent the means $\pm$ S.E. of 22 cells (control, *Ctr*) and 7 cells (PTX and TDα), respectively. The pipette solution contained 0.3 μM Ca^{2+}. ***, $p < 0.001$ *versus* control; *ns*, not significant.

RESULTS

G$\beta\gamma$ Subunits Mediate BK Channel Inhibition by M$_2$ and M$_4$ Receptor Activation—A PTX-sensitive inhibitory effect of muscarinic receptor stimulation on BK channel activity has been demonstrated in ASM cells at the single channel level (27, 28). When we elicited whole cell outward currents (I_{out}) in smooth muscle cells freshly isolated from the mouse trachea (TSMCs) from a holding potential of -10 mV by depolarizing the cells every 5 s for 300 ms from -60 to $+80$ mV, addition of the specific BK channel inhibiting peptide iberiotoxin (300 nM) revealed that more than 90% of I_{out} was generated by BK channels (data not shown). The addition of a maximal effective concentration of CCh (10 μM) decreased current densities at all voltages by $\sim$50% (current densities at $+80$ mV: control, 135.5 $\pm$ 9.2; CCh, 66.7 $\pm$ 10.7 pA pF^{-1}, $p < 0.01$, $n = 8$). In accordance with the data reported by Kume *et al.* (27, 28), the inhibitory effect of CCh was completely abolished when TSMCs were exposed to PTX 5 h prior to CCh application. To dissect the effects induced via M$_2$Rs and M$_3$Rs, we transiently expressed the pore-forming BK channel α subunits (KCNMA1) cloned from bovine trachea in HEK293 cells stably expressing the human M$_2$R (21). Whole cell outward currents were then elicited as described above (Fig. 1*A*). In HEK293 cells not expressing BK channels, a negligible endogenous outward current was obtained which amounted to less than 2% (at $+80$ mV) of the current elicited in transfected cells (data not shown). We therefore consider outward currents in transfected HEK293 cells exclusively as BK currents (I_{BK}). As observed in TSMCs, 10 μM CCh suppressed I_{BK} at all voltages by $\sim$50% (Fig. 1, *A* and *D*), and this inhibition was completely abolished when HEK293 cells were exposed to 500 ng ml^{-1} PTX 5 h prior to CCh application (Fig. 1, *B* and *D*). Because this finding indicates that G$_{i/o}$ proteins also mediate the CCh-induced inhibition of BK chan-

FIGURE 2. **Stimulation of M₄Rs but not of M₃Rs and α_{2A}-ARs are linked to BK channel inhibition.** Whole cell BK outward currents (I_{BK}) were elicited in HEK293 cells transfected with either the M₃R (*A*), the M₄R (*B*), or the α_{2A}-AR (*C*) and the BK channel α subunit. *A–C*, original current traces from three representative cells depolarized every 5 s for 300 ms from −60 to +80 mV in 10-mV increments. Holding potential was −10 mV. Currents before (control) and in the presence of 10 μM CCh or 10 μM NA are shown. *D*, summary of the effects of CCh and NA on current densities of I_{BK} at a membrane potential of +80 mV. The *bars* represent mean values ± S.E. of 6 (M₃R), 8 (M₄R), and 10 (α_{2A}-AR) cells. The pipette solution contained 0.3 μM Ca^{2+}. **, $p < 0.01$ *versus* control (*Ctr*); *ns*, not significant.

nel currents in HEK293 cells, we set up experiments to characterize which subunit of $G_{i/o}$ is responsible for channel inhibition. We therefore co-expressed the BK channel α subunit and TDα, the Gα subunit of the heterotrimeric G protein that mediates signal transduction in vertebrate photoreceptor cells. TDα has been shown to act as a scavenger of G$\beta\gamma$ dimers dissociated from heterotrimeric $G_{i/o}$ proteins (29) and thus specifically antagonizes G$\beta\gamma$-mediated effects, whereas G$\alpha_{i/o}$ actions are preserved (30, 31). As shown in Fig. 1 (*C* and *D*), CCh completely lost its inhibitory effect on BK channel current in TDα-overexpressing cells. When HEK293 cells expressing the M₃R and the BK channel α subunit were exposed to 10 μM CCh by utilizing the same experimental protocol, no steady-state inhibition of I_{BK} was obtained (Fig. 2*A*). Together the findings indicate that the $G_{i/o}$-coupled M₂R, but not the preferentially $G_{q/11}$-coupled M₃R (32), is linked to BK channel inhibition via G$\beta\gamma$ dimers. We therefore tested whether the second $G_{i/o}$-coupled muscarinic receptor, *i.e.* the M₄R, or stimulation of $G_{i/o}$-
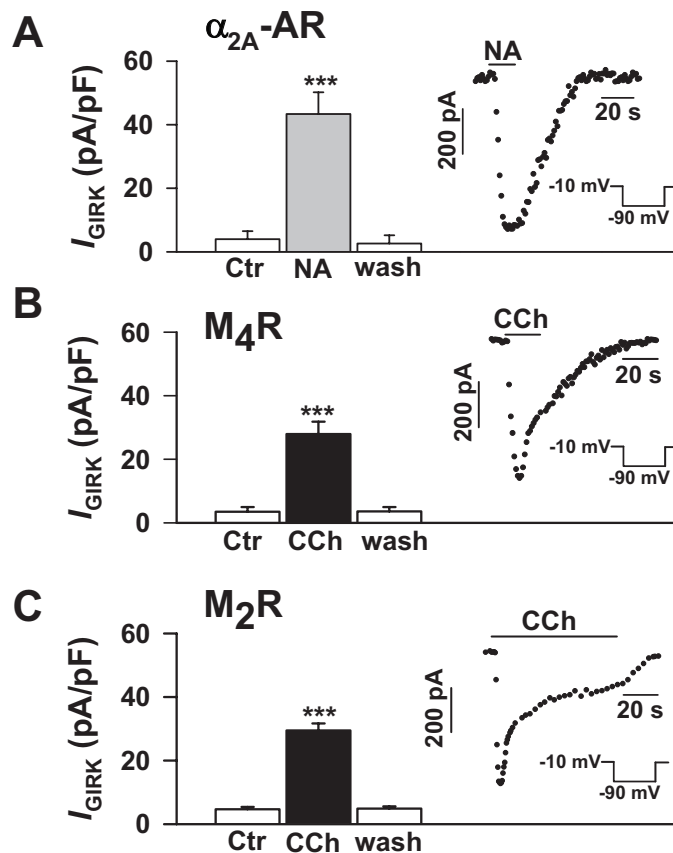
FIGURE 3. **Agonist-induced GIRK currents in HEK293 cells stably expressing the M₂R, M₄R, and α_{2A}-AR.** The cells were transiently transfected with the G protein-gated inwardly rectifying K$^+$ channel subunits GIRK1 and GIRK4. GIRK currents were elicited in the whole cell configuration by 300-ms hyperpolarizing pulses from a holding potential of −10 to −90 mV (20 mM external potassium). Note that the currents (I_{GIRK}) are inward currents. GIRK current densities are strongly enhanced by 10 μM NA in the α_{2A}-AR-expressing cells (*A*; $n = 6$), and by 10 μM CCh in the M₄R-expressing (*B*; $n = 10$) and M₂R-expressing cells (*C*; $n = 7$). *Insets*, time course of agonist-induced GIRK currents. ***, $p < 0.001$ *versus* control (*Ctr*).

coupled receptors of different subfamilies can also inhibit steady-state I_{BK}. Whole cell patch clamp experiments in M₄R- or α_{2A}-AR-expressing cells revealed that application of 10 μM CCh to the M₄R-expressing cells resulted in a significant, approximately 35% decrease of the BK channel current, whereas noradrenaline (10 μM) was ineffective in α_{2A}-AR-expressing cells (Fig. 2, *B–D*). To confirm that α_{2A}-ARs expressed in HEK293 cells effectively couple to $G_{i/o}$ proteins, we transiently transfected these cells and, for comparison, also the cells expressing M₂R and M₄R, with the G protein-gated inwardly rectifying K$^+$ channel subunits GIRK1 and GIRK4. Because functional GIRK channels are activated by G$\beta\gamma$ dimers (33), their activation indicates the agonist-induced release of G$\beta\gamma$ from $G_{i/o}$. Agonist-activated currents were measured as inwardly directed K$^+$ currents generated in the whole cell configuration by 300-ms hyperpolarizing pulses from a holding potential of −10 to −90 mV (20 mM external K$^+$). Application of 10 μM noradrenaline and 10 μM CCh induced large currents in the cells expressing the α_{2A}-AR or one of the two muscarinic receptors, respectively (Fig. 3). Interestingly, stimulation of α_{2A}-ARs with noradrenaline generated a significantly larger GIRK current than CCh in M₄R- or M₂R-expressing cells.
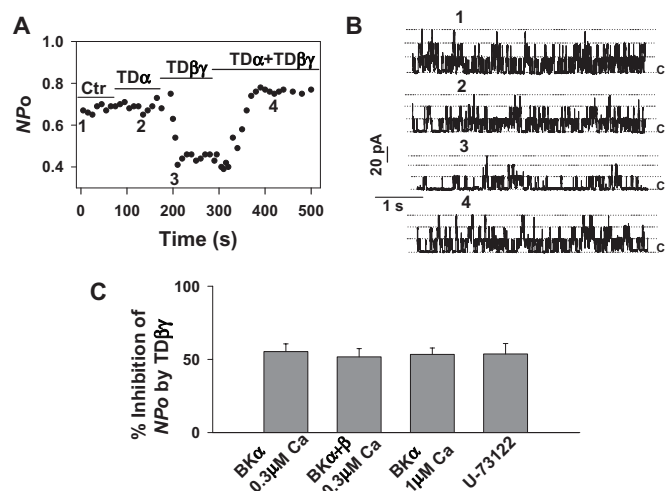
FIGURE 4. **Direct inhibition of BK channel open probability (NP_o) in HEK293 cells by TD$\beta\gamma$.** Single channel NP_o of BK channels was recorded in inside-out patches from HEK293 cells transiently transfected with the BK channel α subunit. Holding potential was +60 mV, and the intracellular (bath) Ca^{2+} concentration was 0.3 μM. *A*, NP_o as influenced by the sequential super-fusion of the cytosolic surface of a single patch with 1 μM TDα and 300 nM TD$\beta\gamma$ alone and in combination. *B*, traces show representative single channel current recordings taken at the times indicated by the corresponding numbers in *A*. The channel openings are upward deflections. The *dashed lines* represent numbers of channels in the open state, and *c* indicates the closed state level. *C*, the inhibitory effect of TD$\beta\gamma$ on NP_o is not influenced by raising the intracellular (bath) Ca^{2+} concentration from 0.3 to 1 μM, by co-transfecting the BK channel α subunit with the β_1 subunit, or by inhibition of PLCβ with 2.5 μM U-73122. The *bars* represent the mean values ± S.E., $n = 15$ (BKα 0.3 μM Ca^{2+}), $n = 6$ (BKα 1 μM Ca^{2+}), $n = 9$ (BK$\alpha + \beta$ 0.3 μM Ca^{2+}), and $n = 7$ (U-73122). *Ctr*, control.

Taken together, the results show that all three $G_{i/o}$-coupled receptors effectively release $G\beta\gamma$ subunits, but only $G\beta\gamma$ released by activation of $G_{i/o}$-coupled muscarinic receptors induces BK channel inhibition. $G\beta\gamma$ dimers regulate the activity of some ion channels such as the GIRK and neuronal voltage-dependent Ca^{2+} channels by direct interaction (34), whereas other effects require stimulation of $G\beta\gamma$-dependent effector enzymes like PLCβ isoforms (35, 36) and the subsequent activation of PKC. We therefore set up experiments to analyze the possible contribution of direct and indirect $G\beta\gamma$-mediated effects to BK channel inhibition.

$G\beta\gamma$ Dimers Directly Inhibit BK Channels—To study the possibility that $G\beta\gamma$ dimers directly inhibit BK channel activity, experiments on inside-out patches from HEK293 cells expressing BK channel α subunits were carried out. In the presence of symmetrically high potassium (140 mM) and at a holding potential of +60 mV, the cytosolic surface of an inside-out patch was sequentially superfused with purified G protein subunits either alone or in combination (Fig. 4, *A* and *B*). When 1 μM TDα was applied first as a negative control, channel open probability (NP_o) did not change. Instead, NP_o rapidly decreased from 0.67 to 0.39 when the superfusion solution contained 300 nM TD$\beta\gamma$. The effect of $G\beta\gamma$ was restricted to BK channel open probability, and the single channel conductance remained unchanged. The inhibitory effect of TD$\beta\gamma$ was stable as long as the G protein was present but returned to the control level (0.74) when 1 μM TDα as scavenger was additionally applied. When the effect of 300 nM TD$\beta\gamma$ alone was evaluated in 15 patches, a decrease of NP_o by 55.3 ± 5.3% was obtained (Fig. 4*C*). This decrease was
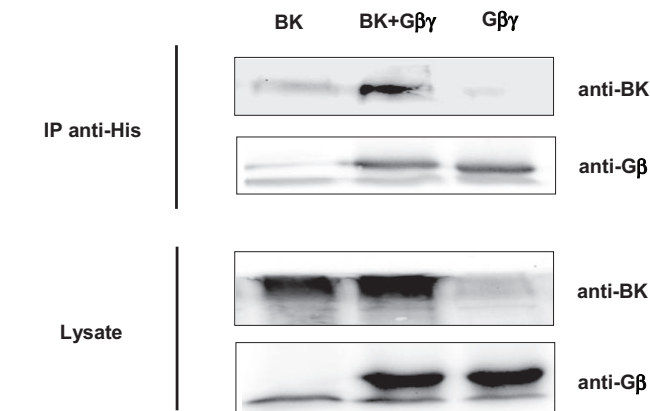

FIGURE 5. **Binding of G$\beta\gamma$ subunits to the BK channel α subunits in HEK293 cells.** HEK293 cells were transfected with plasmids encoding the BK channel α subunit, GHis$\beta_1\gamma_2$ (4 μg of DNA each), and up to 8 μg of an empty pcDNA control vector. After cell lysis, the expressed GHis$\beta_1\gamma_2$ was precipitated with an anti-His antibody and analyzed by immunoblot with a polyclonal anti-Gβ antibody. Co-precipitated BK channel α subunits were detected with a polyclonal anti-BKα antibody. Equal expression of GHis$\beta_1\gamma_2$ and BK channel α subunit constructs in the cell lysates was verified. *IP*, immunoprecipitation.

not significantly altered by increasing the Ca^{2+} concentration of the intracellular (bath) solution to 1 μM (inhibition by 51.7 ± 5.7%, $n = 6$) or by expressing the regulatory β_1- subunit of the BK channel together with BKα (inhibition by TD$\beta\gamma$: 53.4 ± 4.4%, $n = 9$). The experiments presented so far, do not exclude the possibility that PLCβ isozyme(s) sensitive to activation by $G\beta\gamma$ dimers and present in inside-out patches may have mediated TD$\beta\gamma$-induced inhibition of NP_o. Inside-out patches were therefore superfused for 10 min with the PLC inhibitor U-73122 (2.5 μM) first and then additionally with 300 nM TD$\beta\gamma$. The resulting inhibition of NP_o by 53.7 ± 7.1% ($n = 7$) was not significantly different from the inhibition in the absence of the PLC inhibitor (Fig. 4*C*). U-73122 by itself had no significant effect on NP_o (data not shown). Together the results indicate that TD$\beta\gamma$ inhibits BK channels in inside-out patches independent of the intracellular Ca^{2+} concentration, the additional expression of the regulatory β_1 subunit, or an activation of PLCβ isozymes. We thus determined whether free $G\beta\gamma$ dimers physically associate with BK channel α subunits in an intact cellular context by co-immunoprecipitation. Cell lysates from transiently transfected HEK293 cells expressing either recombinant BK channel α subunits, His-tagged $G\beta_1\gamma_2$ (GHis$\beta_1\gamma_2$), or their combination, were used for immunoprecipitation with an anti-His tag antibody. BKα was co-precipitated only from lysates of cells co-expressing GHis$\beta_1\gamma_2$ and BKα (Fig. 5). In summary, the results obtained in inside-out patches and by co-immunoprecipitation suggest that the interaction of $G\beta\gamma$ dimers with BKα or an as yet unidentified adapter protein(s) reduces BK channel open probability.

The M_2R-induced Activation of the PLC/PKC Contributes to the Inhibition of BK Channels—It is well known that M_2Rs couple to the PLC/PKC pathway by $G_{i/o}$-mediated stimulation of $G\beta\gamma$-sensitive PLCβ isoforms (35–37) of which the β_3 isoform is present in HEK293 cells.[4] Although the inhibition by directly applied $G\beta\gamma$ dimers on NP_o was quantitatively similar to the

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[4] M. Schmidt, personal communication.
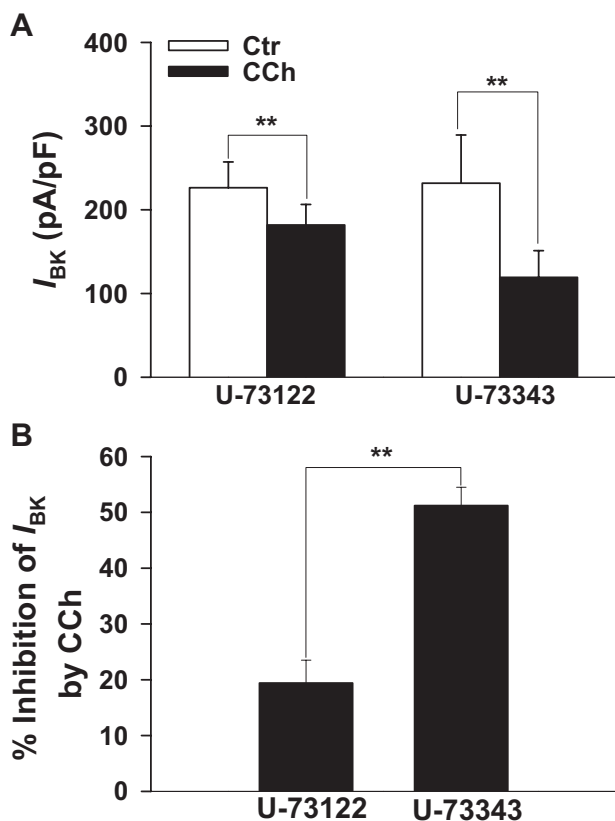
FIGURE 6. **Activation of PLC partially contributes to the CCh-induced inhibition of BK currents in transfected HEK293.** Whole cell BK currents (I_{BK}) were elicited as described. Inhibition of I_{BK} by 10 μM CCh is attenuated by the PLC inhibitor U-73122 (2.5 μM) but remains unopposed in the presence of the inactive analog U-73343 (2.5 μM). *A, bars* represent the mean current densities of cells exposed to CCh in the presence of U-73122 ($n = 9$) or U-73343 ($n = 6$). *B,* same data as in *A* but presented as the percentage of inhibition of current densities. The pipette solution contained 0.3 μM Ca²⁺. **, $p < 0.01$. *Ctr,* control.


FIGURE 7. **Activation of PKC contributes to the CCh-induced inhibition of BK currents in HEK293 cells stably expressing the M₂R.** Whole cell BK currents (I_{BK}) were elicited as described. *A,* attenuation of the CCh-induced inhibition (as a percentage) of I_{BK} by the specific PKC inhibitors Ro 31-8220 (1 μM) and Gö 6976 (1 μM) and by the broad spectrum protein kinase inhibitor staurosporine (0.1 μM). The *bars* represent the mean values ± S.E. of 22 cells (control (*Ctr*), same cells as in Fig. 1*D*), 8 (Ro 31-8220), 7 (Gö 6976), and 10 (staurosporine) cells. *B,* the CCh-induced I_{BK} inhibition is enhanced by increasing the Ca²⁺ concentration in the pipette filling solution from 0.3 to 3 μM. The *bars* represent the mean values ± S.E. of 22 cells (0.3 μM Ca²⁺; same cells as control in *A*) and 8 cells (3 μM Ca²⁺). The cells in *A* and *B* were clamped from -10 to $+80$ mV. *, $p < 0.05$; **, $p < 0.01$. *C* and *D,* effects of 100 nM phorbol 12-myristate, 13-acetate (PMA) on current-voltage relationships of I_{BK} in the absence (*C*) and in the presence of the PKC inhibitor Gö 6976 (1 μM; *D*) are shown. The mean current densities of six cells, respectively, are plotted in *C* and *D* against the respective test potential. Note that Gö 6976 has no effect on I_{BK} of its own. Whole cell currents were evoked by applying a 300-ms depolarizing pulse every 5 s in 10-mV increments from a holding potential of -10 mV. The *insets* in *C* and *D* show representative I_{BK} recordings elicited by 300-ms pulses from a holding potential of -10 to $+80$ mV. The pipette solution contained 0.3 μM Ca²⁺ if not indicated otherwise.

G$\beta\gamma$-mediated inhibition of steady-state BK channel currents (compare Figs. 1*D* and 4*C*) and was unaffected by the PLC inhibitor U-73122 (Fig. 4*C*), this does not exclude the possibility that a PTX-sensitive and G$\beta\gamma$-mediated coupling of the M₂R to the PLC/PKC pathway contributes to the effect of CCh on the steady-state currents in the whole cell configuration. We therefore exposed M₂R-expressing cells to U-73122 or its inactive analog U-73343 and measured whole cell current densities. As shown in Fig. 6, the CCh-induced inhibition at $+80$ mV in U-73343-treated cells (51.2 ± 3.3%, $n = 6$) was not significantly different from inhibition in control cells (Fig. 1). In the presence of U-73122, however, the CCh-induced inhibition was significantly attenuated to 19.4 ± 4.1% ($n = 9$). U-73122 alone induced no direct inhibitory effect on the BK current (data not shown). To examine a possible contribution of the PLC-induced PKC activation to CCh-induced BK channel inhibition, we measured I_{BK} in transfected HEK293 cells exposed to the PKC inhibitors Ro 31-8220 and Gö 6976 and the broad spectrum protein kinase inhibitor staurosporine (Fig. 7*A*). At $+80$ mV, 10 μM CCh inhibited BK channel currents in the presence of 1 μM Ro 31-8220, 1 μM Gö 6976 and 0.1 μM staurosporine by 26.3 ± 3.6% ($n = 8$) 22.7 ± 3.1% ($n = 7$), and 22.6 ± 3.4% ($n = 10$), respectively. Because Gö-6976 selectively inhibits Ca²⁺-

dependent PKC isozymes, and the inhibitors with the broader spectrum did not significantly differ in their efficacy to diminish CCh-induced inhibition, a contribution of protein kinases other than Ca²⁺-dependent PKCs seemed unlikely. To further confirm the role of Ca²⁺-activated PKCs, experiments were performed in HEK293 cells exposed to 10-fold higher Ca²⁺ concentrations in the pipette filling solution or to the PKC activator PMA. Whereas the CCh-induced inhibition of I_{BK} at $+80$ mV was 52.3% in control cells (0.3 μM Ca²⁺), the inhibition significantly increased to 77.8 ± 2.8% ($n = 8$) when the pipette solution contained 3 μM Ca²⁺ (Fig. 7*B*). PMA (100 nM) decreased BK channel currents at all potentials (Fig. 7*C*, 44.7 ± 6.3%, $n = 6$, at $+80$ mV). This inhibitory effect of PMA was completely abolished by 1 μM Gö 6976 (Fig. 7*D*) or 1 μM Ro 31-8220 (data not shown). Taken together, the findings indicate that stimulation of M₂Rs inhibits BK channel currents in HEK293 cells by a dual mechanism that in part requires activation of the G$\beta\gamma$-PLCβ-PKC signaling pathway as well as an interaction of G$\beta\gamma$ with the BK channel complex.

CCh-induced Inhibition of BK Channels in Tracheal Smooth Muscle Cells—To verify that the signal transduction pathways disclosed by the experiments reported above do not only occur in a heterologous expression system but also under physiological conditions in ASM, we tested the influence of PTX, U-73122, Gö 6976, and Ro 31-8220 on CCh-induced inhibition
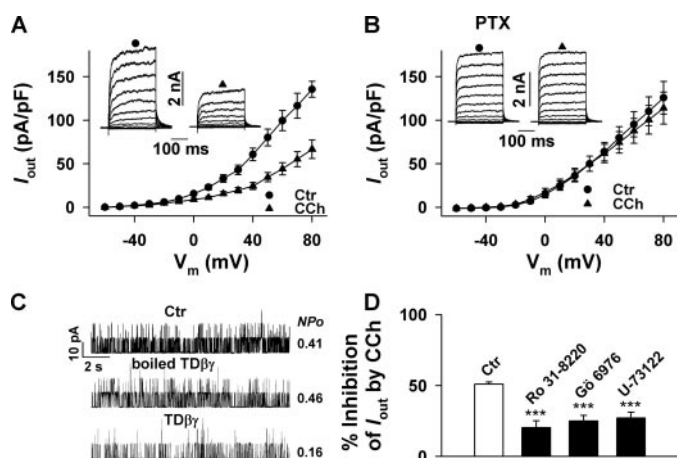
FIGURE 8. **The PTX-sensitive, CCh-induced inhibition of BK channels in mouse tracheal smooth muscle cells involves direct interaction of Gβγ with BK channels and activation of the PLC/PKC pathway.** *A* and *B*, whole cell outward currents (I_{out}) were elicited from a holding potential of -10 mV by depolarizing the cells every 5 s for 300 ms from -60 to $+80$ mV in 10-mV increments. *A*, current-voltage relationships of I_{out} before (control, *Ctr*) and after the application of 10 μM CCh are shown (10 cells from five mice). *B*, abolition of the CCh-induced inhibition of I_{out} after pretreatment of the cells with PTX (15 cells from eight mice). *Insets* in *A* and *B*, representative original current recordings before and in the presence of CCh. *C*, direct inhibition of BK channel open probability (NP_{o}) by TDβγ. Single channel NP_{o} of BK channels was recorded in inside-out patches. Holding potential was $+80$ mV, and the intracellular (bath) Ca^{2+} concentration was 0.3 μM. TDβγ (300 nM) before and after heat denaturation was sequentially applied to the cytosolic surface of a single patch. *Ctr*, control trace. The *traces* show representative single channel current recordings. Channel openings are upward deflections. *D*, attenuation of the CCh-induced inhibition (in percent) of I_{out} by the specific inhibitors U-73122 (2.5 μM), Ro 31-8220 (1 μM), and Gö 6976 (1 μM). The *bars* represent the mean values $\pm$ S.E. of cells in the absence (*Ctr*; 10 cells from four mice) and in the presence of either U-73122 (6 cells from two mice), Ro-31-8220 (6 cells from three mice), or Gö 6976 (7 cells from three mice). The cells were clamped from a holding potential of -10 to $+80$ mV. The pipette solution contained 0.3 μM Ca^{2+}. ***, $p < 0.001$ *versus* control (*Ctr*).

of I_{out} in TSMCs (Fig. 8). As already mentioned, PTX treatment completely abolished the CCh-induced inhibition (51.0 $\pm$ 1.6%, $n = 8$, at $+80$ mV) at all potentials. Application of the PKC inhibitors Ro 31-8220 and Gö 6976 (1 μM each) or the PLC inhibitor U-73122 (2.5 μM) for 10 min did not significantly affect I_{out}. When CCh was added thereafter, inhibition of current densities was significantly smaller in the presence of Gö 6976 (20.4 $\pm$ 4.8%; $n = 6$), Ro 31-8220 (25.0 $\pm$ 4.0%; $n = 7$), and U-73122 (27.1 $\pm$ 4.2%; $n = 6$) than in control cells, resembling partial inhibition in M₂R-expressing HEK293 cells. Importantly, direct application of TDβγ to TSMC in the inside-out configuration inhibited single channel NP_{o} to a similar ($\sim$50%) extent as in transfected HEK293 cells (Fig. 8C). This inhibition was not significantly affected by prior pretreatment with the PLC inhibitor U-73122 (data not shown). These results clearly demonstrate that the inhibitory coupling between muscarinic receptors and BK channels is highly similar in specifically M₂R-expressing HEK293 cells and freshly isolated TSMCs.

DISCUSSION

The results presented in this study demonstrate, for the first time, that Gβγ dimers released from $G_{i/o}$ proteins upon activation of M₂Rs are capable of inhibiting BK channel activity by two different mechanisms. First, they directly interact with the BK channel α subunit or an associated protein and reduce

the channel open probability NP_{o}. Second, they activate Ca^{2+}-dependent PKCs via the PLCβ pathway that additionally contribute to the inhibition of channel activity. We obtained evidence that this dual mechanism occurs similarly in native smooth muscle cells isolated from mouse trachea and in HEK293 cells expressing recombinant receptors together with the pore-forming BK channel α subunits that, after forming tetrameric channels, retain their characteristic biophysical properties and are functionally responsive to various protein kinases (23, 38, 39). Therefore our data indicate that this regulation is a more general phenomenon, and the sensitivity of the M₂R-induced inhibition of BK channel activity to PTX (40) and TDα expression (29) in transfected HEK293 cells demonstrates that the presence of the G protein-coupled receptor and the BK channel α subunit is sufficient to establish this regulation in a background containing $G_{i/o}$ proteins, Gβγ-activated PLCβ isoforms, and Ca^{2+}-activated PKCs.

The evidence for a direct membrane-delimited inhibition of BK channel activity by Gβγ dimers is based on experiments in cell-free inside-out patches from transfected HEK293 cells as well as mouse TSMCs exposed to TDα, TDβγ, or their combination. The results unambiguously demonstrate that TDβγ but not TDα inhibits BK channel open probability by 55%, and this action can be reversed by formation of the inactive heterotrimer. The TDβγ-induced inhibition was not influenced by varying the intracellular (bath) Ca^{2+} concentration, by inhibition of PLCβ, or by co-expressing the α subunit of the BK channel with the auxiliary β_1 subunit (KCNMB1) whose presence in the channel complex confers an increased voltage- and calcium sensitivity toward the pore-forming α subunit (41). These findings indicate that in cell-free inside-out patches, the activation of Ca^{2+}-activated PKC isoforms does not contribute to channel inhibition. Together with the co-immunoprecipitation experiments, the data clearly argue for a mechanism whereby channel inhibition by Gβγ occurs through direct interaction with the α subunit of the BK channel or a closely associated intermediary. BK channels therefore join the group of directly Gβγ-modulated ion channels that so far consist of G protein-coupled inwardly rectifying K^+ channels (GIRK or Kir3) (33), and neuronal voltage-dependent Ca^{2+} channels that give rise to P/Q-, N-, and R-type currents (Ca_v2) (34).

Interestingly, in both of these ion channels, PKC-dependent phosphorylation contributes to the complex regulation (42, 43). There are several reports, mainly on arterial smooth muscle cells (44–46), but also on rat pituitary tumor cells (47), indicating that PKC inhibits BK channels (48). The inhibition likely occurs by phosphorylation of Ser/Thr residues. Several putative PKC phosphorylation sites have been identified within the COOH-terminal "tail" of the BK channel α subunit (23, 49). In the present study, we found that the two PKC inhibitors Ro 31-8220 and Gö 6976 (50) significantly attenuated the CCh-induced BK channel inhibition. In addition, the PKC activator PMA inhibited BK channel activity in transfected HEK293 cells by $\sim$50%, and this effect was completely eliminated by Ro 31-8220 and Gö 6976. Ro 31-8220 is an unspecific blocker of many PKC isozymes, whereas Gö 6976 preferentially inhibits Ca^{2+}-dependent PKCs (50, 51). Because both PKC inhibitors were equally effective, and CCh-induced BK channel inhibition

increased (Fig. 7*B*) when the Ca^{2+} concentration was raised, Ca^{2+}-dependent PKCs are likely involved. Furthermore, the finding that staurosporine, a broad spectrum inhibitor of several protein kinases including PKC, produced the same inhibitory effect as the specific PKC inhibitors likely excludes the additional contribution of other protein kinases. It is well established that the M$_2$R can stimulate the activity of G$\beta\gamma$-sensitive PLCβ isoforms by activation of G$_{i/o}$ proteins (35–37). Because activation of PLCβ and the resulting production of the endogenous PKC activator diacylglycerol and Ca^{2+}-releasing IP$_3$ are thought to be the major source of activation of Ca^{2+}-dependent PKCs, it was not surprising that the PLC inhibitor U-73122 blunted the CCh-induced inhibition of BK channel activity to a similar extent as inhibition of PKCs. In line with this interpretation, the functional coupling of M$_2$Rs to PLC-IP$_3$-induced Ca^{2+} release via G$_{i/o}$ proteins has been clearly demonstrated by CCh-induced Ca^{2+} transients in transfected and fura-2-loaded Chinese hamster ovary cells. In contrast to M$_3$R-expressing Chinese hamster ovary cells, these CCh-evoked Ca^{2+} transients in M$_2$R-expressing cells were sensitive to PTX (52). Taken together, our results show a contribution of the M$_2$R-G$_{i/o}\beta\gamma$-PLCβ-PKC pathway to CCh-induced inhibition of BK channel activity of ∼50%.

Experiments designed to determine whether BK channel inhibition is a general principle of G$_{i/o}$-coupled receptors showed that stimulation of recombinant M$_4$Rs could also inhibit BK channel activity, whereas stimulation of the α_{2A}-AR by NA was without response. This lack was not due to a missing or insufficient coupling between the α_{2A}-AR and G$_{i/o}$ proteins. Within the same cells, α_{2A}-AR stimulation largely activated transiently expressed GIRK channels, which could also be activated by M$_2$R and M$_4$R stimulation. The maximal effect was largest in the α_{2A}-AR-expressing cells, followed by the M$_2$R-expressing cells, and the effect was weakest in the M$_4$R-expressing cells, which strictly correlated to the amount of recombinantly expressed receptors (21, 22). The specificity of G$_{i/o}$-coupled muscarinic receptors to inhibit BK channels is intriguing. The reason for this may be a highly localized signal transduction complex of receptor, G protein, and ion channel (Fig. 9). Indeed, the ability of BK channels to assemble specifically into macromolecular complexes with other transmembrane proteins such as the β_2-adrenoreceptor (53) and voltage-gated calcium channels (54) has been shown before.

BK channels are expressed in many cell types where they play a powerful integrative role in the regulation of electrical excitability by conducting repolarizing outward currents (49, 55). Therefore, the question arises of what is the physiological significance of BK channel inhibition as part of the cell response to muscarinic receptor activation in the ASM. Like in other tissues, BK channels in ASM buffer cell excitation in response to excitatory cell signals such as depolarization and an increased Ca^{2+} concentration. Consequently, inhibition of BK channel activity via M$_2$Rs counteracts this negative feedback loop and thus favors cell excitation in synergism with the M$_2$R-G$_{i/o}$-PLCβ-IP$_3$ induced Ca^{2+} release from the sarcoplasmic reticulum (Fig. 9). Functional experiments support this mechanism. First, CCh-induced contractions in tracheae from mice with targeted deletion of the M$_2$R or the M$_3$R demonstrate that both
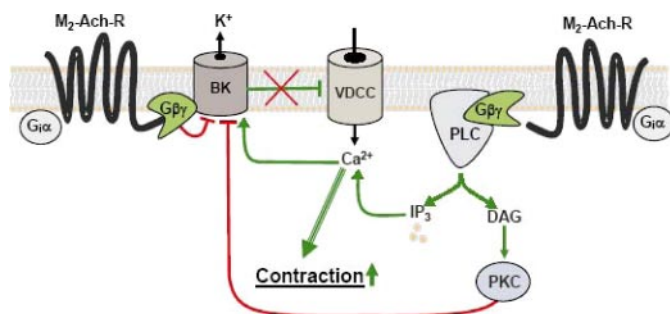
FIGURE 9. **Scheme illustrating the inhibition of the negative feedback regulation of BK channels in ASM by stimulation of M$_2$Rs.** Occupation of M$_2$Rs by an agonist induces the dissociation of heterotrimeric G$_i$ proteins in Gα_i and G$\beta\gamma$ subunits. G$\beta\gamma$ inhibits BK channels (*BK*) by binding directly to the channel protein or to a closely associated protein with modulatory function. In addition, G$\beta\gamma$ stimulates the β-isozymes of PLC, which in turn catalyzes the formation of IP$_3$ and diacylglycerol (*DAG*) from phosphatidylinositol bisphosphate. IP$_3$ releases Ca^{2+} from the sarcoplasmic reticulum through the Ca^{2+}-conducting IP$_3$ receptor. Diacylglycerol accumulates in the plasma membrane and causes, in concert with an elevated cytosolic Ca^{2+} concentration, translocation and activation of calcium-activated PKCs. Activated PKCs inhibit BK channels, probably by phosphorylating specific Ser/Thr residues of the channel protein. Inhibition of BK channels interferes with the function of BK channels as negative feedback regulators, membrane hyperpolarization is prevented, and more Ca^{2+} flows into the cell through voltage-dependent Ca^{2+} channels (*VDCC*). Together with the IP$_3$-mediated Ca^{2+}-release from the SR, BK channel inhibition helps to enhance the cytosolic Ca^{2+} concentration and hence contraction. Stimulatory and inhibitory pathways are shown in *green* and *red*, respectively.

receptor subtypes importantly contribute to contraction (15, 16). Second, inhibition of BK channels in isolated guinea pig tracheae by charybdotoxin reversed the inhibitory effect of PTX on methacholine-induced contraction (17). Furthermore, under physiological conditions, the well known G$\alpha_{i/o}$-dependent inhibitory effect of M$_2$R stimulation on adenylyl cyclase activity that counteracts the β_2-adrenoceptor-induced, cAMP-dependent ASM relaxation agrees well and likely contributes to the contraction-reinforcing properties of this receptor subtype.

An issue that still has to be addressed is the question of why G$\beta\gamma$ subunits inhibit BK channels by a dual mechanism, a direct membrane-delimited pathway, and a more elaborate signaling cascade resulting in the activation of PKC? Although a definite answer cannot be given, the answer might be as simple as that one alone is not enough to cause a physiologically relevant effect. The results in our study are pointing in that direction. On the one hand the inhibition of the PLC/PKC pathway diminishes the inhibitory effect of CCh by ∼50%. On the other hand the activation of the PLC/PKC pathway by CCh in HEK293 cells only expressing the M$_3$R is not sufficient to cause an inhibition of steady-state I_{BK}. Taking further into account that the direct application of G$\beta\gamma$ to the channel induced an ∼50% inhibition of NP_o independent of the intracellular Ca^{2+} concentration, the membrane-delimited direct interaction might be more essential. In line with that view, in a complex formed by a specific G protein-coupled receptor (M$_2$R or M$_4$R), the G$_{i/o}$ protein, and the BK channel (Fig. 9), such a locally delimited pathway would allow for an ultra rapid and efficient coupling, whereas the second messenger formation requiring PKC-dependent inhibition needs more time and thus might be more essential for a second, longer lasting phase of inhibition.

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