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Regulation of BK_{Ca} Channels Expressed in Human Embryonic Kidney 293 Cells by Epoxyeicosatrienoic Acid

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ABSTRACT

Epoxyeicosatrienoic acids (EETs) are arachidonic acid metabolites of cytochrome P450 monooxygenase, which are released from endothelial cells and dilate arteries. Dilation seems to be caused by activation of large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) leading to membrane hyperpolarization. Previous studies suggest that EETs activate BK_{Ca} channels via ADP-ribosylation of the G protein Gαs with a subsequent membrane-delimited action on the channel [*Circ Res* **78:**415–423, 1996; **80:**877–884, 1997; **85:**349–356, 1999]. The present study examined whether this pathway is present in human embryonic kidney (HEK) 293 cells when the BK_{Ca} α-subunit (*cslo*-α) is expressed without the β-subunit. 11,12-EET increased outward K^+ current in whole-cell recordings of HEK293 cells. In cell-attached patches, 11,12-EET also increased the activity of *cslo*-α channels without affecting unitary conductance. This action was mimicked by cholera toxin. The ADP-ribosyltransferase inhibitors 3-aminobenzamide and *m*-iodobenxylguanidine blocked the stimulatory effect of 11,12-EET. In inside-out patches 11,12-EET was without effect on channel activity unless GTP was included in the bathing solution. GTP and GTPγS alone also activated *cslo*-α channels. Dialysis of cells with anti-Gαs antibody completely blocked the activation of *cslo*-α channels by 11,12-EET, whereas anti-Gα*i*/o and anti-G$\beta\gamma$ antibodies were without effect. The protein kinase A inhibitor KT5720 and the adenylate cyclase inhibitor SQ22536 did not reduce the stimulatory effect of 11,12-EET on *cslo*-α channels in cell-attached patches. These data suggest that EET leads to Gαs-dependent activation of the *cslo*-α subunits expressed in HEK293 cells and that the *cslo*-β subunit is not required.

The vascular endothelium modulates tone of the underlying smooth muscle by releasing a number of different contracting and relaxing factors. Among these, endothelin, nitric oxide, and prostacyclin have been particularly well characterized (Furchgott and Vanhoutte, 1989). Recent studies suggest that an additional factor [i.e., epoxyeicosatrienoic acid (EET)], which is a product of the cytochrome P450 pathway, is also synthesized and released from the endothelium (Bauersachs et al., 1994; Hecker et al., 1994; Campbell et al., 1996). Endothelial cells possess cytochrome monooxygenase activity (Abraham et al., 1985; Pinto et al., 1987; Rosolowsky et al., 1990), and several cytochrome P450 isoforms have been described in endothelial cells. In vitro studies have shown that EETs relax coronary, pial, cerebral, caudal, and renal arteries; in some studies, membrane hyperpolarization has been observed (Hecker et al., 1994; Campbell et al., 1996; Fukao et al., 1997; Eckman et al., 1998). These results suggest that EETs contribute to endothelium-dependent relaxation and hyperpolarization in some blood vessels.

The vasodilatory action of EET seems to be due in large part to activation of large conductance Ca^{2+}-activated K channels (BK_{Ca}). Patch-clamp studies have shown that EETs increase the open probability of BK_{Ca} channels in native cells (Campbell et al., 1996; Li and Campbell, 1997; Hayabuchi et al., 1998). Membrane potential measurements in intact blood vessels have reported that EET-induced hyperpolarization is blocked by the BK_{Ca} channel blocker iberiotoxin (Eckman et al., 1998). Finally, EET-induced relaxation can be reduced or abolished by either iberiotoxin or tetraethylammonium (Campbell et al., 1996; Li and Campbell, 1997; Eckman et al., 1998; Li et al., 1999). Previous studies of native cells suggest that EETs enhance BK_{Ca} activity by activating the G protein Gαs (Li and Campbell, 1997) via ADP-ribosylation (Li et al., 1999). Some studies suggest that this is a direct membrane-delimited action of Gαs on the channel (Campbell et al., 1996; Li and Campbell, 1997).

Many details concerning the mechanism by which EETs modulate BK_{Ca} channel activity remain unclear. For exam-

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ABBREVIATIONS: EET, epoxyeicosatrienoic acid; BK_{Ca} channel, large-conductance Ca^{2+}-activated K^+ channel; HEK, human embryonic kidney; PKA, protein kinase A; NP_o, channel open probability; HEDTA, *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid; 3-AM, 3-aminobenzamide; MIBG, *m*-iodobenzylguanidine; PCR, polymerase chain reaction; bp, base pair(s); GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; GDPβS, guanyl-5′-yl thiophosphate; CTX, cholera toxin.

ple, a specific receptor for EETs has yet to be positively identified. Furthermore, the mechanism by which the G protein Gαs leads to activation of the BK_{Ca} channel activity is unknown. It is possible that G protein subunits interact specifically with the α-subunit of the BK_{Ca} channel. Alternatively, the β-subunit may be involved in the process. Expression systems are particularly useful to address these questions, because it is possible to express a known isoform of the BK_{Ca} α-subunit in the absence of the β-subunit and ultimately to manipulate with molecular biology techniques the predicted components of the pathway. The goal of the present study was therefore to determine whether the pathway previously described for EET-induced modulation of BK_{Ca} channels in native cells (Campbell et al., 1996; Li and Campbell, 1997; Li et al., 1999) is present when a known isoform of the α-subunit of the BK_{Ca} channel is expressed in a mammalian expression system (i.e., HEK293 cells). In previous studies, we have shown that BK_{Ca} α-subunits (*cslo-α*) expressed in HEK293 cells give rise to voltage-gated, Ca^{2+}-sensitive currents with electrophysiological and pharmacological features similar to those of native BK_{Ca} (Adelman et al., 1992; Esguerra et al., 1994; Perez et al., 1994; Fukao et al., 1999). Specific experiments in this study were designed to determine whether: 1) EETs enhance *cslo-α* channel activity in this expression system, 2) the G protein αs and/or $\beta\gamma$ subunit is involved, 3) activation involves ADP-ribosylation, and 4) activation of *cslo-α* involves the adenylyl cyclase/protein kinase A (PKA) pathway or a direct membrane-delimited pathway. Our results reveal a striking similarity in the pathway identified previously from experiments in native vascular smooth muscle cells and the pathway present in HEK293 cells expressing *cslo-α*. These results suggest that all of the elements necessary for the EET pathway are present in HEK293 cells expressing *cslo-α* and that the β-subunit of BK_{Ca} is not required for activation. This expression system represents a promising model for future studies of this important regulatory pathway.

Materials and Methods

Expression of *cslo-α* Channels. The cDNA encoding the α-subunit of the canine colonic BK_{Ca} channel (*cslo-α*) was expressed in HEK293 cells as described previously (Fukao et al., 1999).

Electrophysiological Recording. The patch-clamp technique was used to measure membrane currents in whole-cell and isolated patch configurations as previously described (Fukao et al., 1999). Data acquisition and analysis were performed with pClamp software (version 6.0.4; Axon Instruments, Burlingame, CA). Channel open probability (NP_o) in patches was determined from recordings of more than 3 min by fitting the sum of Gaussian functions to an all-points histogram plot at each potential. Single-channel conductance was determined from all-point amplitude histograms using Fechan and P-stat programs (Axon Instruments). Capacitance compensation and series resistance compensation (80%) were performed.

Solutions and Drugs. For whole-cell recordings of HEK293 cells, the bath solution contained 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4, and the pipette solution contained 50 mM KCl, 70 mM KAsp, 8 mM NaCl, 0.826 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM MgATP, 0.1 mM NaGTP, 10 mM HEPES, and 1 mM HEDTA, pH 7.2. For single-channel recordings in the inside-out mode, the bath solution contained 140 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, and 1 mM HEDTA, pH 7.2. The concentration of free Ca^{2+} in the bath solution was varied (range, 10^{-8} to 10^{-4} M) to determine the Ca^{2+} sensitivity of BK_{Ca} channels. The Ca^{2+} concentration was estimated by a computer program (Bers et al., 1994), and the appropriate amounts of $CaCl_2$ were added. The ionized Ca^{2+} concentration was confirmed using a Ca^{2+}-sensitive electrode. The pipette solution contained 140 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4. For single-channel recordings in the cell-attached mode, the bath solution contained 140 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4, and the pipette solution contained 140 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.4. All patch-clamp experiments were performed at room temperature (22°C). 11,12-EET was purchased from Cayman Chemical (Ann Arbor, MI). KT5720, SQ22536, cholera toxin, and all antibodies were obtained from Calbiochem (San Diego, CA). Anti-G$\beta\gamma$ is a polyclonal antibody raised against brain G$\beta\gamma$ (catalog no. 371821). Anti-Gαi/o is a mixture of two antibodies: 1) Anti-Gαi1 and Gαi2 subunit antibody (catalog no. 371723) generated by using a synthetic peptide antigen corresponding to a C-terminal decapeptide sequence (345–354) found in both Gαi1 and Gαi2. 2) Anti-Gαi3 and Gαo-subunit antibody (catalog no. 371726) generated by using a synthetic peptide antigen corresponding to the C-terminal sequence 345–354 of Gαi3. Anti-Gαs antibody (catalog no. 371732) generated by using a synthetic peptide antigen corresponding to the C-terminal sequence 385–394 of Gαs. 3-Aminobenzamide (3-AM) and *m*-iodobenzylguanidine (MIBG) were obtained from Sigma Chemical Co. (St. Louis, MO). 11,12-EET was dissolved in ethanol and KT5720, forskolin in dimethyl sulfoxide. Solvent per se had no effect on channel activity at final concentration (ethanol, 0.03%; dimethyl sulfoxide, 0.1%).

Total RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction. Total RNA was prepared from human jejunum smooth muscle and cultured HEK293 cells using the SNAP Total RNA isolation kit (Invitrogen, San Diego, CA) as per the manufacturer's instructions. First-strand cDNA was prepared from the RNA preparations using the Superscript II reverse transcriptase kit (Life Technologies, Inc., Gaithersburg, MD), 500 μg/μl of oligo(dT) primers were used to reverse transcribe the 1-μg RNA sample. The cDNA reverse transcription product was amplified with β-slo-specific primers by polymerase chain reaction (PCR) (Epperson et al., 1999). The amplification profile for these primer pairs was: 95°C for 10 min to activate the amplitaq polymerase (PE Biosystems, Foster City, CA), 95°C for 15 s, and 60°C for 1 min; each for 40 cycles. The amplified products (5 μl) were separated by electrophoresis on a 4% agarose/1× TAE (Tris, acetic acid, EDTA) gel, and the DNA bands were visualized by ethidium bromide staining. The reverse transcriptase control used a cDNA reaction as template for which the reverse transcriptase was not added, controlling for genomic DNA contamination in the source RNA. The no template control was a PCR amplification for which the template was not added, controlling for nonspecific amplification and spurious primer dimer fragments. These negative controls were subjected to a second round of amplification to assure specificity of the reactions and the quality of the reagents.

Primer Design. The following PCR primers were used (the GenBank accession numbers are given in parentheses for the reference nucleotide sequences used): human *β-slo* (U25138) nucleotides 199–218 and 376–397; amplicon = 199 base pairs (bp), β-actin (V01217) nucleotides 2204–2224 and 2384–2402; amplicon = 198 bp.

Statistics. Data are expressed as mean ± S.E. Statistical significance for repeated measures was determined using analysis of variance. $P < 0.05$ was considered significant.

Results

The endogenous currents of HEK293 cells and the currents recorded in cells expressing *cslo-α* were previously characterized by this group using the whole-cell and inside-out excised patch configurations. Endogenous currents are much smaller in amplitude than those recorded from cells expressing

cloned *cslo-α* channels and thus do not significantly contaminate recordings (Fukao et al., 1999).

Native HEK293 cells were examined for endogenous expression of BK_{Ca} β subunit mRNA and compared with freshly isolated human jejunal smooth muscle cells. Using primers designed to hybridize to conserved regions of β-slo and reverse-transcriptase PCR, no detectable product was observed in HEK293 cells, whereas an abundant message was detected in jejunal cells (Fig. 1). These results suggest that the actions of EET on *cslo-α* subunits can be examined in HEK293 cells in the absence of the *cslo-β* subunit.

Effect of 11,12-EET on Whole-Cell *cslo-α* Currents. Experiments were undertaken to determine the action of 11,12-EET on cells expressing *cslo-α* using the whole-cell, patch-clamp mode. Cytosolic Ca^{2+} concentration was buffered at 10 μM with HEDTA in these experiments. Addition of 11,12-EET (1 μM) to the bathing solution led to a significant increase in whole-cell outward current (Fig. 2A). Steady-state current was obtained after approximately 5 min (Fig. 2B). A representative voltage-current relationship of *cslo-α* current before and after treatment of 11,12-EET is shown in Fig. 2C. In eight cells tested, 11,12-EET significantly ($P < 0.05$) increased outward current amplitude 2-fold at +50 mV (Fig. 2D).

Effect of 11,12-EET on *cslo-α* Channel Activity in Cell-Attached Patches. Additional experiments were performed to determine whether changes also occur in single-channel activity recorded in cell-attached patches. 11,12-EET increased *cslo-α* channel activity in a concentration-dependent manner (Fig. 3A). 11,12-EET at concentrations between 0.1 and 1 μM produced a 2- to 3.5-fold increase in NP_o of *cslo-α* channels (Fig. 3B) but had no effect on the unitary conductance [control, 238 ± 9.5; 11,12-EET (1 μM), 246 ± 10; $n = 7$, $P > 0.05$].

Effect of 11,12-EET on *cslo-α* Channel Activity in Inside-Out Patches. In contrast to the stimulatory effect of 11,12-EET on *cslo-α* channel in cell-attached patches, 11,12-EET did not significantly affect *cslo-α* channel activity in inside-out patches when applied to the cytosolic surface of the membrane ($n = 12$). The unitary conductance of *cslo-α* channels was also unchanged by 11,12-EET in inside-out patches (control, 247 ± 19; EET, 247 ± 10; $n = 11$; $P > 0.05$). Because activation of channels by 11,12-EET may involve phosphorylation, additional experiments were undertaken with ATP. Application of ATP (1 mM) to the bath solution had no effect on *cslo-α* channel activity ($n = 8$). Likewise 11,12-EET was without effect in the presence of ATP ($n = 4$). These results suggest that ATP alone is insufficient to support activation of *cslo-α* channels by 11,12-EET in isolated patches.

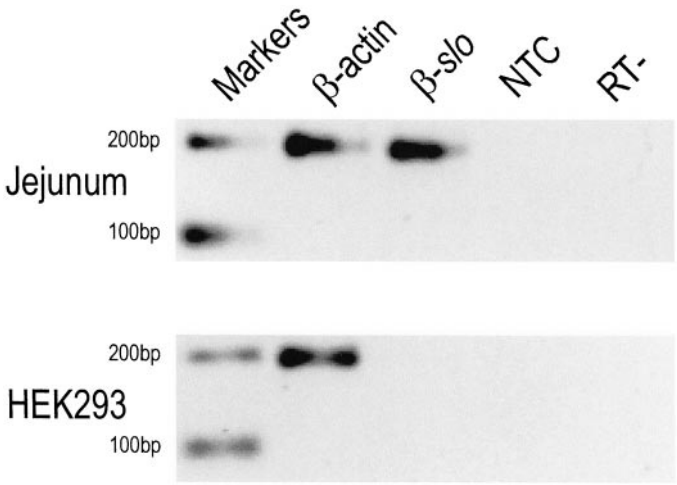

Fig. 1. Lack of *βslo* amplification products in cultured HEK293 cells. Shown is a representative gel containing amplification products from reverse transcription-PCR using human jejunum smooth muscle and cultured HEK293 cells as the source of RNA. Although abundant *βslo* message was detected in human jejunal cells, no message was apparent in HEK293 cells. A single 40-cycle amplification was used to generate the products. The amplicon for *βslo* was 199 bp and, for β-actin, 198 bp. Reverse transcription and no template control represent negative controls testing the quality of the reagents used (see *Materials and Methods*).

Effect of GTP and GTPγS on *cslo-α* Channel Activity in Inside-Out Patches. There is evidence from native cell experiments that EET can lead to activation of BK_{Ca} channels via Gαs (Li et al., 1997). To explore the role of G proteins in our expression system, additional experiments were undertaken with GTP and the nonhydrolyzable analogs GTPγS and GDPβS. GTP (100 μM) significantly increased *cslo-α* channel activity in inside-out patches without affecting the single-channel conductance (Fig. 4, A and B). In the presence of 100 μM GTP, addition of 11,12-EET (1 μM) led to a further increase in *cslo-α* channel activity (Fig. 4, A and B). These data suggest that 11,12-EET activates *cslo-α* via a GTP-dependent mechanism. GDPβS (200 μM), which inhibits GTP-dependent pathways, did not affect the basal *cslo-α* channel activity in inside-out patches (Fig. 4D), but completely blocked the stimulatory effect of GTP as well as GTP plus 11,12-EET (Fig. 4B). The GTP analog GTPγS (10 μM) also increased channel activity of *cslo-α* in inside-out patches (Fig. 4, C and D). This effect was also blocked by GDPβS (Fig. 4D).

Effect of Anti-Gαs Antibody on the Stimulatory Effect of 11,12-EET on *cslo-α* Channels. To further investigate the nature of the GTP-dependent response, antibodies to various G proteins were tested in experiments using the whole-cell configuration. Specific antibodies against Gαs, Gαi/o, and Gβγ were included in the pipette solution to inhibit the effects of these G protein subunits. After obtaining a stable whole-cell current, 11,12-EET was added to the bathing solution to activate *cslo-α* channels. There was no significant difference in the ability of 11,12-EET to stimulate *cslo-α* current in the presence of anti-Gαi/o or anti-Gβγ antibodies. In contrast, 11,12-EET was without effect on *cslo-α* current in the presence of anti-Gαs antibody (Fig. 5A), suggesting that 11,12-EET activated *cslo-α* via Gαs.

Effect of Cholera Toxin on *cslo-α* Channel Activity in Cell-Attached Patches. To provide further evidence for coupling of Gαs to *cslo-α* channels, we tested cholera toxin (CTX), which activates Gαs by ADP-ribosylation (Hopkins et al., 1988). Inclusion of CTX in the bathing solution gave rise to a significant increase in *cslo-α* channel activity in cell-attached patches (Fig. 5B) without a change in single-channel conductance (control, 248 ± 13 pS; cholera toxin, 250 ± 12 pS; $n = 8$). In the presence of CTX, 11,12-EET did not produce a further increase *cslo-α* channel activity (Fig. 5B).

Effect of Mono-ADP-ribosyltransferase Inhibitors on the Stimulatory Effect of 11,12-EET on *cslo-α* Channels. A recent study by Li et al. (1999) has suggested that activation of native BK_{Ca} channels by EET involves ADP-ribosylation of Gαs. To determine whether this same pathway is present in HEK293 cells, we investigated the effect of two different inhib-

itors of mono-ADP-ribosyltransferase, 3-aminobenzamide (Purnell and Whish, 1980) and *m*-iodobenzylguanidine (Smets et al., 1990). 3-AM (1 mM) did not affect basal channel activity in cell-attached patches. However, in the presence of 3-AM the stimulatory effect of 11,12-EET (1 μM) was abolished. Likewise, MIBG (100 μM) was without effect on basal channel activity but blocked the stimulatory effect of 11,12-EET on *cslo-α* channels (Fig. 5C).

Effect of KT5720 and SQ22536 on the Stimulatory Effect of 11,12-EET on *cslo-α* Channels. Our results suggest that 11,12-EET activates BK_{Ca} via ADP-ribosylation of the G protein Gαs. Gαs is a well known activator of the adenylyl cyclase/PKA pathway. To investigate the role of this pathway in the actions of Gαs and 11,12-EET, additional experiments were undertaken with blockers of this pathway. The PKA inhibitor KT5720 (200 nM) was without effect on basal *cslo-α* channel activity in cell-attached patches (Fig. 6). In addition, the stimulatory effect of 11,12-EET on *cslo-α* channel was not inhibited by pretreatment with KT5720 (Fig. 6, A and B). In contrast, KT5720 completely abolished the stimulatory effect of the adenylyl cyclase activator forskolin on *cslo-α* channels (Fig. 6B). The adenylate cyclase inhibitor SQ22536 (200 μM) was also without affect on basal *cslo-α* channel activity (Fig. 6C) in cell-attached patches. Stimulation of BK_{Ca} channels by either 11,12-EET or cholera toxin was unchanged in the presence of SQ22536 (Fig. 6C). These results indicate that the adenylyl cyclase/PKA pathway is present in HEK293 cells but that this pathway cannot represent the predominant mechanism by which 11,12-EET and Gαs activate *cslo-α* channels.

Discussion

11,12-EET is a cytochrome P450 product of the arachidonic acid cascade that is synthesized and released by the vascular endothelium and may serve as one of several different endothelium-derived factors, which relax and hyperpolarize the adjacent smooth muscle (Bauersachs et al., 1994; Hecker et al., 1994; Campbell et al., 1996). In the present study we found that 11,12-EET leads to activation of the cloned α-subunit of the BK_{Ca} channel (*cslo-α*) when expressed in HEK293 cells. This activation involves a novel pathway in which 11,12-EET leads to ADP-ribosylation of the G protein Gαs. Gαs in turn activates *cslo-α* via a membrane-delimited pathway that is independent of PKA and may involve a direct action of Gαs on the channel.

A number of studies have suggested that EETs relax blood vessels by activating BK_{Ca} channels in the smooth muscle (Hecker et al., 1994; Campbell et al., 1996; Li et al., 1997; Eckman et al., 1998). In the present study, 11,12-EET stimulated outward *cslo-α* current in whole-cell recordings and enhanced the activity of *cslo-α* channels in cell-attached patches without a change in single-channel conductance. The sensitivity of cloned *cslo-α* channels to 11,12-EET (i.e., 0.1 μM EET) was between that reported for native BK_{Ca} channels of large arteries (i.e., 0.3–10 μM EET) (Gebremedhin et al., 1992; Hu and Kim, 1993; Eckman et al., 1998) and that of small arteries (1 nM EET) (Li and Campbell, 1997). At present, it is not known what factors contribute to these differences in sensitivity.

The α-slo subunit has been cloned from a variety of tissue sources and species. All are derived from the same gene and exhibit very similar amino acid and nucleotide sequences across species. Northern blot analysis using a cDNA probe containing the conserved core region of *cslo-α* has revealed that *cslo-α* transcripts are ubiquitously expressed in a number of canine vascular muscles, including portal vein, renal artery, and pulmonary artery (Vogalis et al., 1996). Various splice forms have been detected at the transcriptional level, some of which are expressed with tissue specificity. It is not

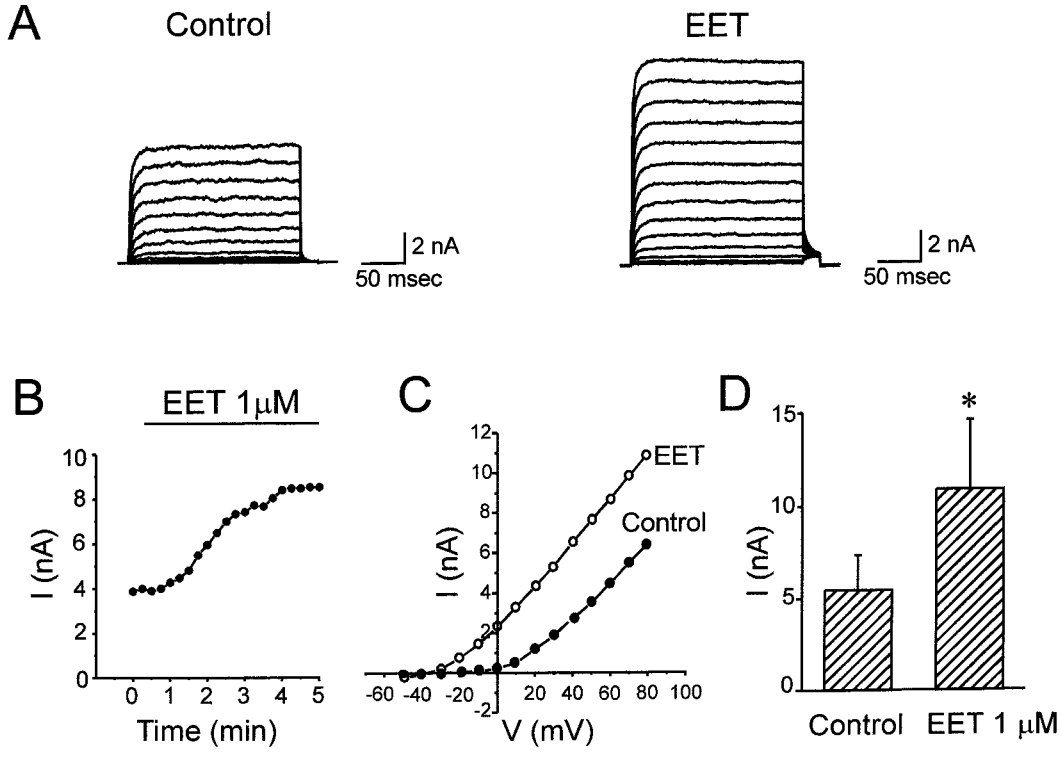

Fig. 2. Effect of 11,12-EET on whole-cell *cslo-α* current expressed in HEK293cells. A, representative traces of *cslo-α* current expressed in HEK293 cells in the absence and presence of 11,12-EET (1 μM). Membrane potential was held at −70 mV and stepped at 15-s intervals to potentials between −50 mV and +80 mV in 10-mV increments for 200 ms and then held at −30 mV for 20 ms. B, time course of the steady-state current amplitude before and after treatment of 11,12-EET (1 μM) at a membrane potential of +50 mV. C, current-voltage relationship of *cslo-α* currents before (●) and after (○) application of 11,12-EET (1 μM). D, averaged *cslo-α* currents of control and after application of 11,12-EET (1 μM). Data shows averaged peak currents at voltage clamp steps from −70 to +50 mV at 30-s intervals. Values are means ± S.E. (n = 8). *P < 0.05 compared with control.

clear to what extent the complete form and the various splice forms are utilized translationally to form functional BK_{Ca} channels in any smooth muscle. In the only complete description of α-slo in artery to date, Salkoff and coworkers detected two alternatively spliced forms of α-slo in human aorta and umbilicus as well as the complete α-slo sequence (McCobb et al., 1995). However, no functional differences between the three forms of α-slo were observed when expressed in oocytes or Chinese hamster ovary cells. Neither of the splice variants described by McCobb et al. (1995) were detected in canine colon (Vogalis et al., 1996). The *cslo-α* clone used in the present study is equivalent to the full-length *hslo* 1.1 form from McCobb et al. (1995). Using this molecular form we were able to mimic the actions of 11,12-EET and Gαs previously described for native vascular BK_{Ca} channels, suggesting that *cslo-α* is functionally indistinguishable from the form(s) expressed in vascular muscles, which give rise to the EET response.

GTP per se, as well as the nonhydrolyzable analog GTPγS, gave rise to a significant increase in *cslo-α* channel activity that was blocked by GDPβS. These data suggest that the predominant action of G proteins on *cslo-α* channels in HEK293 cells is stimulatory. In the presence of GTP, 11,12-EET caused a significant increase in channel activity, and this effect was also blocked by GDPβS. In contrast, in the absence of GTP, 11,12-EET was without effect. Thus, 11,12-EET seems to activate channels via a GTP-dependent mechanism. Because anti-Gαs antibody but not anti-Gi/o or anti-Gβγ antibodies blocked the actions of 11,12-EET, this suggests that 11,12-EET stimulates *cslo-α* via the GTP-binding protein Gαs. The known Gαs activator cholera toxin also enhanced BK_{Ca} channel activity, providing additional evidence for coupling between Gαs and *cslo-α* channels. This conclusion is in agreement with previous studies of 11,12-EET in native bovine coronary artery cells (Li and Campbell, 1997).

Recently it has been suggested that 11,12-EET can activate mono-ADP-ribosyltransferase, which leads to the transfer of ADP-ribose to the 52-kDa G protein Gαs, resulting in activation of BK_{Ca} channels in small bovine coronary arteries (Li et al., 1999). A similar result was previously reported for EET in the rat liver (Seki et al., 1992). In agreement with these data, we observed that the stimulatory effect of 11,12-EET on *cslo-α* was blocked by two different mono-ADP-ribosyltransferase inhibitors, 3-AM and MIBG. This suggests that ADP-ribosylation of Gsα is also important in the regulation of the cloned α-subunit of BK_{Ca} by 11,12-EET. The pathway by which 11,12-EET leads to activation of mono-ADP-ribosyltransferase remains unclear. A high-affinity binding site for 14(*R*),15(*S*)-EET in guinea pig mononuclear membranes has been reported, suggesting that a receptor for EET may exist (Wong et al., 1993). Thus, 11,12-EET may stimulate specific receptors that activate mono-ADP-ribosyltransferase, leading to ADP-ribosylation of Gαs. Interestingly, this action mimics cholera toxin, which is an exogenous ADP-ribosyltransferase that also activates Gαs by ADP-ribosylation (Hopkins et al., 1988).

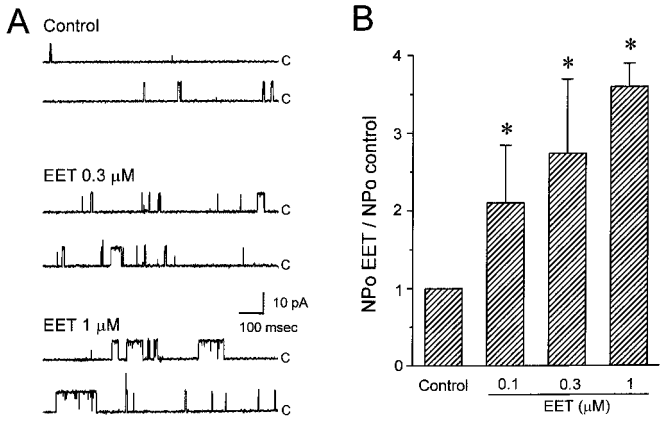

Fig. 3. Effect of 11,12-EET on *cslo-α* channel activity in cell-attached patches. A, representative recording of *cslo-α* channel under control condition (top trace) and after application of 0.3 μM 11,12-EET (middle trace) and 1 μM 11,12-EET (bottom trace) to the bath solution. Cells were held at +40 mV. c, the closed state. B, summary of the effect of 11,12-EET (0.1, 0.3, and 1 μM) on NPo of the *cslo-α* channels (n = 6–13). Values are means ± S.E. $^*P < 0.05$ compared with control.

In both native cell experiments and in expression systems there is good evidence that PKA activation leads to an increase in BK_{Ca} channel activity (Standen and Quayle, 1998) via phosphorylation of serine 869 (Nara et al., 1998). Indeed, BK_{Ca} channel activity was increased in the present study by the adenylyl cyclase activator forskolin. Inhibition of this effect by the PKA inhibitor KT5720 implies the existence of a functional adenylyl cyclase/PKA pathway in HEK293 cells, and we considered the possibility that this pathway might contribute to the Gαs-dependent responses to 11,12-EET. However, in cell-attached patches, the stimulatory effect of 11,12-EET was not blocked by either the adenylyl cyclase inhibitor SQ22536 nor the PKA inhibitor KT 5720, providing direct evidence that the actions of 11,12-EET are independent of the adenylyl cyclase/PKA pathway. This conclusion is in agreement with a study by Campbell et al. (1996) in which 11,12-EET was shown to relax the bovine coronary artery without a significant change in tissue levels of either cAMP or cGMP. In studies of native cells, EET activates BK_{Ca} channels through a PKA-dependent mechanism in renal arteries (Imig et al., 1999), whereas in porcine (Hayabuchi et al., 1998) and bovine coronary arteries (Campbell et al., 1996) a PKA-independent pathway has been proposed. Multiple isoforms of adenylate cyclase and PKA exist (Houslay and Milligan, 1997). The variable role of PKA in the actions of EET may be related to: 1) the presence of different isoforms of adenylyl cyclase and PKA in different cells, 2) the quantity of isoforms present, and 3) the degree of coupling between Gαs and adenylyl cyclase. In HEK293 cells the membrane-delimited actions of Gαs seem to far outweigh those of the adenylyl cyclase/PKA pathway, because SQ22536 was also without effect on cholera toxin, which activates all Gαs within the cell. This suggests very poor coupling between Gαs and adenylyl cyclase in these cells. Thus, in HEK293 cells, 11,12-EET seems to stimulate *cslo-α* via a direct membrane-delimited action of Gαs. The nature of this interaction between channel and G protein requires further investigation but seems to involve ADP-ribosylation of Gαs.

BK_{Ca} channels play a fundamental role in the regulation of membrane potential in smooth muscle, particularly under circumstances where intracellular calcium is elevated (Brayden and Nelson, 1992). In recent years it has become apparent that the activity of these channels can be importantly modulated by a variety of different physiological stimuli, including EET. Native BK_{Ca} channels are composed of pore-

forming α-subunits (i.e., α-slo) plus a regulatory β-subunit [predominantly β1 in smooth muscle (Jiang et al., 1999)] raising the possibility that the β-subunit plays a role in regulation of BK_{Ca} channel activity by Gαs. HEK293 cells transfected with specific BK_{Ca} subunits provide an excellent system to investigate this issue, because endogenous currents in general are small and there are no endogenous BK_{Ca} currents (Yu and Kerchner, 1998; Fukao et al., 1999) or message encoding the *cslo-β* subunit. Accordingly, we interpret our results as indicating the regulation of *cslo-α* channels expressed in the absence of β-subunits. Further evidence of the lack of β-subunits is that the voltage for half-maximal activation of the expressed BK_{Ca} currents with 10 μM free Ca^{2+} is +20 mV (Fukao et al., 1999), similar to values reported by others for activation of the *α-slo* subunits in the absence of the *β-slo* subunits (Toro et al., 1998)). Thus, the present study suggests that 11,12-EET activates BK_{Ca} channels through a direct action on the α-subunit independent of the β-subunit. However, we do not rule out a modulatory role for the β subunit in this process.

In summary, we have shown that the *cslo-α* expressed in HEK293 cells is activated by 11,12-EET in both the whole-cell and single-channel configuration. Activation involves ADP-ribosylation of Gαs but is independent of the adenylyl cyclase/PKA pathway, suggesting a direct, membrane-delimited pathway. These results agree well with previous studies

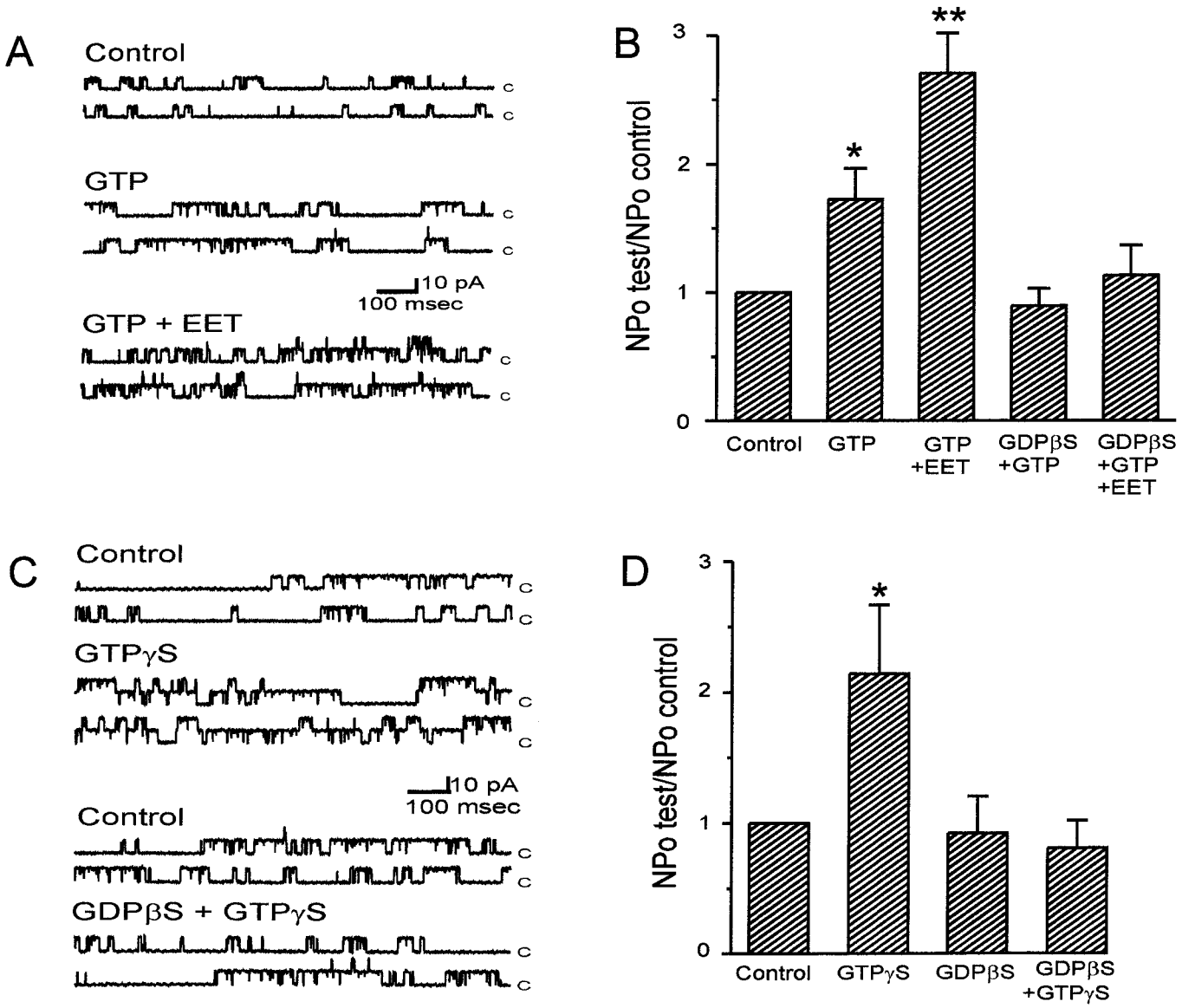

Fig. 4. Effect of GTP and GTPγS on *cslo-α* channel activity in inside-out patches. A, representative recording of *cslo-α* channel in inside-out patches at membrane potential of +40 mV. GTP (100 μM) increased the *cslo-α* channel activity in inside-out patches. In the presence of GTP, 11,12-EET gave rise to a further increased the channel activity. Cytosolic Ca^{2+} concentration was maintained at 10^{-6} M. c, the closed state. B, summary of the effect of GTP (n = 16), GTP + 11,12-EET (n = 5), GDPβS (200 μM) + GTP (n = 6), and GDPβS + GTP + 11,12-EET (n = 5) on *cslo-α* channel activity. C, representative recordings of *cslo-α* channel activity before (top trace) and in the presence of GTPγS (10 μM). GTPγS increased the *cslo-α* channel activity in inside-out patches (second trace). In a different patch, GTPγS was without effect on *cslo-α* channel activity when added in the presence of GDPβS (200 μM). D, summary of the effect of GTPγS (n = 6), GDPβS (n = 6), and GDPβS + GTPγS (n = 5) on *cslo-α* channel. Responses under the various conditions were compared with the control condition (normalized to 1). Values are means ± S.E. *$P < 0.05$ compared with control. **$P < 0.05$ compared with GTP.

of native cells (Campbell et al., 1996; Li and Campbell, 1997; Li et al., 1999) and suggest that the β-subunit of BK_{Ca} is not required for this pathway. The HEK293 expression system seems to be a promising model for future studies of this important regulatory pathway.

Acknowledgments

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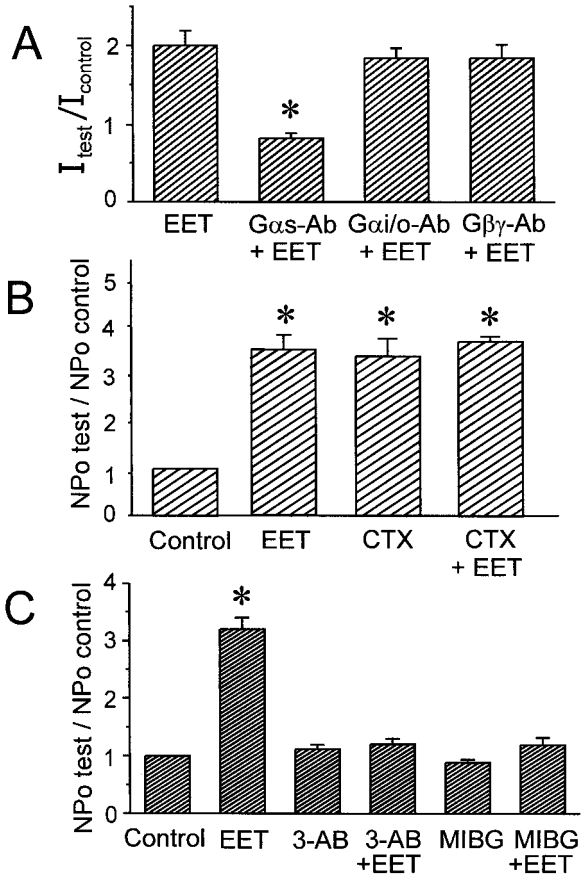

Fig. 5. Effect of various agents that modify G protein function on responses to 11,12-EET in HEK293 cells. A, effect of G protein antibodies. The bar graph shows a summary of the effect of anti-Gαs, anti-Gαi/o, and anti-Gβγ antibodies on the stimulatory effect of 11,12-EET of *cslo-α* current in whole-cell, patch-clamp mode. Antibodies were included in the pipette solution and dialyzed into cells. Peak current attained during a voltage step from −70 to +50 mV in the presence of 11,12-EET was normalized to the current obtained in the absence of EET. Only the anti-Gαs antibody produced a significant reduction in the stimulatory effect of 11,12-EET on *cslo-α* channel activity. Antibodies were diluted to produce a final concentration that was two times greater than that necessary for a Western blot (1:500) (n = 6–10). B, effect of the Gαs activator cholera toxin (CTX). The bar graph shows a summary of the effect of 11,12-EET (1 μM, n = 13), CTX (100 ng/ml, n = 8), and CTX + 11,12-EET (1 μM, n = 5) on *cslo-α* channel activity in cell-attached patches. Channel activity following 15-min exposure to cholera toxin was compared with the control channel activity (normalized to 1). The membrane potential was clamped at +40 mV. C, effect of ADP-ribosyltransferase inhibitors on the stimulatory effect of 11,12-EET on *cslo-α* channel activity in cell-attached patches. Channel activity in the presence of inhibitor plus 11,12-EET was compared with the activity obtained with 11,12-EET in the absence of inhibitor (normalized to 1). The ADP-ribosyltransferase inhibitors, 3-aminobenzamide (3-AM, n = 6) and *m*-iodobenzylguanidine (MIBG, n = 7) both blocked the stimulatory effect of 11,12-EET on *cslo-α* channel. Membrane potential was clamped at +40 mV. Values are means ± S.E. $^*P < 0.05$ compared with control.

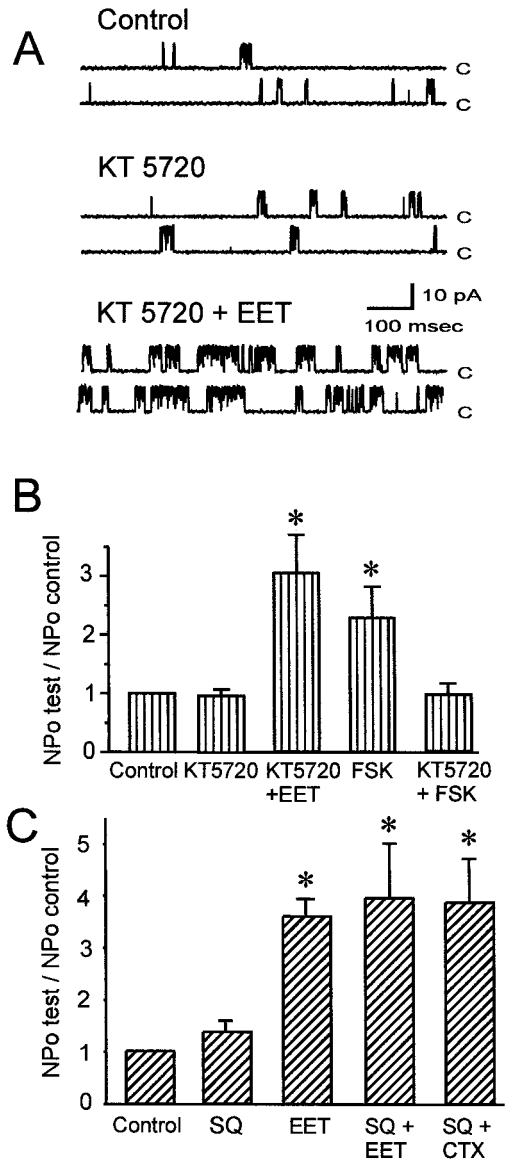

Fig. 6. Effect of blockers of the adenylyl cyclase/PKA pathway on the stimulatory effect of 11,12-EET on *cslo-α* channel activity in cell-attached patches. A, representative recording of *cslo-α* channel recorded at a membrane potential of +40 mV (top trace). The PKA inhibitor KT5720 alone (200 nM) was without effect on channel activity (middle trace), and 11,12-EET (1 μM) still enhanced *cslo-α* channel activity in the presence of KT5720 (bottom trace). Cytosolic Ca^{2+} concentration was maintained at 10^{-6} M. c, the closed state. B, summary of the effect of KT5720 (n = 16), KT5720 + 11,12-EET (n = 9), forskolin (10 μM, n = 7), and KT5720 + forskolin (n = 6) on *cslo-α* channel activity. C, effect of the adenylyl cyclase inhibitor SQ22536 on the actions of 11,12-EET and cholera toxin on HEK293 cells. The bar graph shows a summary of the effects of SQ22536 (200 μM, n = 17), 11,12-EET (1 μM, n = 13); SQ22536 + 11,12-EET (n = 7) and SQ22536 + CTX (100 ng/ml; n = 6) on *cslo-α* channel activity in cell-attached patches. Effects on channel activity were compared with the control channel activity (normalized to 1). Values are means ± S.E. (n = 6–10) $^*P < 0.05$ compared with control.

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