Caveolae Localize Protein Kinase A Signaling to Arterial ATP-Sensitive Potassium Channels

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Abstract—Arterial ATP-sensitive K⁺ (Kₐtp) channels are critical regulators of vascular tone, forming a focal point for signaling by many vasoactive transmitters that alter smooth muscle contractility and so blood flow. Clinically, these channels form the target of antianginal and antihypertensive drugs, and their genetic disruption leads to hypertension and sudden cardiac death through coronary vasospasm. However, whereas the biochemical basis of Kₐtp channel modulation is well-studied, little is known about the structural or spatial organization of the signaling pathways that converge on these channels. In this study, we use discontinuous sucrose density gradients and Western blot analysis to show that Kₐtp channels localize with an upstream signaling partner, adenylyl cyclase, to smooth muscle membrane fractions containing caveolae, a protein found exclusively in cholesterol and sphingolipid-enriched membrane invaginations known as caveolae. Furthermore, we show that an antibody against the Kₐtp pore-forming subunit, Kir6.1 co-immunoprecipitates caveolin from arterial homogenates, suggesting that Kir6.1 and caveolin exist together in a complex. To assess whether the colocalization of Kₐtp channels and adenylyl cyclase to smooth muscle caveolae has functional significance, we disrupt caveolae with the cholesterol-depleting agent, methyl-β-cyclodextrin. This reduces the cAMP-dependent protein kinase A–sensitive component of whole-cell Kₐtp current, indicating that the integrity of caveolae is important for adenylyl cyclase–mediated channel modulation. These results suggest that to be susceptible to protein kinase A–dependent activation, arterial Kₐtp channels need to be localized in the same lipid compartment as adenylyl cyclase; the results also provide the first indication of the spatial organization of signaling pathways that regulate Kₐtp channel activity. (Circ Res. 2004;95:1012-1018.)

Key Words: Kₐtp channel ■ adenylyl cyclase ■ caveolae ■ compartmentation ■ protein kinase A

Arterial tissues were obtained from adult male Wistar rats (~300 g) (Charles River Laboratories, Inc, Margate, UK) killed by stunning rather than the 300 g described in the original text.

Materials and Methods

Animals
Arterial tissues were obtained from adult male Wistar rats (~300 g) (Charles River Laboratories, Inc, Margate, UK) killed by stunning.
and rapid cerebral dislocation. The care and euthanasia of animals conformed to the requirements of the UK Animals (Scientific Procedures) Act 1986.

Antibodies, Polyacrylamide Gel Electrophoresis, and Immunoblotting

The following primary antibodies were used: anti-adaptin, anti-caveolin 1, anti-caveolin 2, anti-caveolin 3, and anti-protein kinase RIβ (BD Transduction Laboratories); anti-adenylly cyclase 3, anti-adenylly cyclase 5/six, anti-Kir6.1 (R-14; sc-11224), and associated blocking peptide (sc-11224P) and anti-protein kinase RIIα (Santa Cruz Biotechnology); anti-smooth muscle α-actin (Sigma-Aldrich). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were from Jackson Immunchemicoal Laboratories. HRP-conjugated goat secondary was from Sigma-Aldrich. Protein extracts were resolved by SDS-polyacrylamide gel electrophoresis on 12% polyacrylamide-Tris gels and transferred electrophotically onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech). Immunoblotting was performed as described previously.17

Fractionation of Caveolin-Enriched Membrane and Assay for Cholesterol

Rat aortic smooth muscle cells were isolated enzymatically using methodology described previously.17 Cells were cultured in high-glucose Dulbecco Modified Eagle Media (DMEM) supplemented with 15% fetal calf serum, penicillin (50 U/mL), streptomycin (50 μg/mL), and fungizone (2.5 μg/mL). All media and reagents were from Life Technologies, Inc. Cells were maintained at 37°C in a humidified atmosphere (10% CO2) and harvested between passages 12 to 15. Smooth muscle cell identity was confirmed by immunocytochemical staining with a smooth muscle–specific α-actin antibody. Buoyant caveolae-enriched membrane fractions were isolated under detergent-free conditions from rat aortic smooth muscle cell homogenates by ultracentrifugation on discontinuous sucrose gradients using a method adapted from18 as described by.19 Briefly, four large (175 cm2) flasks of confluent aortic smooth muscle cells were laid with 4 mL of 35% sucrose prepared in MBS with 250 mmol/L NaCl, pH 6.5) and loaded in a polyethylene-terephthalate thin-walled ultracentrifuge tube. The sample was overlaid with 4 mL of 35% sucrose prepared in MBS containing a hydrophobic core capable of solubilizing cholesterol and extracting it from membranes. After treatment with methyl-β-cyclodextrin, the degree of cholesterol depletion was assessed by staining a sample of smooth muscle cells with the cholesterol-binding agent filipin according to methodology described previously.21

Electrophysiology

Single smooth muscle cells were isolated enzymatically from small branches of the rat mesenteric artery, as described previously.22 Cells were stored at 4°C, and used on the day of preparation. Whole-cell K+ currents were recorded from single smooth muscle cells using an Axopatch 200b amplifier (Axon Instruments). Recorded membrane currents were filtered at 5 kHz, digitized using a Digidata 1320A interface (Axon Instruments), and analyzed using pCLAMP software. Patch pipettes were pulled from borosilicate glass (outer diameter 1.5 mm, inner diameter 0.86 mm; Clarke Electromedical) and fire polished to give a final resistance of ~5 MΩ when filled. The pipette-filling solution contained (in mmol/L) 110 KCl, 30 KOH, 10 HEPES, 1 MgCl2, 0.1 CaCl2, 1 NaATP, 0.5 GTP; adjusted to pH 7.2. The 6 mmol/L K+ extracellular solution contained (in mmol/L) 134 NaCl, 6 KCl, 1 MgCl2, 0.1 CaCl2, 0.1 MgCl2, 1 NaATP, 10 HEPES; adjusted to pH 7.4. To separate KATP currents, we recorded at ~60 mV to minimize activation of voltage-dependent K+ channels, and raised extracellular [K+] to 140 mM/L to give a substantial inward driving force for K+. In addition, 140 mmol/L K+ extracellular solution contained (in mmol/L) 140 KCl, 1 MgCl2, 0.1 CaCl2, 10 HEPES, 10 glucose; pH 7.4. Pinacidil, glibenclamide, GDPβS, propranolol, 8-SPT, and CGRP 8-37 were from Sigma-Aldrich. 2′,5′-Dideoxyadenosine and Rp-cAMPS (the Rp isomer of adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt) were from Calbiochem. Pinacidil and glibenclamide were dissolved in dimethylsulphoxide (DMSO). The final concentration of DMSO was less than 0.2%. Experiments were conducted in a temperature-controlled bath at 25°C. Results are expressed as mean±SEM. Intergroup differences were analyzed using ANOVA followed by the Student-Newman-Keuls test for multiple comparisons or Student t test for simple comparisons.

Results

We have shown previously that, even in the absence of vasodilators, PKA exerts a sustained, steady-state activation of KATP channels.23 This tonic drive could originate at a number of different levels in the PKA signaling pathway: namely constitutive activity of a G protein–coupled receptor (GPCR), of the G proteins, or of adenylly cyclase itself. To investigate where this sustained activity originates, we compared the effects of the PKA inhibitor Rp-cAMPS on whole cell KATP currents in cells where the PKA signaling pathway had been arrested at specific points. Under control conditions, the addition of 100 mmol/L Rp-cAMPS caused a 21.2±3.7% inhibition (mean±SEM; n=8) of whole cell current induced by the KATP channel opener pinacidil (10 mmol/L) in cells.
PKA-dependent activation of KATP channels arises from tonic inhibition of PKA (Rp-cAMPS inhibition 1.0 μmol/L) (A), a cocktail of antagonists to Gs-coupled receptors (1 μmol/L CGRP 8-37 and 10 μmol/L 8-SPT, 100 μmol/L propanolol) (B), or 500 μmol/L of the adenylyl cyclase inhibitor 2’,5’-dideoxyadenosine (D). Dashed line indicates the zero current level. In all recordings, the cells were dialyzed with a 140 mmol/L K+ solution to increase the inward driving force for K+. Pinacidil (10 μmol/L), Rp-cAMPS (100 μmol/L), and the KATP channel blocker, glibenclamide (10 μmol/L) were applied as indicated. E, Mean inhibition by Rp-cAMPS, and F, mean amplitude of glibenclamide-sensitive current in experiments like those of A through D. (n=8,7,7,6 cells; **P<0.001 *P<0.05, ANOVA followed by Student-Newman-Keuls test).

Isolated from rat mesenteric artery (Figure 1A). This PKA-sensitive component was not affected by intracellular application of the nonhydrolyzable GDP analogue, GDPβS (1 mmol/L) (Figure 1B), or by exposure to a cocktail of antagonists to the major Gs-coupled receptors that functionally regulate KATP channels (1 μmol/L CGRP 8-37 for the CGRP receptor, 10 μmol/L 8-SPT for adenosine receptors, 100 μmol/L propanolol for the β-adrenergic receptor; Figure 1C). These data suggest that the sustained PKA-dependent drive on KATP channels does not originate from constitutive receptor or G-protein activity. In contrast, intracellular application of the adenylyl cyclase inhibitor 2’,5’-dideoxyadenosine (500 μmol/L) significantly reduced pinacidil-evoked KATP current (Figure 1F), and rendered it nearly insensitive to inhibition of PKA (Rp-cAMPS inhibition 1.0±0.5%, n=6, Figure 1D and 1E). These findings indicate that the tonic PKA-dependent activation of KATP channels arises from sustained cAMP production originating from basal adenylyl cyclase turnover. In cardiac myocytes, where adenylyl cyclase also exhibits constitutive activity, basal intracellular cAMP levels are controlled through the activity of phosphodiesterases (PDEs), which limit not only the signaling lifespan of cAMP but also the distance it can diffuse from its site of production. We have previously demonstrated that the downstream target of cAMP, PKA, is anchored in proximity to KATP channels through the action of an A-kinase anchoring protein. If the diffusion range of cAMP is restricted within smooth muscle cells, it follows that KATP channels and their associated kinases must be in the vicinity of cAMP production. Adenylyl cyclase is a comparatively rare component of the plasma membrane, constituting only 0.001% of the total membrane protein, and in smooth muscle is found predominantly in caveolae. Therefore we investigated whether KATP channels also localize to these lipid microdomains.

The unusual lipid composition of caveolae (enriched with cholesterol and sphingolipids) gives them distinct properties as compared with the bulk of the plasma membrane—namely a highly reduced “buoyant” density. We isolated buoyant membrane fractions under detergent-free conditions from rat aortic smooth muscle cell homogenates by ultracentrifugation on discontinuous sucrose gradients. To assess the purity of caveolar and noncaveolar fractions, we used Western blot analysis to determine the distribution of specific marker proteins, and an assay to determine the level of cholesterol within each fraction. As a marker for the buoyant caveolar fraction, we used the protein caveolin. Caveolins bind cholesterol and represent the major structural components of caveolae, coating the whole of the cytoplasmic surface of these organelles. They comprise a family of three distinct 21- to 24-kDa isoforms; caveolin-1 and -2 are widely expressed and most likely form a heterooligomeric complex, whereas caveolin-3 is a muscle-specific isoform. Figure 2A shows Western blot analysis of ten 1-mL fractions collected from top to bottom of the sucrose density gradient. Caveolin-1, was found predominantly in fraction 4 of the gradient, with small residual amounts either side of this layer in fractions 3 and 5. In contrast, β-adaptin, a marker protein for clathrin-coated pits, was largely excluded from the caveolar fractions and localized to the bottom layers of the gradient. Measurement
Kir6.1 with caveolin. Figure 4B shows that antibodies specific to Kir6.1 and caveolin may be via specific protein-protein interactions. This ability to isolate caveolin from smooth muscle homogenates was specific to the Kir6.1 antibody because antibodies directed against the clathrin-coated pit protein, β-adaptn, a protein associated with clathrin-coated pits, was primarily localized to the noncavolar membrane fractions (Figure 3A). In agreement with the work of Ostrom et al,11 adenylyl cyclase isoforms 3 and 5/6 also localized primarily to the caveolar membrane fractions (Figure 3B). Other proteins such as the regulatory subunit of PKA were more evenly distributed between the caveolar and noncaveolar membrane regions.

K<sub>ATP</sub> channels form as 4+4 octomers of Kir6 pore-forming subunits and sulfonylurea receptor (SUR) proteins.27 The dominant channel in most vascular smooth muscle most likely comprises Kir6.1/SUR2B subunits.28 To determine the distribution of K<sub>ATP</sub> channels between the isolated membrane fractions, we used an antibody directed against the Kir6.1 subunit. We detected the presence of Kir6.1 predominantly in the buoyant, caveolin-enriched fraction of the membrane (Figure 4A, top). Importantly, preincubation of the Kir6.1 antibody with a blocking peptide representing the unique antigenic carboxyl-terminal sequence of Kir6.1 specifically reduced the ability of the antibody to detect this protein band (Figure 4A, bottom).

To verify our membrane fractionation results by an independent method, we undertook coimmunoprecipitation experiments. Aside from being the major structural component of caveolae, caveolins also act as scaffold proteins that interact with many caveolar-localized signaling molecules.29 We therefore hypothesized that if Kir6.1 was indeed resident within caveolae it might be possible to coimmunoprecipitate Kir6.1 with caveolin. Figure 4B shows that antibodies specific to Kir6.1 were able to isolate all three isoforms of caveolin from rat aortic homogenates, suggesting that Kir6.1 and caveolin exist together in a complex in intact cells. This complex may arise through direct protein-protein interaction between Kir6.1 and caveolin or by virtue of the fact that these proteins localize to a membrane microdomain that remains intact throughout the immunoprecipitation process. If this latter case is true, the interaction between Kir6.1 and caveolin should be abolished by the disruption of the caveolae membrane compartment. The integrity of lipid microdomains such as caveolae is heavily dependent on cholesterol. Cholesterol depletion profoundly alters caveolar structure leading to a flattening, or total disappearance, of these membrane invaginations and dissociation of sequestered proteins from these domains.15,32 We pretreated strips of rat aorta with the cholesterol-depleting agent, methyl-β-cyclodextrin19,20 for 1 to 2 hours before performing the coimmunoprecipitation assay and found that cholesterol depletion had no effect on the ability of the antibody against Kir6.1 to isolate caveolin (Figure 4B, bottom), suggesting that association between Kir6.1 and caveolin may be via specific protein-protein interactions. This ability to isolate caveolin from smooth muscle homogenates was specific to the Kir6.1 antibody because antibodies directed against the clathrin-coated pit.
protein β-adaptin failed to coprecipitate any caveolin isoforms (Figure 4C).

Biochemical data therefore suggest compartmentation of both K<sub>ATP</sub> channels and adenylyl cyclase in smooth muscle caveolae. To determine whether this colocalization has functional significance, we investigated the effect of disrupting caveolae on the PKA-dependent tonic activation of K<sub>ATP</sub> channels. We pretreated suspensions of isolated smooth muscle cells with methyl-β-cyclodextrin for 1 to 2 hours before recording whole-cell currents induced by the K<sub>ATP</sub> opener, pinacidil (10 μmol/L). Near complete inhibition of tonic PKA drive by the potent blocker of adenylyl cyclase, 2',5'-dideoxyadenosine, did produce a significant reduction in evoked K<sub>ATP</sub> current (Figure 1F). We suggest that to be susceptible to PKA-dependent regulation, K<sub>ATP</sub> channels need to be localized in the same lipid compartment as adenylyl cyclase, and that disruption of the caveolae by cholesterol depletion reduces the PKA-sensitive component of the K<sub>ATP</sub> current by functionally uncoupling adenylyl cyclase and the channel.

**Discussion**

Our findings suggest a functional compartmentation of K<sub>ATP</sub> channels and their upstream signaling partner adenylyl cyclase in specialized signaling “pockets” formed on the surface of smooth muscle cells by caveolae. Arterial K<sub>ATP</sub> channels are subject to sustained tonic activation by cAMP-dependent protein kinase (PKA), which we show originates from the constitutive activity of the enzyme adenylyl cyclase. Physiologically, this tonic K<sub>ATP</sub> channel activation is likely to maintain a background level of channel activity that contributes a vasodilating drive to resting vascular tone. The evidence that K<sub>ATP</sub> channel activity lowers resting vascular resistance is especially clear in the coronary circulation, where K<sub>ATP</sub> channel blockade increases vascular resistance and reduces coronary blood flow in several species including man. These specialized lipid microdomains have previously been implicated in generating subcellular signaling compartments by aggregating interacting signaling molecules. Furthermore, removal of the resting vasodilator contribution of K<sub>ATP</sub> channels in transgenic mice leads to arterial hypercontractility and hypertension.

In smooth muscle cells, adenylyl cyclase resides predominantly in caveolae, small vesicular invaginations of the plasma membrane that associate with the cholesterol-binding protein caveolin. These specialized lipid microdomains have previously been implicated in generating subcellular signaling compartments by aggregating interacting signaling molecules. We find that, like adenylyl cyclase, K<sub>ATP</sub> channels localize to the same cholesterol-enriched smooth muscle membrane fractions as the caveolae marker caveolin. Additionally, an antibody specific to the pore-forming subunit of K<sub>ATP</sub> channels communoprecipitates caveolin from arterial homogenates, suggesting that K<sub>ATP</sub> channels and caveolin exist together in a complex within cells. This association could be via specific protein-protein interactions between Kir6.1 and caveolin, a known scaffold protein that interacts with many caveolae-localized signaling molecules.
or by virtue of the fact that both proteins reside in a lipid compartment that remains intact throughout the immunoprecipitation process. The voltage-gated potassium channel, Kv1.5, for example, targets to caveolae and coimmunoprecipitates with caveolin-1 through Kv1.5-mediated immunolocalization of a protein-lipid complex harboring caveolin. Our results show that exposure to the cholesterol-depleting agent methyl-β-cyclodextrin has no effect on the ability of antibodies directed against Kir6.1 to coprecipitate caveolin, suggesting a tight association between the proteins is maintained following disruption of their native lipid environment. This points to a direct protein-protein interaction between Kir6.1 and caveolin, an idea that is supported by the finding that antibodies against another caveola-resident protein, adenylyl cyclase, failed to coprecipitate caveolin (data not shown).

The integrity of the membrane compartments generated by caveolae seems important in maintaining normal KATP channel regulation. This is demonstrated by the finding that disruption of caveolae by cholesterol depletion significantly reduces the PKA-sensitive component of KATP channel current, indicating that tonic PKA-dependent channel activation may rely on the spatial confinement of adenylyl cyclase and KATP channels. This would be consistent with previous findings in cardiac muscle that show that the distance cAMP can diffuse from its site of production (ie, adenylyl cyclase) is severely restricted by the action of phosphodiesterases. The major target for cAMP, PKA, is therefore a pivotal enzyme in both tonic and receptor-driven regulation of KATP channels and its localization to caveolae, which in other tissues have been shown to be highly enriched with both G protein-coupled receptors and G proteins, makes it likely that larger, more elaborate signaling complexes exist within these domains. Caveolae have already been implicated as integration sites for smooth muscle Ca2+ signaling because of their ability to aggregate proteins involved in Ca2+ regulation and excitation-contraction coupling. The subcellular distribution of the major receptors that couple to arterial KATP channels is largely unknown, but activated receptors for angiotensin II have been shown to be highly enriched with both G protein-coupled receptors and G proteins, making it likely that larger, more elaborate signaling complexes exist within these domains. Caveolae have already been implicated as integration sites for smooth muscle Ca2+ signaling because of their ability to aggregate proteins involved in Ca2+ regulation and excitation-contraction coupling.

The compartmentation of adenylyl cyclase and KATP channels presumably represents just one element of the signaling machinery surrounding these channels. Opening of arterial KATP channels causes membrane hyperpolarization, a decrease in Ca2+ influx through voltage-dependent L-type Ca2+ channels and vasorelaxation. Thus, vasodilators open KATP channels, whereas vasoconstrictors close them, and such modulation probably represents a major component of their physiological regulation. Many vasodilators elevate KATP channel activity by acting at Gs-coupled receptors to stimulate adenylyl cyclase and activate PKA. Additionally, vasoconstrictors such as angiotensin II that activate Gq/11-coupled receptors to inhibit KATP channels via stimulation of protein kinase C may also suppress KATP channel activity by Gq-mediated inhibition of adenylyl cyclase and a reduction in steady-state PKA-dependent channel phosphorylation. Adenylyl cyclase is therefore a pivotal enzyme in both tonic and receptor-driven regulation of KATP channels, and its localization to caveolae, in which other tissues have been shown to be highly enriched with both G protein-coupled receptors and G proteins, makes it likely that larger, more elaborate signaling complexes exist within these domains. Caveolae have already been implicated as integration sites for smooth muscle Ca2+ signaling because of their ability to aggregate proteins involved in Ca2+ regulation and excitation-contraction coupling.
References