Calcineurin Aα but Not Aβ Augments $I_{\text{Cl(Ca)}}$ in Rabbit Pulmonary Artery Smooth Muscle Cells*

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Activation of Ca$^{2+}$-dependent Cl$^-$ currents ($I_{\text{Cl(Ca)}}$) increases membrane excitability in vascular smooth muscle cells. Previous studies showed that Ca$^{2+}$-dependent phosphorylation suppresses $I_{\text{Cl(Ca)}}$ in pulmonary artery myocytes, and the aim of the present study was to determine the role of the Ca$^{2+}$-dependent phosphatase calcineurin on chloride channel activity. Immunocytochemical and Western blot studies with isoform-specific antibodies revealed that the α and β forms of the CaN catalytic subunit are expressed in PA cells but that only the α variant translocated to the cell periphery upon a rise in intracellular [Ca$^{2+}$]. $I_{\text{Cl(Ca)}}$ evoked by pipette solutions containing a [Ca$^{2+}$] set at 500 nM was considerably larger when the pipette solution included constitutively active CaN containing the α catalytic isoform. This stimulatory effect was lost by boiling the enzyme or by the inclusion of a specific CaN inhibitory peptide and was not shared by the inclusion of the β form of the catalytic subunit. In the absence of constitutively active CaN, cyclosporin A, an inhibitor of CaN, suppressed $I_{\text{Cl(Ca)}}$ evoked by 500 nM Ca$^{2+}$ when the current amplitude was relatively large but was ineffective in cells with smaller currents. In perforated patch recordings, cyclosporin A consistently inhibited $I_{\text{Cl(Ca)}}$ evoked as a consequence of Ca$^{2+}$ influx through voltage-dependent calcium channels. These novel data show that in PA myocytes activation of $I_{\text{Cl(Ca)}}$ is enhanced by Ca$^{2+}$-dependent dephosphorylation and that the regulation of this conductance is highly isoform-specific.

Smooth muscle cells actively accumulate chloride resulting in an equilibrium potential ~30 mV less negative than the resting membrane potential (1). Consequently, the activation of Cl$^-$ channels leads to Cl$^-$ efflux and membrane depolarization. Ca$^{2+}$-dependent Cl$^-$ currents ($I_{\text{Cl(Ca)}}$) have been recorded from a wide number of smooth muscle cells where they have been implicated in agonist-induced and spontaneous contractions (2, 3). In all of the smooth muscle cells, the generation of $I_{\text{Cl(Ca)}}$ has an obligatory requirement for increased intracellular [Ca$^{2+}$] with a threshold of ~200 nM (4, 5). However, in tracheal and arterial smooth muscle cells, the activation of $I_{\text{Cl(Ca)}}$ was augmented by inhibitors of Ca$^{2+}$/calmodulin-dependent kinase, (5, 6) and internal dialysis with constitutively active CaMKII suppressed $I_{\text{Cl(Ca)}}$ in pulmonary artery myocytes (6). These data revealed an inhibition of channel activity contemporaneously with the generation of the current.

However, these studies did not take into account other possible Ca$^{2+}$-dependent pathways. The aim of the present study was to assess whether $I_{\text{Cl(Ca)}}$ activity in rabbit pulmonary artery (PA) myocytes is also influenced by Ca$^{2+}$-dependent dephosphorylation. Calcineurin (CaN) is a heterodimeric serine/threonine protein phosphatase that is involved in a number of cellular responses (7–9). CaN is composed of a catalytic subunit (CaNA) that is activated by Ca$^{2+}$–binding to its regulatory subunit (CaNB) and by the binding of the Ca$^{2+}$/calmodulin complex (Ca$^{2+}$/CaM). The catalytic subunit of calcineurin (CaN) exists in three distinct isoforms (α, β, and γ), each encoded by a separate gene, and isoform-specific substrates have been identified (10). Western blot analysis indicated that the α and β isoforms are expressed in pulmonary arteries but that only the CaNA-α isoform translocates from the cytosol to the membrane following an elevation of intracellular Ca$^{2+}$ concentration. Intracellular dialysis with constitutively active CaNA-α produced a large enhancement of $I_{\text{Cl(Ca)}}$ elicited by 500 nM Ca$^{2+}$-containing pipette solutions that was attenuated by the co-dialysis with a peptide inhibitor of CaN. In comparison, CaNA-β had no stimulatory effect on $I_{\text{Cl(Ca)}}$ in PA myocytes. Consequently, through the use of recombinant calcineurin, we show that the regulation of $I_{\text{Cl(Ca)}}$ by this phosphatase is highly dependent on the calcineurin isoform. This study, in association with our earlier work with CaN inhibitors in coronary artery smooth muscle cells (11), reveals the crucial influence of calcineurin on calcium-activated chloride channels and highlights the complex pathways that govern Ca$^{2+}$-dependent Cl$^-$ activity in vascular myocytes.

The abbreviations used are: $I_{\text{Cl(Ca)}}$, Ca$^{2+}$-dependent Cl$^-$ currents; CaN, calcineurin; CaMKII, calcium/calmodulin-dependent kinase II; CaA, cyclosporin A; pF, picosfarsad; PA, pulmonary artery; TRITC, tetramethylrhodamine isothiocyanate; BAPTA, 1,2-Bis(2-aminophenoxy)ethane-$N,N',N$-tetraacetic acid.

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**MATERIALS AND METHODS**

Single Cell Electrophysiology—Cells were prepared from the main and second branch pulmonary arteries isolated from New Zealand White rabbits (2–3 kg) as described previously (6, 11–13). After isolation, cells were stored in a low Ca$^{2+}$-physiological salt solution at 4°C and used within 6 h. The composition of the modified physiological salt solution was as follows: NaCl (120 mM); NaHCO$_3$ (25 mM) (pH 7.4 after bubbling with 95% O$_2$, 5% CO$_2$ gas); KCl (4.2 mM); KH$_2$PO$_4$ (1.2 mM); MgCl$_2$ (0.05 mM); CaCl$_2$ (0.05 mM); CsCl (0.05 mM); NaH$_2$EDTA (0.1 mM); and glucose (11 mM). $I_{\text{Cl(Ca)}}$ were recorded predominantly in the whole-cell voltage clamp mode and were evoked directly by pipette solutions containing free Ca$^{2+}$ set at 500 nM.

For experiments in which $I_{\text{Cl(Ca)}}$ was elicited by 500 nM Ca$^{2+}$, the external solution contained: NaCl (126 mM); Hepes-NaOH (10 mM), pH 7.4; glucose (20 mM); CaCl$_2$ (1.8 mM); MgCl$_2$ (1.2 mM); and tetrathylammonium chloride (20 mM). The pipette solution contained: tetrathylammonium chloride (20 mM); CsCl (106 mM); Hepes (5 mM); BAPTA (10 mM); MgATP (3 mM); GTP (0.2 mM); and MgCl$_2$ (0.42 mM) and pH was set to 7.2 by the addition of CaOH. Free Ca$^{2+}$ was set by adding an appropriate amount of CaCl$_2$ (7.5 mM) determined by the EQCAL buffer program (Biosoft, Ferguson, MO) and was independently verified using a Ca$^{2+}$-sensitive electrode (Thermo Orion, Model 93–20, Beverly, MA) and calibrated Ca$^{2+}$-solutions available from a commercial source (World Precision Instruments, Inc., CALBIF-2, Sarasota, FL). Sustained Ca$^{2+}$-activated Cl$^-$/currents generated by this technique have been characterized fully in previous studies (6, 11, 12). The main voltage step protocol used in these experiments was the same as that used in earlier studies to characterize $I_{\text{Cl(Ca)}}$. In the perforated patch experiments, the pipette solution contained CsCl (126 mM), Hepes (5 mM), EGTA (5 mM), amphotericin (300 µg ml$^{-1}$ from a 60 mg ml$^{-1}$ stock in dimethyl sulfoxide), and pH set to 7.2 by the addition of CaOH. For those experiments, the external solution was identical to that used in experiments in which $I_{\text{Cl(Ca)}}$ was evoked by 500 nM Ca$^{2+}$ as described above. All of the enzymes, MgATP, BAPTA, ionomycin, and ML-7, were purchased from Sigma. Cyclosporin A was purchased from Calbiochem (EMD Biosciences, San Diego, CA), and calcineurin inhibitory peptide (CaN-AIP) was from Biomol Research Laboratories (Plymouth Meeting, PA).

**Synthesis of Constitutively Active Calcineurin**—Constitutively active calcineurin isoforms were created by introducing stop codons into the cDNA for the catalytic subunit, CaNA, causing the translated CaNA subunits to truncate immediately C-terminal of the CaM-binding domain and delete the auto-inhibitory domain. All methodologies for cDNA manipulation, baculovirus screening, and purification of CaN using monolayer cultures of SF21 cells have been described previously (7, 10, 14).

**Immunofluorescence and Confocal Imaging**—The immunocytochemical detection of CaN isoforms in single PA myocytes was performed as described for coronary artery myocytes by Ledoux et al. (11) using polyclonal goat anti-calcineurin A-α and A-β antibodies (Santa Cruz Biotechnology) both at a dilution of 1:50. These antibodies are reported by Santa Cruz Biotechnology to recognize a C-terminal epitope in human, rat, and mouse CaNA-α or CaNA-β, respectively. However, the amino acid sequences of rabbit CaNA-α (GenBank™ accession number AAN23152) and CaNA-β (GenBank™ accession number AAN23153) are identical to the human CaNA-α and CaNA-β amino acid sequences, so it is very possible that these antibodies specifically recognized rabbit CaNA-α and CaNA-β in our experiments. Cells were imaged at rest and after stimulation with ionomycin and 500 nM Ca$^{2+}$ to raise intracellular [Ca$^{2+}$] and create an internal environment similar to the conditions of the electrophysiological experiments. Contraction of the myocytes was prevented by incubation with the myosin light chain kinase inhibitor, ML-7 (5 µM). The primary antibodies were diluted in phosphate-buffered saline containing 1% normal donkey serum and 0.04% Triton X-100. Negative control experiments were performed by repeating the above steps in the absence of primary antibodies. Coverslips containing the cells were washed three times in phosphate-buffered saline and exposed to a Cy3-coupled anti-mouse antibody (Alexa 647; Molecular Probes) and a TRITC-coupled anti-rabbit antibody (Alexa 546; Molecular Probes) at a dilution of 1:400 for 1 h in the dark at room temperature. Solutions of both secondary antibodies were prepared in goat and diluted in 1% normal donkey serum and 0.04% Triton X-100 (Jackson Immunoresearch Laboratories, Inc.). The bar graphs shown in Fig. 2, C and D, are the mean ± S.E. ratio of membrane/cytosol fluorescence intensity for CaNA-α and CaNA-β taken from a cross-sectional line scan.

**Fig. 1. Western blot analysis of CaNA-α and CaNA-β from PA smooth muscle**. Western blot analysis was performed using antibodies specific for CaNA-α or CaNA-β on PA smooth muscle lysates (lane 1, 50 µg) and purified CaNA-α (1 µg) or CaNA-β (0.5 µg) expressed in SF9 cells (lane 2).

**Fig. 2. Immunostaining of CaNA-α and CaNA-β in freshly isolated PA myocytes.** Panels A and B show representative images of immunocytochemistry experiments on PA myocytes using antibodies specific for CaNA-α (green) and CaNA-β (red). In panel A, cells were bathed in a normal extracellular solution, whereas in B, the cells had been preincubated in the external solution containing 80 nM ionomycin, 3 µM ML-7, and 500 nM Ca$^{2+}$ for 15 min prior to fixation to raise intracellular [Ca$^{2+}$]. Quantification of data from the experiments shown in A and B is shown in panels C and D. Panel C represents the mean results of experiments where CaNA-α and CaNA-β antibodies were applied individually (CaNA-αControl (n = 4), 500 nM Ca$^{2+}$ (n = 6) and CaNA-β-Control (n = 5), 500 nM Ca$^{2+}$ (n = 5)). Panel D shows the mean ± S.D. data from dual labeling experiments (Control (n = 4), 500 nM Ca$^{2+}$ (n = 5)). For panels C and D, the open and solid bars refer, respectively, to myocytes exposed to normal external solution (1.8 mM Ca$^{2+}$) labeled with the CaNA-α or CaNA-β antibodies, whereas the right-slanted and left-slanted hatched bars correspond to myocytes exposed to 500 nM of free Ca$^{2+}$ solution containing 80 nM ionomycin and 3 µM ML-7, which were dually labeled with CaNA-α and CaNA-β antibodies.
of an arbitrary region of the cell located outside the nuclear region. Fluorescence intensity of ~5–10 pixels wide on the two sides of the membrane spanned by the line scan was averaged and normalized to averaged fluorescence intensity in the cytoplasm.

Western Blot Analysis of Rabbit Pulmonary Artery Smooth Muscle—50 μg of protein from the 1000 × g supernatant of homogenized rabbit PA smooth muscle was separated by 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose sheets. Protein concentrations were determined by the Bradford assay using bovine γ-globulin as standard. The blots were probed with Santa Cruz Biotechnology goat anti-CaNA-α or goat anti-CaNA-β antibodies (1:10,000 dilution) followed by horseradish peroxidase-conjugated rabbit anti-goat IgG (1:50,000 dilution) antibodies. Immunodetection was carried using ECL Advance from Amersham Biosciences, and the TIFF images were collected with a CCD camera imaging system (Labworks, UVP Inc.). Densitometry was carried out using Un-Scan-It (Silk Scientific).

Statistics—All of the data are the mean ± S.E. of n cells from at least two different animals. Significance was taken with p values below 0.05.

RESULTS

Endogenous Expression of Distinct Isoforms of Calcineurin A—Western blot analysis was performed using tissue lysates derived from endothelium-denuded pulmonary arteries. Antibodies selective for CaNA-α and CaNA-β revealed that both isoforms were expressed, and a comparison with purified CaN standards showed that ~0.56 μg of CaNA-α and 0.3 μg of CaNA-β were present (Fig. 1). Consequently, PA smooth muscle expresses both the α and β variants of CaNA but the α appears to be slightly more abundant. The use of a CaN antibody that was non-specific for these isoforms revealed that this enzyme translocated to the membrane of coronary artery myocytes upon a rise of intracellular [Ca2+]i (11). We used antibodies that were specific for the α and β isoforms of CaNA to examine the cellular distribution of the individual isoforms in freshly dissociated PA myocytes under resting conditions and after raising intracellular [Ca2+]i. Under control conditions, both isoforms appeared evenly distributed between the cytosol and membrane (Fig. 2A). After the cells were exposed to a medium containing 80 nM ionomycin, 3 μM ML-7 (to inhibit myosin light chain kinase and thus contraction; see Ref. 11) and 500 nM Ca2+ designed to raise [Ca2+]i, CaNA-α translocated toward the plasma membrane, whereas CaNA-β remained for the most part distributed homogeneously throughout the cytoplasm and membrane (Fig. 2A). Consequently, the ratio of optical density of immunofluorescence intensity for CaNA-α labeling at the membrane over cytosol was ~2.5 regardless of whether the immunodetection involved single (Fig. 2C) or dual (Fig. 2D) antibody experiments. The preferential localization of CaNA-α was not observed when the extracellular solution did not contain Ca2+. These results reveal that in PA cells CaN translocation in response to an elevation of intracellular Ca2+ concentration is isoform-specific.

Effect of CaN Inhibition on Ca2+-activated Cl− Channels in PA Myocytes—The influence of endogenous CaN-mediated dephosphorylation on I_cl(Ca) was investigated using the CaN-specific blocker, cyclosporin A (CsA), which is a neutral lipophilic cyclic undecapeptide that binds to cyclophilin A to form a complex that suppresses CaN activity by interfering with the active site of the catalytic domain (8, 9). Initial experiments were performed using the perforated patch variant of the whole-cell recording configuration, and I_cl(Ca) was elicited by promoting Ca2+ influx through voltage-dependent calcium channels as characterized in previous studies on various types of smooth muscle cell (e.g. 15–17). Calcium channels were opened by depolarization from the holding potential of −60 mV, and I_cl(Ca) was manifest as an outward current at test potentials positive to the theoretical chloride equilibrium (−0 mV) and as a slowly declining inward current upon repolarization to the holding potential (Fig. 4A). The application of 2 μM CsA for 180 s had a small insignificant effect (p = 0.09) on the voltage-gated calcium current at +10 mV (Fig. 3, A and B) but pro-
Fig. 4. Variable effects of cyclosporin A on sustained $I_{\text{Cl(Ca)}}$ evoked by 500 nM Ca$^{2+}$ in PA myocytes. A shows representative $I_{\text{Cl(Ca)}}$ evoked by 500 nM Ca$^{2+}$ recorded under control conditions at different times upon achieving whole cell access ($t = 0, 40,$ and 80 s). Voltage-dependent relaxations were generated by depolarization from -50 to +70 mV followed by repolarization to -80 mV. Scalars show 250 ms and 200 pA. B shows the mean rundown of $I_{\text{Cl(Ca)}}$ over the first 2 min of recording. The vertical axis shows the normalized amplitude of the outward relaxation as defined in panel A. The horizontal axis is the time after the rupture of the membrane. Each point is the mean of 22 cells ± S.E. C shows a family of $I_{\text{Cl(Ca)}}$ currents evoked by 500 nM Ca$^{2+}$ in the absence and presence of 2 μM CsA. Scalars represent 2.5 pApF$^{-1}$ and 500 ns. Cells were held at -50 mV and stepped to potentials between -90 and +110 mV. Panels D and E show the mean current voltage relationship for currents evoked by 500 nM Ca$^{2+}$ in the absence (▲) and presence (◆) of 2 μM CsA. D shows data from an initial study taken from eight cells isolated from three animals. E shows data from a second study again involving cells from two animals ($n = 6$ cells). F is a comparison of control currents measured in the absence of CsA during studies 1 (filled triangles) and 2 (open triangles). All of the data shown in panels D–F were collected after 6 min of cell dialysis. All of the points are the mean ± S.E. of 6–8 cells.

duced a 39 ± 10% inhibition of the inward Cl$^{-}$ tail current at -60 mV ($n = 5; p = 0.006, \text{Fig. 3C}$) that was associated with an increase in its rate of decay (Fig. 3D).

To isolate an effect of CsA on CaN-mediated dephosphorylation of the underlying Cl$^{-}$ channel as opposed to possible effects on the voltage-dependent calcium channels or Ca$^{2+}$-homeostatic mechanisms, experiments were performed where $I_{\text{Cl(Ca)}}$ was evoked directly by pipette solutions containing 500 nM Ca$^{2+}$ and 3 mM ATP. With this pipette solution, the rupture of the cell membrane in PA myocytes to achieve whole-cell mode resulted in a large inward current at the holding potential of -50 mV and the generation of time-dependent outward relaxations following depolarization to +70 mV (Fig. 4). Under the ionic conditions used, these currents represent sustained $I_{\text{Cl(Ca)}}$ that have been characterized extensively in vascular smooth muscle cells including PA myocytes (6, 11–13). In accord with previous observations (12), the amplitude of $I_{\text{Cl(Ca)}}$ at -50 mV declined progressively to a steady-state level over ~2 min following rupture that was associated with a reduction in the amplitude of the outward relaxation at +70 mV (Fig. 4, A and B). Following the initial period of rundown, the amplitude of $I_{\text{Cl(Ca)}}$ remained at a constant level ~20% of the initial amplitude for the remainder of the experiment. Currents were elicited in cells bathed in normal external solution and alternated with cells incubated in 2 μM CsA for 10 min before rupturing the membrane seal to gain whole-cell access. Incubation of cells in CsA significantly attenuated the amplitude of $I_{\text{Cl(Ca)}}$ generated by 6 min of cell dialysis with 500 nM Ca$^{2+}$ in seven cells from three animals (Fig. 4D), and the mean late current at +70 mV was 501 ± 70 and 263 ± 51 pA in the absence and presence of CsA ($p = 0.043$). In an additional study, CsA had no apparent effect on the amplitude of $I_{\text{Cl(Ca)}}$ (Fig. 4E). However, in these cells, the control current was significantly smaller (see Fig. 4F) that was associated with a more prominent rundown of $I_{\text{Cl(Ca)}}$ upon membrane rupture. These data show that CsA was able to inhibit sustained $I_{\text{Cl(Ca)}}$ and the effectiveness of this agent was proportional to the amplitude of the control currents. Overall, these experiments show that the suppression of endogenous CaN by CsA diminishes the amplitude of $I_{\text{Cl(Ca)}}$ in PA smooth muscle cells but also suggest that CaN is particularly labile in the whole-cell configuration resulting in variable effects of the phosphatase inhibitor.

**Effect of Dialysis with Constitutively Active Forms of CaN**—To circumvent any variable influence due to endogenous CaN and to shift the cellular status in favor of dephosphorylation, we undertook experiments using pipette solutions enriched with constitutively active recombinant CaN isoforms. The inclusion of CaNA–α in a pipette solution containing 500 nM Ca$^{2+}$ attenuated the initial rundown observed upon rupture of the cell membrane (Fig. 5A), and this was followed by a progressive enhancement of current amplitude over the next 20 min. Consequently, intracellular dialysis with CaNA–α augmented considerably the amplitude of $I_{\text{Cl(Ca)}}$ (Fig. 5, B and C). After a 6-min recording, the mean current at the end of a step

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Fig. 5. Effects of constitutively active CaN on $I_{\text{Cl(Ca)}}$. Panel A shows the time course of changes of $I_{\text{Cl(Ca)}}$ activated by 500 nM Ca$^{2+}$ in the absence (●) or presence of 500 nM CaN-α (○). The point of membrane rupture is shown by an asterisk. The vertical axis shows the amplitude of $I_{\text{Cl(Ca)}}$, recorded at the end of a 750-ms depolarization to +90 mV normalized to cell size. The horizontal axis is the recording time following cell access. Panel B shows examples of $I_{\text{Cl(Ca)}}$ activated by 500 nM Ca$^{2+}$ in the absence (Control) or presence of 500 nM CaN-α 6 min after achieving whole cell access. Cells were held at −50 mV and stepped to potentials between −90 and +110 mV. C shows the mean amplitude of $I_{\text{Cl(Ca)}}$ at the end of the test pulse in the presence of CaN-α (●), boiled CaN-α (■), or under control conditions (○). Each point is the mean ± S.E. of 7, 12, and 4 cells, respectively, with error bars denoting the mean ± S.E. D shows the time constant for current activation at +130 mV and current decay at −80 mV in the absence (stripes) and presence (open) of CaN-α. For panels C and D, the data were all collected after 6 min of cell dialysis. Each column is the mean ± S.E. of 7–9 cells.

Similar to previous studies (6, 12), currents evoked by 500 nM Ca$^{2+}$ in the absence and presence of CaN-α reversed ($E_{\text{rev}}$) close to the theoretical chloride equilibrium potential ($+2$ mV) at +6 ± 2 and +6 ± 1 mV (n = 4), respectively. Replacement of external NaCl with sodium thiocyanate shifted $E_{\text{rev}}$ to −40 ± 1 and −42 ± 5 mV, respectively (Fig. 6). These data show that the large current recorded in the presence of CaN-α was not due to the de novo activation of a contaminating current but was due to an enhanced activation of $I_{\text{Cl(Ca)}}$.

The stimulatory effects of CaN-α were abolished by co-dialysis with CaN-AIP (Fig. 7A), a peptide inhibitor of CaN that mimics the auto-inhibitory domain (6, 10). In these experiments, $I_{\text{Cl(Ca)}}$ at +90 mV with CaN-α alone was 68 ± 14 pA pF$^{-1}$ (n = 6), but in combination with 100 μM CaN-AIP, the mean current was +90 mV was 19 ± 4 pA pF$^{-1}$ (n = 5). In contrast to the striking data with CaN-α, the β isoform failed to enhance $I_{\text{Cl(Ca)}}$. In these experiments, a pipette solution containing 500 nM CaN-α was alternated with one containing 500 nM CaN-β and the mean $I_{\text{Cl(Ca)}}$ values at +90 mV were 56 ± 11 and 15 ± 4 pA pF$^{-1}$, respectively (n = 9 both groups, Fig. 7B). These data not only reveal a high degree of isoform specificity but also show that the stimulation produced by CaN-α was not the result of a nonspecific effect due to the cell dialysis of a foreign protein. This point was supported by the observation that CaN-α did not enhance currents evoked by pipette solutions containing 10 mM BAPTA with no added Ca$^{2+}$ (effectively zero Ca$^{2+}$, Fig. 7D). These experiments also reveal that CaN-dependent dephosphorylation “alone” cannot stimulate $I_{\text{Cl(Ca)}}$, and establish that CaN is a crucial regulator of Cl$^{-}$ channel activity but is not the impetus for channel activation.

**DISCUSSION**

The findings of the present study reveal CaN to be an important regulator of $I_{\text{Cl(Ca)}}$ in PA smooth muscle cells and demonstrate that the modulation of the underlying channels is mediated solely by CaN-α. Our data show that the inhibition of endogenous CaN with the specific agent, cyclosporin A, reduced the amplitude of $I_{\text{Cl(Ca)}}$ evoked either directly by pipette solutions containing 500 nM Ca$^{2+}$ or as a consequence of Ca$^{2+}$ influx through voltage-dependent Ca$^{2+}$ channels. These data are consistent with our previous observation in coronary cells that endogenous CaN is an important regulator of $I_{\text{Cl(Ca)}}$ in vascular smooth muscle cells (11). The enrichment of the pipette solution with a constitutively active form of CaN-α augmented markedly the amplitude of $I_{\text{Cl(Ca)}}$ allied with a considerable change in the voltage-dependent kinetics. Remarkably, this effect was not shared by the β isoform of CaN, although Western blot analysis revealed that both α and β isoforms are expressed at significant levels in the PA. Consistent with a specific role of CaN-α for regulating $I_{\text{Cl(Ca)}}$ in PA cells was the observation that this isoform translocated toward
the plasma membrane following elevation of intracellular Ca\(^{2+}\) levels, a property not shared by CaN-B.

**Specific Regulation of I\(_{Cl(Ca)}\) by CaN-alpha in Pulmonary Artery Smooth Muscle Cells**—CaN-dependent dephosphorylation influences a number of cellular processes including the regulation of transcription factors, synaptic vesicle recycling, and cardiac muscle hypertrophy (8, 9). CaN has also been shown to modulate a number of cation channels including voltage-dependent and ATP-sensitive K\(^{+}\) channels in smooth muscle cells (18, 19), but data on Cl\(^{-}\) channels are sparse. Recently, we reported that inhibitors of CaN reduced the amplitude of I\(_{Cl(Ca)}\) in coronary artery myocytes in a Ca\(^{2+}\)-dependent manner and that these agents decreased the apparent binding affinity of the Cl\(^{-}\) channel for Ca\(^{2+}\) (11). This study shows that the CaN inhibitor CsA reduces the amplitude of I\(_{Cl(Ca)}\) in PA cells activated as a consequence of Ca\(^{2+}\) influx through voltage-dependent calcium channels or evoked directly by pipette solutions containing 500 nM free Ca\(^{2+}\). Moreover, CsA was observed to be relatively ineffective on currents recorded from cells where the control currents were relatively small. These data suggest that the activity of CaN may vary among populations of myocytes and that these agents decrease the apparent binding affinity of the Cl\(^{-}\) channel for Ca\(^{2+}\). This study demonstrates that the apparent binding affinity of the Cl\(^{-}\) channel for Ca\(^{2+}\) is decreased by CaN inhibitors in PA cells but has no effect on this current in coronary artery cells (6), although effects are observed when the channel is stimulated by 1 \(\mu\)M free Ca\(^{2+}\) (11). However, peptide and inorganic inhibitors of CaN consistently inhibited I\(_{Cl(Ca)}\) elicited by 500 nM Ca\(^{2+}\) in coronary artery myocytes (11). Consequently, CaN-mediated dephosphorylation in PA myocytes may be subjugated by an overwhelming activity of CaMKII (and perhaps other kinases). The rundown of I\(_{Cl(Ca)}\) activity seen in PA cells following activation is likely to reflect a change in the kinase/phosphatase balance in the vicinity of the channel. This hypothesis was corroborated by the use of constitutively active CaN. It is worth stressing that in perforated patch experiments CsA consistently suppressed inward tail I\(_{Cl(Ca)}\) and altered deactivation kinetics without influencing peak I\(_{Cl(L)}\). These data lend support to the notion that I\(_{Cl(Ca)}\) may be physiologically regulated by CaN in conditions minimizing intracellular dialysis and infer that CaN may have a relatively greater impact on I\(_{Cl(Ca)}\) regulation when the stimulating rise in [Ca\(^{2+}\)]\(_i\) is transient. Under these conditions, the activation of both CaMKII and CaN by Ca\(^{2+}\)/CaM would be less than that observed with a sustained rise in [Ca\(^{2+}\)]; however, CaN has a greater Ca\(^{2+}\) sensitivity than CaMKII (8, 20), the influence of the phosphatase is likely to dominate.

**Unique Regulation of I\(_{Cl(Ca)}\) by Calcineurin A-alpha**—One novel finding of our study was the isoform-specific enhancement of I\(_{Cl(Ca)}\) produced by the inclusion of a constitutively active form of CaN-alpha. The reversal potential of currents evoked by 500 nM Ca\(^{2+}\) in the absence (A and B) or presence of (C and D) CaN-alpha were determined using a two-pulse protocol as shown at the bottom. All of the recordings were obtained after 15 min of cell dialysis. Cells were bathed initially in an external solution containing 126 mM NaCl (A and C) and then replaced by one containing 126 mM sodium thiocyanate (B and D). Arrows indicate the potential at which the current was close to the reversal potential.
of CaN. Whereas both CaNA-α and CaNA-β were shown to be expressed in pulmonary arteries, only intracellular dialysis with CaNA-α modulated $I_{\text{Cl(Ca)}}$, although the α and β forms of the catalytic A domain are 81% identical at the amino acid level (21) and have a similar $Ca^{2+}$ dependence. Co-application of CaNA-α with a peptide fragment analogous to the auto-inhibitory domain confirmed that the effects of CaNA-α were due to a specific phosphatase action. Moreover, immunocytochemical experiments revealed that only endogenous CaNA-α, but not CaNA-β, translocated toward the membrane under conditions mimicking our patch clamp experiments with internal $Ca^{2+}$ clamped at 500 nM. CaN heterodimers containing CaNA-α and CaNA-β catalytic subunits exhibit a similar $Ca^{2+}$ dependence, as this is conferred by the B subunit, but display different substrate affinities and catalytic activities in vitro (10). Moreover, trans- genetic approaches have revealed specific functions for the CaNA isoforms. For example, CaNA-β is required for T-cell proliferation as well as cardiac hypertrophy (22, 23), whereas CaNA-α(-/-) mice display hyperphosphorylated $\tau$-proteins in the brain and altered post-synaptic de-potentiation in the hippocampus (24, 25). This study is the first to show isoform-selective effects on a native ion channel and suggest that the expression of the α isoform of CaNA is the crucial determinant of the phosphorylation status of the $Ca^{2+}$-activated Cl$^{-}$ channel. Consequently, the relative expression of CaNA-α or differences in the α:β ratio of CaN heterodimers between different vascular smooth muscle tissues would be expected to alter the level of $I_{\text{Cl(Ca)}}$ regulation by this phosphatase. This exquisite property allows the regulation of $I_{\text{Cl(Ca)}}$ by CaN to be fine-tuned by the cell through alterations in the composition of CaN heterodimers. Moreover, the inability of CsA to suppress $I_{\text{Cl(Ca)}}$ when the control amplitude was relatively small suggests strongly that the generation of $I_{\text{Cl(Ca)}}$ was reliant upon the level of CaN activity. The precise mechanism conferring specificity of CaNA-α on $I_{\text{Cl(Ca)}}$ cannot be deduced from our data and will require further investigation. Fig. 8A shows the basic features of the structural domains of CaN and the percentage of amino acid sequence identity of the rat brain α and β isoforms (26). The greatest sequence divergence between these two isoforms is observed at the N and C termini (<30%) and to a lesser extent at the Linker I region (60%). An interesting characteristic of the β isoform is the presence of 10 proline residues at the N terminus, a sequence not shared by the α isoform (Fig. 8B). Such a sequence could make this domain a target of interacting proteins, which could obstruct or limit access of the phosphatase to the target protein. Future studies will be undertaken to compare the effects of various chimeric constructs of the two isoforms on the anion current.

As yet, the molecular nature of the protein underlying $I_{\text{Cl(Ca)}}$ remains undefined (27) and therefore precise information as to how dephosphorylation heightens $I_{\text{Cl(Ca)}}$ activity can only be speculated. Our data show unequivocally that $Ca^{2+}$-dependent dephosphorylation does not gate the opening of the Cl$^{-}$ channel by a rise in $[Ca^{2+}]$, as CaNA-α failed to generate $I_{\text{Cl(Ca)}}$ when the internal solution contained 10 mM BAPTA only (i.e. pipette $[Ca^{2+}]$ was in the low nanomolar range). The channel properties underlying the kinetics of $I_{\text{Cl(Ca)}}$ elicited by the technique used in this study have been characterized in non-muscle cells.
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A

| N-term | phosphatase domain | CnB-BD | linker I | CnM-BD | linker II | Inh-D | C-term |
|--------|--------------------|--------|---------|--------|----------|-------|--------|
| 29%    | 91%                | 100%   | 60%     | 96%    | 81%      | 84%   | 27%    |

Percentage of amino acid sequence identity

B

N terminus

CaNA-α

1

M S E T P A I D K L ST T R V V K A V P F P

CaNA-β

M A P E P A R A A P P P P P F P L G A R V V K A V P F P

C terminus

CaNA-α

484

F P R K D A M P S E A M N S G L K N L A G S T N G D S N G S N S S N I Q

CaNA-β

494

F P R K D A V Q C C - P N S L I N A H T T E N C H C G N H S A Q

521

525

Panel A shows the basic structural domains of calcineurin and the percentage of amino acid sequence identity for the rat brain CaNA-α and CaNA-β based on the report by Kuno et al. (26). N-term and C-term, N- and C-terminal ends, respectively; CnB-BD, calcineurin B-binding domain; CnM-BD, calcineurin M-binding domain; Inh-D, auto-inhibitory domain. Panel B shows the alignment of the amino acid sequence of the N and C termini of rat brain CaNA-α and CaNA-β. The numbers above or below the sequences indicate the linear sequence order of the amino acid for each isoform. Identical amino acids are highlighted by boxes. Notice the long sequence of proline residues at the N terminus of CaNA-α, which is not present in CaNA-β.

(28, 29). The voltage-dependent outward relaxation reflects an increase in open probability due to an increase in the binding affinity for Ca^{2+} and a slower rate of channel closure, whereas the exponentially declining inward current at negative potentials is a simple approximation of the channel deactivation. As CaNA-α increased the rate of activation at positive potentials and slowed that rate of decay at negative potentials, these observations suggest that the removal of phosphate groups either increases the apparent binding affinity or slows the rate of channel closure. These questions will be addressed in future experiments.

This study shows that CaN is a crucial regulator of I_{Cl(Ca)} in PA cells and that this regulation exhibits a high degree of isoform selectivity. However, the effects of CaN rest in a delicate balance with the suppressive effects of CaMKII and probably other kinases and it is the relative contribution of these enzymes that dictates the amplitude of I_{Cl(Ca)}. A corollary to this point is that the relative dominance of CaMKII and CaN probably differs between smooth muscles and will alter with different [Ca^{2+}]. In view of its greater sensitivity to Ca^{2+}, CaN would be expected to dominate I_{Cl(Ca)} regulation at lower [Ca^{2+}], whereas CaMKII will predominate when [Ca^{2+}] is raised (i.e. during agonist stimulation). A necessary caveat to this generalized hypothesis is that other Ca^{2+}-independent phosphatases that have not been tested in this study may also regulate I_{Cl(Ca)}.

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