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Inhibition of L-Type $Ca_v1.2$ Ca^{2+} Channels by 2,(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) and 2-[1-(3-Dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1*H*-indol-3-yl) Maleimide (Gö6983)

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ABSTRACT

Phosphatidylinositol 3-kinase (PI3-K) is involved in physiological processes of cellular proliferation and inflammation and, as postulated recently, in the regulation of L-type Ca^{2+} channels. The latter conclusion arose in part from the inhibitory action of the compound 2,(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002), which has been established as a selective PI3-K inhibitor (IC_{50} = 1.4 μM). Herein we show, however, that LY294002 and an inhibitor of protein kinase C (PKC), 2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1*H*-indol-3-yl) maleimide (Gö6983), act as direct Ca^{2+}-channel inhibitors, with IC_{50} values of approximately 20 and 10 μM, respectively. Because both drugs are commonly used at concentrations of approximately 10 μM or higher, the interpretation of such experiments is questionable with respect to a regulatory action of PI3-K or PKC on L-type Ca^{2+} channels.

L-type Ca^{2+}-channel activity is crucial for heartbeat and smooth muscle tonus. Recent evidence has shown that, in both tissues, L-type Ca^{2+} channels are modulated by the phosphatidylinositol 3-kinase (PI3-K) pathway, which is mainly known to be involved in cell proliferation and inflammation (Wymann et al., 2003). In smooth muscle, PI3-K was reported to trigger activation of L-type Ca^{2+} channels after stimulation of acetylcholine and angiotensin-II receptors (Quignard et al., 2001; Callaghan et al., 2004; Le Blanc et al., 2004). In heart muscle, PI3-K has been proposed to regulate excitation-contraction coupling (McDowell et al., 2004). However, most of the evidence either for coupling of cholinergic stimulation to Ca^{2+}-channel activity or for regulating excitation-contraction coupling via PI3-K comes from the inhibitory effect of the compound LY294002, which has been introduced as a selective PI3-K inhibitor (Vlahos et al., 1994). In the present study, we show that LY2934002 and the protein kinase C (PKC) inhibitor Gö6983 directly inhibit L-type Ca^{2+}-channel activity. Therefore, a cautionary interpretation is recommended when these drugs are used to confirm an influence of the PI3-K and PKC pathways on biological effects.

Materials and Methods

Generation of $Ca_v1.2$-Deficient Mice. All experiments complied with the animal protection laws of Germany. The generation of control (CTR) mice and mice deficient in the smooth muscle $Ca_v1.2$ calcium channel (smooth muscle α_{1c}-subunit calcium-channel knockout, SMACKO) has been described previously (Moosmang et al., 2003).

Cell Transfection and Culture. HEK 293 cells were stably transfected with the α_{1b} ($Ca_V1.2b$) and the β2a subunit of the smooth muscle L-type calcium channel (GenBank accession numbers X55763 and X64298, respectively) or with only a carboxy-terminal–truncated version of the α_{1b} subunit, as described previously (Seisenberger et al., 1995).

Western Blot. Western blot analysis was performed on transfected HEK 293 cells and on brain tissue from mice using an antibody against the p110 subunit of PI-3Kγ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as described previously (Rong et al., 2003).

Tension Recordings. Muscles strips from murine urinary bladder were prepared as described previously (Wegener et al., 2004). Tension was recorded isometrically at 36 ± 1°C using the myograph 601 (DMT A/S, Aarhus, Denmark).

Current Recordings. L-type Ca^{2+}-channel current was measured in transfected HEK 293 cells by the whole-cell configuration of

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ABBREVIATIONS: PI3-K, phosphatidylinositol 3-kinase; LY294002, 2,(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; Gö6983, 2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1*H*-indol-3-yl) maleimide; Gö6976, 12-(2-cyanoethyl)-6,7,12,12-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole; PKC, protein kinase C; CTR, control; HEK, human embryonic kidney; SMACKO, smooth muscle α_{1c}-subunit calcium-channel knockout.

the patch-clamp technique. Experiments were performed at room temperature using Ba^{2+} as the charge carrier. The bath solution contained 104 mM NaCl, 20 mM tetraethylammonium chloride, 5.4 mM CsCl, 5 mM $BaCl_2$, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 10 mM glucose, and 5 mM HEPES, pH 7.4 (NaOH). The pipette solution contained 112 mM CsCl, 1 mM $MgSO_4$, 3 mM Na_2ATP, 10 mM EGTA, and 5 mM HEPES; pH was adjusted to 7.4 with CsOH. The holding potential was −80 mV. Trains of test pulses were to 0 or +10 mV for 100 ms with 0.2 Hz. Cumulative dose-inhibition curves were measured using two to three different drug concentrations per cell. Drugs were freshly diluted from the stock solution into the bath solution on each experimental day. IC_{50} values were calculated by fitting the averaged dose-inhibition curves to the Hill equation.

Chemicals. All chemicals used were at least of reagent grade and were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. LY294002, wortmannin, Gö6983, and Gö6976 were obtained from Calbiochem (San Diego, CA).

Evaluation of Results. Data are presented as original recordings or expressed as means ± S.E.M. Effects of the drugs were obtained in quasi–steady-state conditions. Concentration-response curves were fitted using Prism 4.0 software (GraphPad Software Inc., San Diego, CA). Correlation coefficients were >0.95.

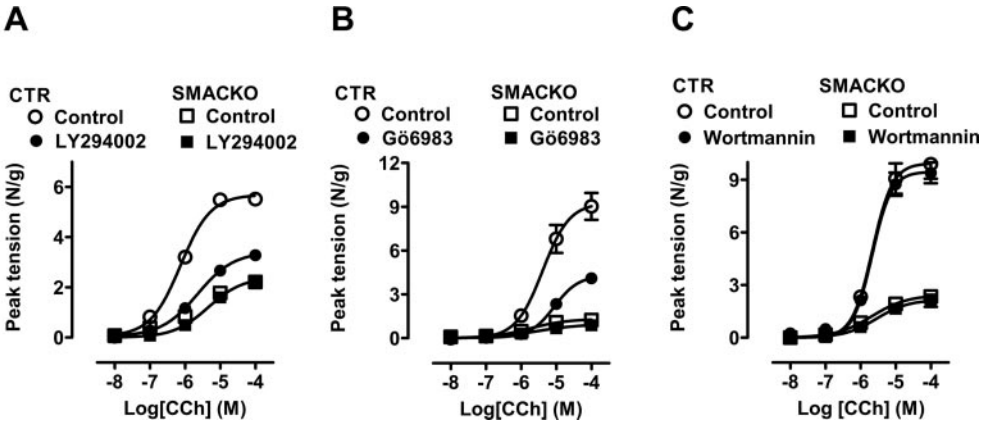

Fig. 1. Concentration-dependent effects of carbachol (CCh) on tension in urinary bladder muscle from CTR (circles and triangles) and SMACKO (squares) mice. The muscles were exposed to increasing concentrations of CCh in the absence (open symbols) and presence (closed symbols) of test substances (repeated-measurements design). Test substances, LY294002 (20 μM; A), Gö6983 (10 μM; B), and wortmannin (100 nM, C) were applied 10 min before initiation of contraction. Data points represent means ± S.E.M. (n = 4–12).

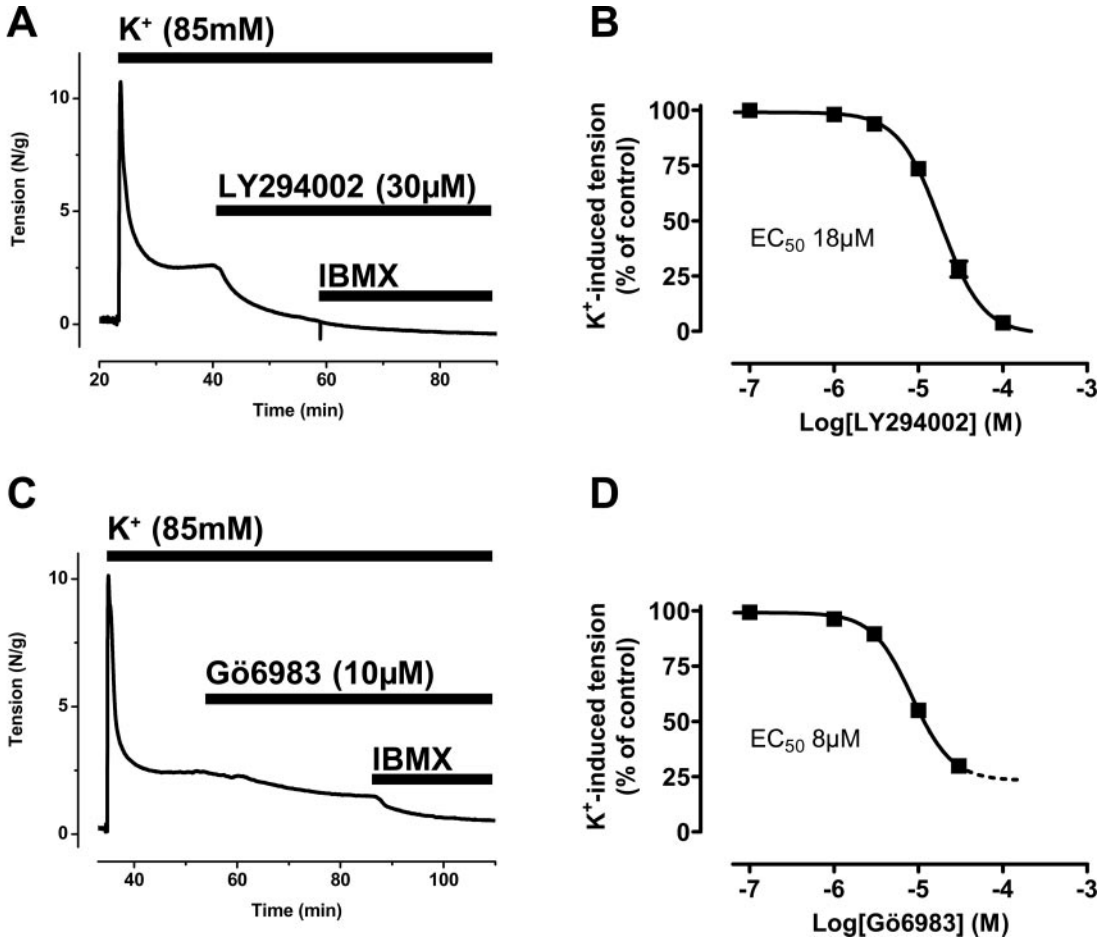

Fig. 2. Effects of LY294002 (A and B) and Gö6983 (C and D) on K^+-induced tension in urinary bladder muscle from CTR mice. A and C, original recordings of tension. Muscles were depolarized by exchanging 85 mM Na^+ with 85 mM K^+. Bars indicate the presence of 85 mM K^+, 30 μM LY294002 (A), 10 μM Gö6983 (C), and 100 μM isobutyl-1-methylxanthin (IBMX), which was used to induce maximal relaxation. B and D, concentration-response curves of LY294002 (B) and Gö6983 (D). Data points represent means ± S.E.M. (n = 4–8).

Results and Discussion

L-type $Ca_v1.2$ calcium-channel activity mediates contraction induced by stimulation of muscarinic receptors in urinary bladder smooth muscle from mice (Wegener et al., 2004). We tested the possibility that the PI3-K pathway is involved in this cascade as described for vascular smooth muscle (Quignard et al., 2001; Callaghan et al., 2004). Therefore, we examined the effects of LY294002, an inhibitor of PI3-K (Vlahos et al., 1994), and of Gö6983, an inhibitor of PKC isoforms including the PKCζ isoform (Gschwendt et al., 1996). Both LY294002 (20 μM) and Gö6983 (10 μM) inhibited carbachol-induced contractions of bladder muscles from control mice to approximately 50% but not from SMACKO mice (Fig. 1, A and B). The protein kinase C inhibitor Gö6976 (10 μM), which does not act on the PKCζ isoform (Gschwendt et al., 1996), was without effect (data not shown). These results implied that the PI3-K/PKCζ pathway is involved in the coupling of muscarinic receptors to $Ca_V1.2$ Ca^{2+} channels, leading to contraction in urinary bladder muscle. However, wortmannin, another inhibitor of PI3-K (IC_{50} = 5 nM) (Arcaro and Wymann, 1993), did not inhibit carbachol-induced contractions at 100 nM (Fig. 1C). Higher concentrations of wortmannin (10 μM) reduced contractions (data not shown), probably because of inhibition of myosin light-chain kinase (IC_{50} = 200 nM) (Burdyga and Wray, 1999) but not Ca^{2+} current (Fig. 4C). In addition, in control experiments in which contraction was induced by K^+ depolarization, both LY294002 and Gö6983 reduced contractions, with IC_{50} values of approximately 18 and 9 μM, respectively (Fig. 2). Identical results were obtained with muscle rings from aorta

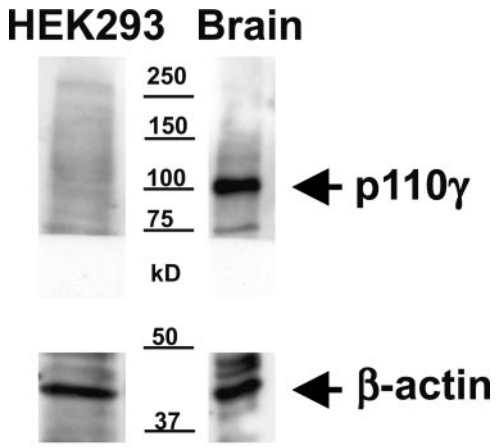

Fig. 3. Western blot analysis of PI3-Kγ protein expression. Arrows indicate the positions of the p110γ subunit and β-actin, respectively. β-Actin was used as loading control. The samples were prepared from transfected HEK 293 cells and murine brain.

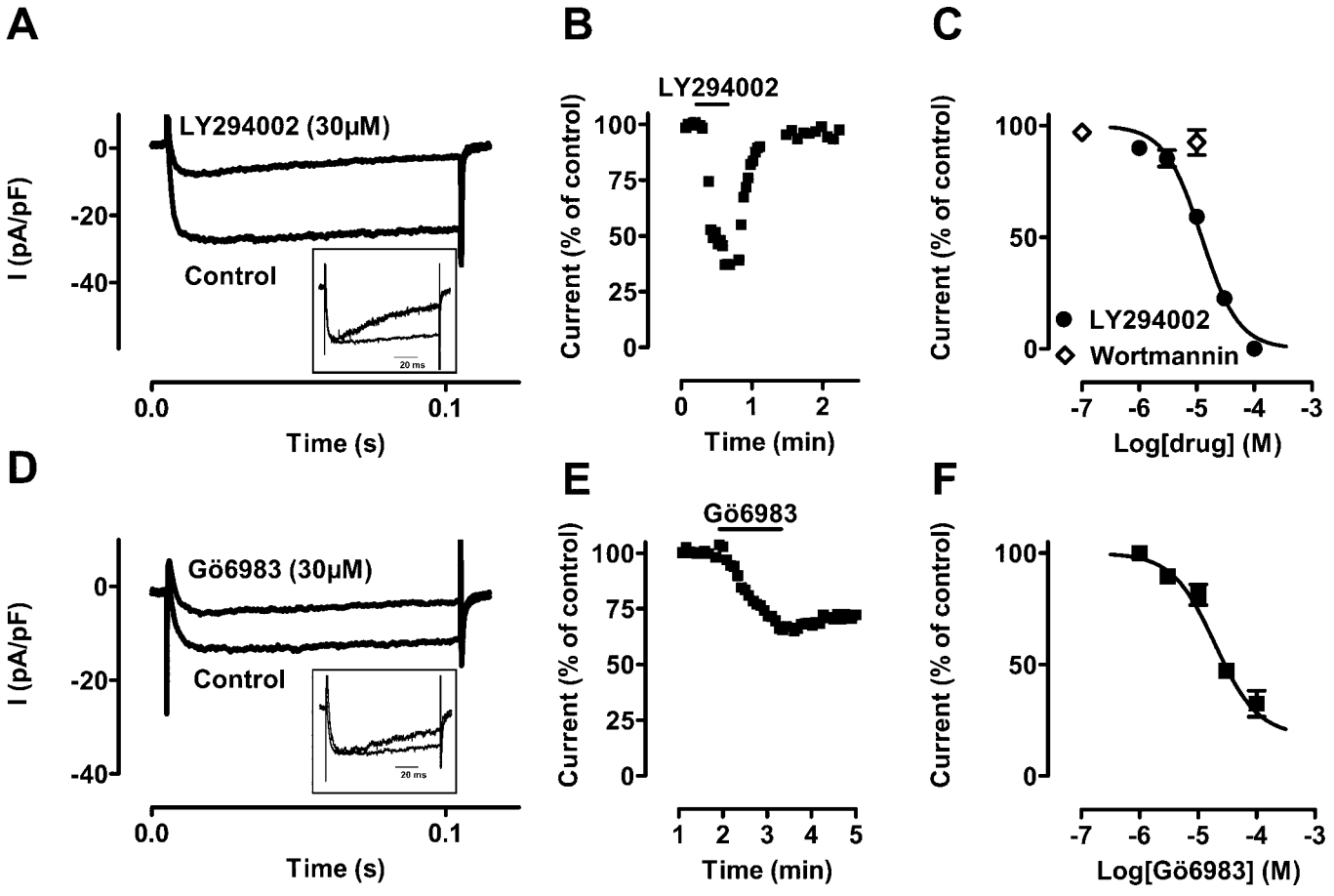

Fig. 4. Effects of LY294002 (A, B, and C), wortmannin (C) and Gö6983 (D, E, and F) on L-type Ca^{2+}-channel currents in HEK 293 cells transfected with the α_{1b} plus β_{2a} subunit of the $Ca_V1.2$ calcium channel. A and D, original recordings in control conditions and in the presence of 30 μM LY294002 (A) and 30 μM Gö6983 (D) are graphically superimposed. Insets show current traces normalized to the maximum. B and E, time course of current block by 20 μM LY294002 (B) and by 10 μM Gö6983 (E). Bars indicate the presence of the respective drug. Effects of LY294002 were almost reversible by washout, whereas those of Gö6983 were only partially reversible. C and F, concentration-response curves of LY294002 (C) and Gö6983 (F). Effects of wortmannin at 0.1 and 10 μM are included in C. Data points represent means ± S.E.M. (n = 3–11).

(data not shown). Atropine (1 μM), an unselective muscarinic antagonist, did not affect K^+-induced contractions in bladder muscle, confirming that muscarinic receptors are not involved in this response. These results suggested a more direct inhibitory action of the drugs on L-type Ca^{2+} channels because contraction induced by depolarization is completely blocked by dihydropyridines and, thus, primarily mediated by this channel type. Therefore, we tested the effects of both drugs on HEK 293 cells expressing functional L-type Ca^{2+} channels. Because there exist contrary reports whether or not HEK 293 cells contain endogenous PI3-K activity (Naga Prasad et al., 2001; Brock et al., 2003), Western blot analysis on our transfected HEK cells was performed; no p110 protein of the PI3-Kγ isoform, which is believed to influence Ca^{2+} channels (Viard et al., 2004), could be detected (Fig. 3). LY294002 and Gö6983, but not wortmannin, inhibited voltage-activated Ca^{2+}-channel currents in these cells being transfected with α_{1b} plus β_{2a} subunit with IC_{50} values of 12 and 20 μM, respectively (Fig. 4). A similar IC_{50} value for LY294002 was measured for cells only expressing the α_{1b} subunit (data not shown). No frequency- (0.1 versus 1 Hz) or voltage-dependent effects (-80 versus -40 mV holding potential) of the drugs were observed. However, current inactivation was accelerated by LY294002 and by Gö6983, indicating, at least partially, an interaction with the open channel (Fig. 4); time constants were 0.07 $\pm$ 0.005 s ($n = 5$) and 0.27 $\pm$ 0.09 s ($n = 3$) in the presence of LY294002 (30 μM) and Gö6983 (30 μM), respectively.

In summary, the present study shows that both LY294002 and Gö6983 exhibit Ca^{2+} antagonistic properties in the concentration range usually applied to selectively block PI3-K (Quignard et al., 2001; Northcott et al., 2002; Wang et al., 2002; Callaghan et al., 2004; McDowell et al., 2004) and PKC (Tao et al., 2003), respectively. Therefore, evidence of a functional coupling of the PI3-K signaling pathway to L-type Ca^{2+}-channel activity is hampered by the inhibitory effects of the compounds LY294002 and Gö6983 on L-type Ca^{2+} channels. The side effects of the putative PI3-K inhibitor LY294002, together with its recently described effect on K^+ currents (El-Kholy et al., 2003; Sun et al., 2004), limits the usefulness of this drug in the analysis of PI3-K function.

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