β1-Subunits are required for regulation of coupling between Ca²⁺ transients and Ca²⁺-activated K⁺ (BK) channels by protein kinase C

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Intracellular Ca²⁺ can regulate both contraction and relaxation of smooth muscle cells. The means by which the same second messenger can generate paradoxical physiological responses are not completely understood, but Ca²⁺ appears to regulate relaxation of smooth muscle cells through localized Ca²⁺ release from the sarcoplasmic reticulum. In many types of smooth muscle cells, these events are coupled to activation of clusters of Ca²⁺-activated K⁺ channels in the plasma membrane (e.g., Refs. 15, 19). Localized Ca²⁺ release results from openings of ryanodine-sensitive Ca²⁺ channels (Ca²⁺ sparks; Ref. 7) or from Ins (1,4,5) trisphosphate (IP₃) receptor-operated channels (Ca²⁺ puffs; Ref. 6). Localized Ca²⁺ release can generate concentrations in the range of 10 μM close to the plasma membrane (20), and due to the Ca²⁺-induced Ca²⁺ release properties of ryanodine and IP₃ receptors, bursts of Ca²⁺ can initiate Ca²⁺ waves that regenerate and spread along the plasma membrane (9).

Cellular responses to Ca²⁺ transients depend, in part, upon expression of Ca²⁺ dependent conductances in the plasma membrane. In smooth muscles, the first reports of coupling between Ca²⁺ transients and membrane currents described spontaneous transient outward currents (STOCs; Refs. 4, 19); however, inward currents (STICs) can also be generated depending upon driving forces and whether Ca²⁺-dependent chloride or nonselective cation conductances are in close proximity to Ca²⁺ release sites (e.g., Ref. 30). STOCs can consist of currents from several types of K⁺ channels, including small, intermediate, and large conductance Ca²⁺-activated K⁺ channels (i.e., SK, IK, and BK, respectively). For example, in murine and guinea pig colonic myocytes, both SK and BK channels contribute to STOCs; however, the largest amplitude events are typically due to BK channels (1, 16).

We have found that the strength of coupling between localized Ca²⁺ transients and currents due to BK channels is regulated by a Ca²⁺ influx-dependent protein kinase C (PKC) in colonic myocytes (2). If Ca²⁺ influx via a nonselective cation conductance was reduced, or if PKC inhibitors were applied, Ca²⁺ transients were unaffected, but these events coupled to more frequent and greater amplitude STOCs. In contrast, a PKC activator decreased the amplitude and frequency of STOCs.

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pharmacology, kinetics, and Ca\(^{2+}\)/voltage sensitivity (13, 17, 18, 28). Recent studies on genetically engineered mice lacking \(\beta_1\)-subunits have demonstrated the importance of these subunits in smooth muscle reactivity. Mice lacking \(\beta_1\)-subunits generated normal Ca\(^{2+}\) sparks in vascular smooth muscle cells, but these events were poorly coupled to STOCs (5). Loss of this activity was associated with a hypertensive phenotype. In the present study, we have studied whether regulation of coupling strength between Ca\(^{2+}\) transients and STOCs by PKC is preserved or lost in mice lacking \(\beta_1\)-subunits. We tested whether blockers of nonselective cation channels and inhibitors/activators of PKC affect the amplitude and frequency of Ca\(^{2+}\) puffs and STOCs and the open probabilities of BK channels in myocytes of \(\beta_1\)-knockout mice.

**EXPERIMENTAL PROCEDURES**

**Cell preparation.** BALB/c, C57Bl/6, and \(\beta_1^{-/-}\) mice (60- to 90-days-old) of either sex were anesthetized with Isoflurane and killed by decapitation. Mice were maintained and the experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the Institutional Animal Use and Care Committee at the University of Nevada, Reno.

After the mice were killed, colons were removed and opened along the mesenteric border. Luminal contents were removed by washing with Krebs-Ringer bicarbonate buffer (KRB; see Solutions and drugs). Tissues were pinned to the base of a Sylgard-coated dish, and the mucosa and submucosa were dissected away. The tissues were equilibrated in Ca\(^{2+}\)-free solution for 60 min and then digested at 37°C for 16 min without agitation in an enzyme solution containing collagenase F (Sigma Chemical) (3). At the end of the digestion period, the tissues were washed four times with Ca\(^{2+}\)-free Hanks’ solution to remove the enzyme and then triticated with blunt pipettes of decreasing tip diameter to free single smooth muscle cells.

**Confocal microscopy.** Suspensions of cells were placed in a specially designed 0.5-mL chamber with a glass bottom. The cells were incubated for 35 min at room temperature in Ca\(^{2+}\)-free solution for 60 min and then digested at 37°C for 16 min without agitation in an enzyme solution containing collagenase F (Sigma Chemical) (3). All measurements were made within 45 min of restoring extracellular Ca\(^{2+}\).

An Odyssey XL confocal laser scanning head (Noran Instruments, Middleton, WI) connected to a Nikon Diaphot 300 microscope with \(\times 60\) water immersion lens (numerical aperture = 1.2) was used to image the cells. The cells were scanned using INTERVISION software (Noran Instruments, Middleton, WI) running on an Indy workstation (Silicon Graphics, Mountain View, CA). Changes in the fluo 4 fluorescence (indicating fluctuations in cytosolic Ca\(^{2+}\)) were recorded for 20-s test periods using T-series acquisition and a laser wavelength of 488 nm (excitation for FITC). Six-hundred frames were acquired per test period (one frame every 33 ms), creating 20-s movie files.

**Ionic currents of single cells.** Ionic currents were measured in isolated muscle cells using the whole cell, perforated-patch (Amphotericin-B) configuration of the patch-clamp technique. An Axopatch 200B amplifier with a CV 203BU headstage (Axon Instruments, Foster City, CA) was used to measure currents, and recording of currents was performed with pCLAMP software (Version 7.0, Axon Instruments) while holding cells at \(-30\) or \(-40\) mV (after correction for a \(-11\) mV junction potential). Currents were digitized at 1 kHz. In some experiments, patch-clamped cells were simultaneously scanned for fluorescence changes in cells preloaded with fluo 4 as described above. All experiments were performed at room temperature (22–25°C).

Unitary currents resulting from openings of single ion channels were also measured in cell-attached patches. Cells were bathed in solutions with high K\(^+\) (140 mM) and containing either 1 mM or 0.01 mM free Ca\(^{2+}\). High-resistance seals (>10 GΩ) were obtained by using borosilicate electrodes (7–12 MΩ). The data were digitized at 2 kHz and filtered at 1 kHz by using pCLAMP Software (Version 6.0, Axon Instruments). Voltage ramp protocols in which patch potential was ramped over 4 s from \(-100\) to \(+100\) mV or from \(-50\) to \(+150\) mV were used. Ramps were repeated every 30 s, and responses to 6 ramps were averaged to generate current-voltage relationship (I-V) curves. Open probability of BK channels at \(+100\) mV was measured during 10- to 15-s steps from 0 to \(+100\) mV. All single-channel recordings were conducted with 4-aminopyridine (5 mM) and nicardipine (1 μM) in the pipette and bath solutions to decrease contamination from delayed rectifier K\(^+\) and voltage-dependent Ca\(^{2+}\) channels. A few control experiments were performed with charybotoxin (ChTX; 200 nM) in the pipette solution, and under these conditions, I-V curves were linear and small in amplitude, suggesting that most of the current response to depolarization was due to openings of BK channels.

**Solutions and drugs.** The standard Krebs-Ringer bicarbonate (KRB) used in this study contained 120 mM NaCl, 5.9 mM KCl, 2.5 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), 15.5 mM NaHCO\(_3\), 1.2 mM NaH\(_2\)PO\(_4\), and 11.5 mM dextrose. This solution had a final pH of 7.3–7.4 after equilibration with 97% O\(_2\)-3% CO\(_2\). The bathing solution used in confocal microscopy studies and whole cell patch-clamp studies contained 134 mM NaCl, 6 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM glucose, and 10 mM HEPES (pH 7.4). The enzyme solution used to disperse cells contained 1.3 mg/ml collagenase F, 2 mg/ml papain, 1 mg/ml BSA, 0.154 mg/ml l-DTT, 134 mM NaCl, 6 mM KCl, 1 mM MgCl\(_2\), 10 mM glucose, and 10 mM HEPES (pH 7.4). The pipette solution used in whole cell patch-clamp experiments contained 110 mM potassium aspartate, 30 mM KCl, 10 mM NaCl, 1 mM MgCl\(_2\), 10 mM HEPES, 0.05 mM EGTA (pH 7.2), and 250 μg/ml amphotericin B. Bath and pipette solution with free Ca\(^{2+}\) equal to 1 mM used in cell attached single channel contained 140 mM KCl, 1 mM MgCl\(_2\), 10 mM glucose, 1 mM CaCl\(_2\), and 10 mM HEPES. Solution with free Ca\(^{2+}\) at 0.01 mM was calculated by using the Max Chelator, provided by Dr. Chris Patton (www.stanford.edu/~cpaton/max.html), to contain 140 mM KCl, 1 mM MgCl\(_2\), 10 mM glucose, 0.755 mM CaCl\(_2\), 1 mM HEDTA, and 10 mM HEPES.

4-Aminopyridine, charybotoxin, apamin, and nicardipine were obtained from Sigma. 1-(β3-[4-Methylphenyl]propoxy)-4-methoxyphenethyl)-1H-imidazole hydrochloride (SKF 96365), ibetoxin, GF 109203X, and phorbol 12-myristate 13-acetate (PMA) were obtained from Tocris Cookson. We used concentrations of drugs recommended from the literature or empirically determined concentrations effective on murine colonic myocytes.

**Analysis of data.** Image analysis was performed by using custom analysis programs using interactive data language software (Research Systems, Boulder, CO), as previously.
Fig. 1. Relationship between spontaneous Ca\(^{2+}\) transients and spontaneous transient outward currents (STOCs). Spontaneous Ca\(^{2+}\) transients and STOCs from colonic myocytes of C57Bl/6 (A) and \(\beta_1^{-/-}\) mice (B) are shown. In A, traces 1 and 2 show spontaneous Ca\(^{2+}\) transients at 2 sites. Multiple Ca\(^{2+}\) transients were recorded from each site during a 20-s scan. The Ca\(^{2+}\) transients were associated with STOCs, as shown in simultaneous whole cell current recordings (bottom trace in A). Spontaneous Ca\(^{2+}\) transients also occurred in \(\beta_1^{-/-}\) myocytes. In B, multiple Ca\(^{2+}\) transients occurred at 2 sites during a 20-s scan. The Ca\(^{2+}\) transients in \(\beta_1^{-/-}\) myocytes were also associated with STOCs (bottom trace in B); however, STOCs were smaller in amplitude in \(\beta_1^{-/-}\) myocytes. C: a plot of STOC amplitude as a function of Ca\(^{2+}\) transient amplitude. Ca\(^{2+}\) transients of a given amplitude elicited larger STOCs in BALB/c (black filled triangles; \(n = 7\) cells) and C57Bl/6 mice (red filled triangles; \(n = 4\) cells) than in \(\beta_1^{-/-}\) (blue open triangles; \(n = 6\) cells). There was a nonlinear relationship between Ca\(^{2+}\) transient amplitude recorded at different sites within the same cell and STOCs in BALB/c and C57Bl/6 mice, whereas a linear relationship existed between Ca\(^{2+}\) transients and STOCs in \(\beta_1^{-/-}\) myocytes (blue line shows best fit by linear regression analysis). D: the amplitude of Ca\(^{2+}\) transients compared with STOCs in wild-type and \(\beta_1^{-/-}\) myocytes. There were no significant differences in Ca\(^{2+}\) transients of BALB/c, C57Bl/6, and \(\beta_1^{-/-}\) mice (black bars; means ± SE), but STOCs were significantly reduced in \(\beta_1^{-/-}\) myocytes compared with BALB/c and C57Bl/6 cells (red bars; means ± SE).

Described (1). Baseline fluorescence (\(F_0\)) was determined by averaging 10 images (out of 600) with no activity. Ratio images were then constructed and replayed for careful examination to detect active areas where sudden increases in \(F/F_0\) occurred. \(F/F_0\) vs. time traces were further analyzed in Microcal Origin (Microcal Software, Northampton, MA) and AcqKnowledge Software (Biopac Systems, Santa Barbara, CA) and represent the averaged \(F/F_0\) from a box region of \(2.2 \times 2.2 \mu m\) centered in the active area of interest to achieve the fastest and sharpest changes.

Statistical analysis. Results are expressed as means ± SE where applicable. Statistical analysis was made with SigmaStat 2.03 software (Jandel Scientific Software, San Rafael, CA). STOC amplitudes were measured by using the Mini Analysis Program (Synaptosoft, Leonia, NJ) with a threshold for detection set at 5 or 15 pA. The distributions of STOC amplitude were strongly skewed, resembling those of single-channel dwell times or survival curves. Accordingly, we have illustrated changes in STOC amplitudes in control and test conditions as cumulative distributions where the y-axis is the fraction of STOCs of amplitude greater than the pA value on the x-axis (2). We report \(P\) values from the log rank tests with \(n\) being the number of cells. Open probability was calculated by dividing the average current of 10 episodes, minus the closed current, by the open current.

Quantitative RT-PCR. Sequence searches for murine PKCa and PKCb were performed using GenBank, and primers specific for PKCa and \(\beta\) (but nonspecific for \(\beta_1\) isoforms) were designed. The primers used for quantitative PCR were as follows: \(\beta\)-actin (5'-ATGGTGGAATGGGTCA-GAAGG-3', 5'-GCTCATTGTAGAAGGTGTGGTGCC-3'), PCR

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\[^{1}\]The nucleotide sequence for the mouse PKC \(\alpha\), \(\beta\), and \(\beta\)II gene has been deposited in the GenBank database under GenBank accession nos. NM_011101, X59274, and X53532.
product length 307 bp); PKC \( \alpha \) (5'-GTGTCTCAGCTAAT-GAAGATGCCG-3', 5'-GGTTGTGGATGGTGGCTTTCTGTC-3', PCR product length 206 bp); and PKC \( \beta \) (5'-GTGTCTGAGAT-GGCTGTTCAAGTT-3', 5'-TGTCTTTTCTTCTGGAGCCTT-GGTAC-3', PCR product length 155 bp). Total RNA was extracted from mouse colon muscles stripped free of mucosa and brain by using the TRizol method (8). First-strand cDNA was synthesized by using 200 U SuperScript II RNase H+RT (GIBCO BRL) at 42°C for 60 min in the presence of 1 \( \mu \)g of total RNA in a 20-\( \mu \)l reaction volume. Real-time quantitative PCR was performed by using Syber Green chemistry on an ABI Prism 5700 sequence detector (PE Biosystems, Foster City, CA). Regression analysis of the mean values of the log\(_{10}\) diluted cDNA was used to generate standard curves. Unknown quantities relative to the standard curve for a set of PKC primers were calculated, yielding the transcriptional quantitation of PKC relative to the endogenous \( \alpha \)-actin standard. The data were calculated by using Excel Chart Wizard and graphed by using the GraphPad Prism. The PCR samples were analyzed on a 2% agarose gel.

Genotyping. \( \beta_1^{-/-} \) mice were confirmed by genotyping each animal studied. Genomic DNA was isolated from previously frozen mouse tails snipped into \(~2\)-mm pieces by the HotSHOT method as described (Ref. 26). PCR was completed on a Gene Amp PCR System 2700 (Applied Biosystems). Primers used in PCR were chosen to target GadPH, a housekeeping gene, \( \beta_1\),\( \beta_2 \)-wild type, and/or \( \beta_1\),\( \beta_2 \)-knockout genes. Primers that targeted \( \beta_1\),\( \beta_2 \)-knockout gene were designed from information provided by Rick Aldrich (Stanford University). PCR products were separated on a 2% agarose gel running at 70 V for 45 min and then visualized by using an ethidium bromide stain.

RESULTS

Macroscopic difference between \( \beta_1^{-/-} \) and BALB/c colon. There were some gross differences in bowel structure and function apparent in colons of \( \beta_1^{-/-} \) mice compared with wild-type mice (i.e., either BALB/c or C57Bl/6 strain-matched controls). Upon opening colons of these animals, loose fecal matter was always found in the distal colon of the \( \beta_1^{-/-} \) mice, whereas hard fecal pellets, typical of the murine colon, were found in

![Figure 2: STOCs in C57Bl/6 and \( \beta_1^{-/-} \) mice](http://ajpcell.physiology.org/)
wild-type animals. The structural integrity of the connective tissues of \( \beta_1^{-/-} \) mice also appeared to be weaker because it was much easier to separate the mucosa and submucosa from the tunica muscularis in colons of \( \beta_1^{-/-} \) mice than in wild-type mice.

\textbf{Ca\textsuperscript{2+} transients and STOCs.} As previously reported (1), colonic myocytes of BALB/c and C57Bl/6 mice produced spontaneous, transient elevations in intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) that occurred either as highly localized events (Ca\textsuperscript{2+} puffs) or more widely spreading Ca\textsuperscript{2+} waves (Fig. 1A). Colonic myocytes from \( \beta_1^{-/-} \) mice also displayed Ca\textsuperscript{2+} transients that were indistinguishable in amplitude and frequency from those of C57Bl/6 and BALB/c mice (Fig. 1, A, B, and D). In experiments in which cells were imaged under the conditions of whole cell voltage clamp, however, we noted significant differences in the coupling between Ca\textsuperscript{2+} transients and STOCs in myocytes of \( \beta_1^{-/-} \) mice (Fig. 1, B–D) compared with control cells (Fig. 1, A, C, and D). Ca\textsuperscript{2+} puffs originated from multiple sites in cells of each group of animals, but the effectiveness of the transients to produce STOCs was greatly reduced in \( \beta_1^{-/-} \) cells (Fig. 1, C and D). In C57Bl/6 cells, Ca\textsuperscript{2+} puffs originating at different sites, but of similar magnitude, coupled to STOCs of widely varying amplitude. Such variable coupling has been previously demonstrated in BALB/c cells (2). In myocytes of \( \beta_1^{-/-} \) mice, Ca\textsuperscript{2+} puffs at different sites were coupled to smaller STOCs of more consistent amplitude (Fig. 1B), and a relatively linear relationship existed between the amplitudes of Ca\textsuperscript{2+} transients and STOCs (Fig. 1C). The variability in coupling between Ca\textsuperscript{2+} transients and STOCs was previously shown to be due regulation of BK channels by a Ca\textsuperscript{2+} influx-dependent PKC (see Ref. 2), so further experiments were performed to determine whether this mechanism of regulating coupling between Ca\textsuperscript{2+} transients and STOCs was expressed by myocytes of \( \beta_1^{-/-} \) mice.

STOCs in colonic myocytes from BALB/c mice arise from the activation of BK and SK channels, and this was also true in cells from C57Bl/6 mice. STOCs were not fully inhibited by iberiotoxin (100 nM) in cells of C57Bl/6 mice, and remaining STOC activity was inhibited by apamin (300 nM). Cells of \( \beta_1^{-/-} \) mice also produced STOCs that were blocked by combination of iberiotoxin and apamin (Fig. 2).

In colonic myocytes, coupling between Ca\textsuperscript{2+} transients and STOCs is dramatically altered by either reducing the driving force for Ca\textsuperscript{2+} influx or blocking basal Ca\textsuperscript{2+} influx pathways (2). This means of regulating coupling between Ca\textsuperscript{2+} transients and STOCs in myocytes of \( \beta_1^{-/-} \) mice and strain-matched controls (C57Bl/6 mice) was investigated. In the control cells, lowering extracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{o}) from 2 to 1 mM caused a significant increase in STOC amplitude (Fig. 3, A, B, and E). In cells of \( \beta_1^{-/-} \) mice, lowering [Ca\textsuperscript{2+}]\textsubscript{o} did not affect STOC amplitude (e.g., from 6.5 ± 0.2 to 6.7 ± 0.2 pA; \( n = 5, P = 0.9264, \) and

![Figure 3](http://ajpcell.physiology.org/)
frequency from 82.8 ± 29.1/min to 127.4 ± 21.8/min; 

\[ n = 5; P = 0.075; \text{Fig. 3, C, D, and F}. \]

SKF 96365 (10 μM), a blocker of Ca\(^{2+}\) influx channels, had no effect on spontaneous Ca\(^{2+}\) transients in C57Bl/6 myocytes but increased the amplitude and frequency of STOCs (Fig. 4, A, B, and E). SKF 96365 (10 μM) had no significant effect on STOC amplitude in cells of \( \beta_{1}^{-/-} \) mice (e.g., from 13.2 ± 1.5 to 11.1 ± 1.4 pA; \( n = 3, P = 0.2635 \), and frequency from 77.3 ± 7.0/min to 78.3 ± 9.3/min; \( n = 3, P = 0.784 \); see Fig. 4, C, D, and F).

Ca\(^{2+}\) entry into colonic myocytes through nonselective cation channels regulates the sensitivity of BK channel through activation of PKC. Therefore, we measured STOC amplitudes before and after treatment with GF 109203X (1 μM), an inhibitor of PKC. GF 109203X had no effect on localized Ca\(^{2+}\) transients (not shown), but it mimicked the effects of reduced [Ca\(^{2+}\)]\(_o\) and SKF 96365 by greatly increasing the amplitude and frequency of STOCs (Fig. 5, A, B, and E). GF 109203X had no significant effect on STOC amplitude or frequency in myocytes of \( \beta_{1}^{-/-} \) mice (e.g., from 9.4 ± 0.16 to 9.7 ± 0.21 pA; \( n = 8, P = 0.6723 \); frequency from 94.0 ± 15.0/min to 99.4 ± 16.4/min, \( n = 8, P = 0.639 \); Fig. 5, C, D, and F).

The low amplitudes of STOCs in \( \beta_{1}^{-/-} \) myocytes (e.g., 9.8 ± 2.6 pA compared with an average of 55.2 ± 13.8 pA in BALB/c and 44.4 ± 9.26 pA in C57Bl/6; \( P = 0.012 \) and \( P = 0.002 \), respectively; Fig. 1D) was likely due to the shift in the Ca\(^{2+}\) sensitivities of BK channels in the absence of \( \beta_{1} \)-subunits (c.f., Ref. 5). The reduced relative open probability of BK channels in \( \beta_{1}^{-/-} \) cells and specifically to determine whether PKC regulates channels in the absence of \( \beta_{1} \)-subunits.

Single-channel experiments. BK channel activity in on-cell patches was recorded, and openings of BK channels were tabulated in response to repetitive steps from 0 to +100 mV for 15 s. In cells of C57Bl/6 mice, addition of GF 109203X (1 μM) to the bath solution increased open probability of BK channels from 0.091 ± 0.018 to 0.17 ± 0.022 (\( n = 7, P < 0.001 \); Fig. 6, A, B, and E).

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**Fig. 4. Effects of SKF 96365 on STOCs.**

**A:** STOCs recorded from a C57Bl/6 myocyte held at −40mV. The amplitude of STOCs was greatly increased by the addition of SKF 96365 (10 μM; B). C and D: the same experiment performed on a \( \beta_{1}^{-/-} \) myocyte. STOCs of \( \beta_{1}^{-/-} \) myocytes were not affected by SKF 96365. Survival plots representing STOC amplitude before and after SKF96365 are shown for C57Bl/6 (E, \( n = 3 \)) and \( \beta_{1}^{-/-} \) myocytes (F, \( n = 3 \)).
Under the same conditions, GF 109203X did not affect the open probability of BK channels in patches from \( \beta_1^{-/-} \) myocytes (i.e., from 0.053 ± 0.0066 to 0.054 ± 0.0043, \( n = 5 \), \( P = 0.717 \); Fig. 6, C–E).

The activity of BK channels was also studied with ramp potential protocols. On-cell patches were held at 0 mV and ramped from -100 to +100 mV every 30 s. Responses from six ramps were averaged as in Fig. 6, F and G. The voltage ramps were repeated after addition of GF 109203X (1 \( \mu \)M). This compound caused a leftward shift in the \( I-V \) relationship relative to the averaged response before the drug and resulted in significantly more current at any given potential above the threshold of activation (e.g., at +80 mV there was an average of 9.36 ± 1.12 pA under control conditions and 25.23 ± 2.32 pA after GF 109203X; \( n = 5 \), \( P < 0.001 \); Fig. 6F). When the same experiments were performed on cells of \( \beta_1^{-/-} \) mice, GF 109203X (1 \( \mu \)M) did not cause a shift in the \( I-V \) relationship (Fig. 6G). Because the \( Ca^{2+} \) sensitivity of BK channels was less than in wild-type cells, we ramped potentials from 50 to 150 mV in these experiments and compared the amplitudes of current at +125 mV to produce comparisons of currents of similar amplitudes. Before GF 109203X, the amplitude of current at 125 mV was 10.24 ± 3.40 pA, and after the drug we measured 7.76 ± 2.66 pA at the same potential (\( n = 6 \); \( P = 0.175 \)).

Ramp protocols demonstrated that PMA caused a significant rightward shift in the \( I-V \) curves for BK channels of BALB/c and C57Bl/6 myocytes (\( n = 6 \), \( P < 0.01 \); Fig. 7F). However, no change was noted in the \( I-V \) curve after addition of PMA (1 \( \mu \)M) to cells of \( \beta_1^{-/-} \) mice (\( n = 7 \), \( P = 0.453 \); Fig. 7E).

Additionally, we performed control experiments in which ChTX (200 nM) was added to the pipette solution to block all BK channels in patches. Under these conditions, most of the channels activated by step protocols from 0 to +100 mV were blocked and ramp
potentials elicited only small, linear currents (data not shown).

Expression of Ca\(^{2+}\)-dependent PKC. Loss of PKC-dependent regulation of coupling strength between Ca\(^{2+}\) transients and STOCs might have been due to changes in the expression of Ca\(^{2+}\)-dependent PKC in β\(_1^-/-\) mice. Therefore, we tested expression of PKC α and β, which are both expressed in the colon muscles and brain of β\(_1^-/-\) and C57Bl/6 mice. Relative transcriptional expression of PKC α and β in murine colon and brain are shown in Fig. 8. The normalized relative levels of PKC α and PKC β (nonspecific for β\(_1^+\) and β\(_1^-\)) were not significantly different in C57Bl/6 and β\(_1^-/-\) colonic muscles.

**DISCUSSION**

A previous study suggested that a Ca\(^{2+}\)-influx-dependent PKC regulates coupling between localized Ca\(^{2+}\) transients and BK channels in murine colonic myocytes (2). Others have shown that open probabilities of BK channels can be reduced by PKC (e.g., Refs. 23, 24); however, the actual means of regulation of BK channels by PKC has not been elucidated. In the present study, we tested whether drugs and conditions expected to reduce Ca\(^{2+}\) influx or stimulate or block PKC affected coupling between localized Ca\(^{2+}\) transients and BK channels (i.e., STOCs) in myocytes of wild-type and β\(_1^-/-\) mice. These agents greatly altered coupling between Ca\(^{2+}\) transients and STOCs in wild-type mice, but each test failed to affect coupling in myocytes of β\(_1^-/-\) mice. Thus our experiments suggest that the Ca\(^{2+}\)-influx-dependent PKC regulation of coupling between Ca\(^{2+}\) transients and STOCs is dependent upon the expression of β\(_1^+\)-subunits.

Coexpression of the β-subunits with pore-forming α-subunits affects the function of the α-pore-forming subunits of BK channels (e.g., Ref. 28). Loss of β-subunits does not appear to affect the overall density of BK channels (12, 21). Smooth muscle tissues express only β\(_1^+\)-subunits, so it is likely that murine colonic myocytes from β\(_1^-/-\) mice lack regulation from all β-subunits. This would be expected to alter the pharmacology of channel blocking drugs, activation/inactivation kinetics, and Ca\(^{2+}\) sensitivity of BK channels (13, 17, 18, 28). We noted approximately an 80% reduction in STOCs in myocytes of β\(_1^-/-\) mice compared with wild-type myocytes, but the localized Ca\(^{2+}\) transients in both cell types were approximately equivalent (Fig. 1).
The difference in coupling between Ca\(^{2+}\) transients and STOCs can be attributed to the decrease in the Ca\(^{2+}\) sensitivity of BK channels in \(\beta_1^{-/-}\) myocytes [as observed by Brenner and coworkers (5) in studies of vascular smooth muscle cells]. In colonic myocytes, we have previously reported an 86-mV shift in activation as a result of the loss of \(\beta_1\)-subunits (in 10 \(\mu\)M Ca\(^{2+}\)), and a 34-mV shift was observed at 100 nM Ca\(^{2+}\). It is difficult to measure the concentration of Ca\(^{2+}\) near the inner surface of the membrane at the peak of a Ca\(^{2+}\) puff, but others have calculated that Ca\(^{2+}\) rises locally to at least 10 \(\mu\)M during Ca\(^{2+}\) sparks (20). At a holding potential of -30 mV (used in our studies), 10 \(\mu\)M Ca\(^{2+}\) reached locally during puffs would result in about 75\% activation of wild-type BK channels in close proximity to Ca\(^{2+}\) release sites (12). In contrast, only about 5\% of BK channels lacking \(\beta_1\)-subunits would be activated at an equivalent holding potential and [Ca\(^{2+}\)]. Thus we believe the decrease in coupling between observed in \(\beta_1^{-/-}\) myocytes can be explained by the decrease in Ca\(^{2+}\) sensitivity of BK channels lacking \(\beta_1\)-subunits.

BK channels, and the coupling between Ca\(^{2+}\) transients and STOCs, are regulated by PKC (2). Regulation by PKC was abolished by loss of \(\beta_1\)-subunits. Other studies have also demonstrated differences in responses to drugs that are dependent upon \(\beta_1\)-subunits. For example, the nongenomic actions of estrogen...
lead to enhancement in the open probability of BK channels only when β1-subunits were coexpressed in HEK-293 cells (27). Similar regulation of canine colonic smooth muscle BK channels expressed by HEK-293 cells by tamoxifen was observed only when regulatory β1-subunits were present (11), and regulation of BK channels by [xeno]estrogens is also inhibited in β1−/− mice (12). Finally, ATP inhibits BK channels consisting only of α-subunits, but this inhibitory effect is lost upon coexpression α- and β-subunits (10). These observations show that the influence of β-subunits on BK channels extends beyond basic biophysical properties of the channels. β-subunits also participate in determining pharmacological responses of BK channels to a variety of agents.

The mechanism(s) by which β1-subunits convey sensitivity to regulation of BK channels by PKC is unknown. It is unlikely that regulation of BK channels by PKC occurs directly through phosphorylation of β1-subunits because no consensus sequences for phosphorylation by PKC are available in this protein. β1-subunits convey enhanced Ca2+ sensitivity on BK channels (29). There are 15 consensus sequences in PKC phosphorylation in the murine α slo2 (14), although 3 of these sites can be eliminated due to predicted inaccessibility within the folded protein (22). Phosphorylation of α-subunits by PKC might reduce the interaction between α- and β1-subunits and thus decrease the relative Ca2+ sensitivity of BK channels. Channels lacking β1 would, therefore, lack PKC-dependent regulation. It is also possible that loss of β1-subunits affects the sensitivity of α-subunits to phosphorylation by PKC. Finally, it is also possible that regulation of BK channel gating by PKC is mediated via a subsidiary protein, not yet identified, that depends upon β1-subunits for its influence on BK channels.

There were interesting phenotypic changes observed in β1−/− mice that might have resulted from reduced Ca2+ sensitivity and subsequent reduced open probability of BK channels. Brenner and coworkers (5) found that the β1-subunits were essential for effective coupling of Ca2+ sparks to BK channels in vascular smooth muscle cells. β1-subunits, therefore, facilitate regulation of arterial smooth muscle tone, and β1−/− mice displayed a phenotype of elevated arterial tone and blood pressure. We observed changes in colonic function in β1−/− mice, and fecal material was atypically loose and not compacted into pellets. This could be the result of motility changes or changes in the water recovery function of the colon. In general, reduction in Ca2+ sensitivity of BK channels should reduce open probability at the voltages experienced by colonic smooth muscle cells, and this should result in greater smooth muscle excitability and stronger colonic contractions. This may reduce the reservoir function of the colon and reduce the efficiency of water recovery. It is

2The amino acid sequence of murine α slo protein can be accessed through NCBI Protein Database under NCBI Accession no. 034740.

hard, however, to develop a complete rationale for the whole organ consequences of loss of β1-subunits without further experiments because the expression and contributions of this conductance are widespread in gastrointestinal cells.

In summary, regulation of coupling between Ca2+ transients and BK channels occurs via a Ca2+ influx-dependent PKC in murine colonic myocytes. This form of regulating the coupling between Ca2+ release and membrane currents is lost in animals lacking β1-subunits. PKC might affect the Ca2+ sensitivity of BK channels by affecting the interactions between α- and β1-subunits, or β1-subunits may affect the ability of PKC to phosphorylate α-subunits. Additional experiments are needed to determine the sites phosphorylated in α-subunits by PKC and whether β1-subunits influence the degree of phosphorylation of α-subunits by PKC.

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DISCLOSURES

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REFERENCES


