Limits of isolation and culture: intact vascular endothelium and BKca

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Sandow SL, Grayson TH. Limits of isolation and culture: intact vascular endothelium and BKca. Am J Physiol Heart Circ Physiol 297: H1–H7, 2009. First published May 1, 2009; doi:10.1152/ajpheart.00042.2009.—The potential physiological role of plasmalemmal large-conductance calcium-activated potassium channels (BKca) in vascular endothelial cells is controversial. Studies of freshly isolated and cultured vascular endothelial cells provide disparate results, both supporting and refuting a role for BKca in endothelial function. Most studies using freshly isolated, intact, healthy arteries provide little support for a physiological role for BKca in endothelial function. Several studies using freshly isolated, intact, healthy arteries provide little support for a physiological role for BKca in endothelial function, although recent work suggests that this may not be the case in diseased vessels. In isolated and cultured vascular endothelial cells, the autocrine action of growth factors, hormones, and vasoactive substances results in phenotypic drift. Such an induced heterogeneity is likely a primary factor accounting for the apparent differences, and often enhanced BKca expression and function, in isolated and cultured vascular endothelial cells. In a similar manner, heterogeneity in endothelial BKca expression and function in intact arteries may be representative of normal and disease states. BKca being absent in normal intact artery endothelium and upregulated in disease where dysfunction induces signals that alter channel expression and function. Indeed, in some intact vessels, there is evidence for the presence of BKca, such as mRNA and/or specific BK subunits, that is consistent with the potential for rapid upregulation, as may occur in disease. This perspective proposes that the disparity in the results obtained for BKca expression and function from freshly isolated and cultured vascular endothelial cells is largely due to variability in experimental conditions and, furthermore, that the expression of BKca in intact artery endothelium is primarily associated with disease. Although answers to physiologically relevant questions may only be available in atypical physiological conditions, such as those of isolation and culture, the limitations of these methods require open and objective recognition.

expression; large-conductance calcium-activated potassium channel; methodology; smooth muscle

The use of freshly isolated vascular cells and vascular cell and organ culture provides fundamental data on the mechanisms that underlie ion channel and receptor function and calcium modulation, activities that are critical for the control of vascular tone and blood flow. At the same time, as with all models, recognizing and highlighting how closely such data reflect that of the intact vessel require careful consideration. In many studies, the descriptions of cells as “freshly isolated” or experiments performed “in situ” are ambiguous. This is because “freshly isolated” is often used simply to describe cells that are not an authenticated cell line but that have been isolated from native tissues and cultured before use. In a similar manner, “in situ” refers to studies performed on material in the place where it occurs but does not by itself provide any detail about whether the tissue has been cultured, dissociated, or treated in some way before use. Although many studies imply that data from freshly isolated cells and cultured cells and organs are directly relevant to intact vascular function, this is not necessarily the case, as such cells and organs can undergo significant phenotypic drift (3). Indeed, many aspects of intact vascular endothelial and smooth muscle cell phenotype, such as ion channel and receptor function and calcium modulation mechanisms, change dramatically after isolation and propagation (1, 6, 20, 29, 34, 39, 40, 44, 45, 55, 58, 61, for example; see also Tables 1 and 2), a fact that is often ignored. Consequently, data gathered in this manner should be interpreted with caution (3, 28).

In addition to phenotypic drift, a number of factors need to be considered in interpreting the physiological relevance of functional data. Variation in the mechanisms controlling vascular function occurs between species, and within and between vascular beds and in disease (21), and consequently some reports of such variability arise as a result of inappropriate comparisons between different beds, species, and experimental states. For example, in relation to experimental states and patch-clamp data, a key question that arises from the summary in Table 1 is, How much apparent phenotypic change arises as a result of the patch-clamp technique, particularly when it is used on isolated and cultured cells, compared with data derived from intact tissue? Indeed, such effects may be related to digestive enzyme and other treatments involved in cell preparation for patch clamp (43, 60). Furthermore, reports in which cells are described as “primary” cultures indicate nothing about the number of passages to which cells have been subjected to before study; and as phenotype has been shown to be altered with passaging (see references cited above; and Tables 1 and 2), a lack of such critical information may limit the usefulness and validity of conclusions from such studies. Indeed, insufficient details in the methods of many previous studies (such as where and how cells were derived) limit the comparative relevance of data from different studies. Unfortunately, these questions are beyond the scope of the present review to fully address here.

A possible explanation for the differences in channel and receptor function in freshly isolated and cultured cells compared with intact artery endothelial cells is that the isolated cells release factors (growth factors, hormones, and dilatory and constrictor substances, for example) in a different manner and environment to that of an intact artery, thereby changing the composition of the media in which they are growing and altering their phenotype (29, 57; and Tables 1 and 2). For example, growth factors have been suggested to modulate vascular large-conductance calcium-activated potassium channel (BKca) function in cell type-specific manner (67). Such phenotypic changes are also associated with endothelial cell...
with cell and organ isolation and culture, as well as potentially in vascular disease.

Many studies have looked for evidence of functional BK$_{Ca}$ in intact vascular endothelium, and although isolated and passaged vascular endothelial cells may express BK$_{Ca}$, the expression and function of such channels have not been conclusively demonstrated in the endothelium of intact vascular tissue. Indeed, although BK$_{Ca}$ may have an as-yet undescribed intracellular role or be present, but nonfunctional (such as in an immature form), the potential plasmalemmal form of BK$_{Ca}$ is currently recognized as being of primary importance to vascular function and is therefore the focus of this perspective.

In a recent study, Simon et al. (54) use commercial rat lung passage 3–8 microvascular endothelial cells to suggest that C-type natriuretic peptide (CNP) causes endothelial cell hyperpolarization via the activation of BK$_{Ca}$ located on these cells, with CNP thus acting as an endothelium-derived hyperpolarizing factor in those cells. It was concluded that their data are relevant for the biology of intact vessels in general and of pulmonary vessels in particular. In the case of endothelium-derived hyperpolarization, CNP does not participate in such activity in intact vessels (11, 37, 53), although the potential physiological role of BK$_{Ca}$ in vascular endothelial cells is controversial (1, 45), as Simon et al. (54) acknowledge.
Indeed, the BK_{Ca} literature demands careful examination. For example, a study of BK_{Ca} protein in intact rat gracilis muscle arteriole, which apparently demonstrates endothelial BK_{Ca} expression (Fig. 4 in Ref. 64), provides no evidence that the protein samples on Western blots were derived from a preparation predominantly containing endothelial cells. Such evidence should include in parallel a detection with antibodies specific for constitutively expressed endothelial-specific markers, such as von Willebrand factor, vascular endothelial cadherin, PECAM, and ICAM. In addition, there are no molecular weight markers on the blot images and only a narrow area of the blot showing the band of apparent interest is presented. Thus it is not possible to assess the size of the stained bands. These issues are unfortunately common for the presentation of the Western blot data. For this reason, authors should be encouraged to submit images containing molecular size markers and the full Western blot, perhaps as supplementary data if space is an overriding issue (22, 50). Furthermore, in terms of the immunohistochemical data, the patency of the endothelium is not evident in the section images presented (Fig. 4 in Ref. 64). Thus whether BK_{Ca} are present in the endothelium of the rat gracilis arteriole is not confirmed. Furthermore, the specificity of antibodies to BK-α and -β, [the pore forming and regulatory vascular BK_{Ca} subunits, respectively (38)] is not well characterized. Of the antibodies to BK-α and BK-β1, tested and characterized using Western blot analysis (see Table 3) in rat cremaster arteriole and mid-cerebral artery (69), only Alomone APC-107 and Merck [to amino acids 118–132 (33)], respectively, label bands at the appropriate molecular weight in intact rat cremaster arteriole and mid-cerebral artery (69), only Alomone APC-107 and Merck [to amino acids 118–132 (33)], respectively, label bands at the appropriate molecular weight.

### Table 2. Examples of endothelial and smooth muscle cell and intact artery channel and receptor characteristics and effects of methodology

<table>
<thead>
<tr>
<th>Species/Organ/Artery/Cell</th>
<th>Preparation Type/Time to Use</th>
<th>Western blot and molecular analysis</th>
<th>Na:K:2Cl membrane cotransporter expression decreases with passaging</th>
<th>24 h hypoxia suppresses HMEC, but not HUVEC A2 stimulation</th>
<th>Low voltage- and two L-type high-voltage Ca²⁺-activated currents with differential activity (fresh compared to culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine corneal endothelium</td>
<td>Culture, 7 days, then passages 3–5</td>
<td>β-Adrenergic receptor, adenosine A2 activity, RT-PCR, cAMP assay and hypoxia</td>
<td>Adenosine A2A predominates in HUVEC, A2B in HMEC</td>
<td>VRAC activation slower, inactivation faster, current density lower in culture compared to explant</td>
<td>K_{Ca} and K_{v} activity predominates in fresh and culture, respectively</td>
</tr>
<tr>
<td>Human</td>
<td>Culture, passages 2–4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrovascular (HUVEC)</td>
<td>(Ref. 68)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microvascular (HMEC)</td>
<td>Culture, passages 32–41 (Ref. 68)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Murine aortic endothelium</td>
<td>Isolated cells up to passage 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat caudal smooth muscle</td>
<td>Fresh, within 12 h</td>
<td>Patch clamp</td>
<td>K_{Ca} and K_{v} activity predominates in fresh and culture, respectively</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat smooth muscle</td>
<td>Fresh, 1 day old</td>
<td>Patch clamp; IHC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat coronary (whole artery)</td>
<td>Intact, immediate; organ culture, 24 h</td>
<td>Wire myography; IHC</td>
<td>Endothelial endothelin-B receptors downregulated in culture (mimicking disease)</td>
<td>Smooth muscle endothelin-B receptors upregulated in culture</td>
<td>Risk reduction higher, and contribution to calcium transient smaller in culture, with differences in RyR distribution</td>
</tr>
<tr>
<td>Rat cerebral (whole artery)</td>
<td>Organ culture, ≤3 days</td>
<td>Patch clamp; wire myography; Western blot, RT-PCR, IHC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat mesenteric (whole artery)</td>
<td>Isolated (0 h) compared to organ culture, 2 to 3 days</td>
<td>RT-PCR, IHC (total artery expression)</td>
<td>TRPC1/6 activity increased and TRPC3 decreased as culture time increased</td>
<td>TRPC6 increased approximately twofold in culture</td>
<td></td>
</tr>
<tr>
<td>Rat mesenteric (whole artery)</td>
<td>Intact, immediate; organ culture, 6–48 h</td>
<td>Wire myography, RT-PCR</td>
<td>5-HT_{2A}, and angiotensin-1 receptors up- and downregulated, respectively, in organ culture</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HMEC, human microvascular endothelial cells; VRAC, volume-regulated anion channel; K_{v}, voltage-activated potassium channel; RyR, ryanodine receptor; TRPC1/3/6, transient receptor potential cation channel, subfamily C, member 1/3/6; 5-HT_{2A}, 5-hydroxytryptamine type 2A receptors.
reduce the effectiveness with which the antibody would detect all possible BK-
which may not be expressed in all tissues (a critical point for tissues or disease states for which the precise nature of BKCa splicing is not known); and
therefore the risk of nonspecific staining;

<table>
<thead>
<tr>
<th>Antibody (Anti)</th>
<th>Raised Against Species/Antigen</th>
<th>Western Dilution</th>
<th>IHC Dilution</th>
<th>Antigen Peptide Block</th>
<th>Supplier/Catalog Number</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kcα 1.1 (BK-α)</td>
<td>Mse AA 1098–1196*†</td>
<td>NE</td>
<td>NE</td>
<td>Yes</td>
<td>Alomone (APC-021)</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Kcα 1.1 (BK-α)</td>
<td>Mse AA 1184–1200*</td>
<td>1.500</td>
<td>1:100</td>
<td>Yes</td>
<td>Alomone (APC-107)</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Kcα 1.1 (BK-β₁)</td>
<td>Hu AA 90–103</td>
<td>1:500</td>
<td>NE</td>
<td>Yes</td>
<td>ABR (PA1-124)</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Kcα 1.1 (BK-β₁)</td>
<td>Hu AA 90–103</td>
<td>1:1000</td>
<td>NE</td>
<td>Yes</td>
<td>Abcam (ab3587)</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Kcα 1.1 (BK-β₁)</td>
<td>Rat AA 2–17</td>
<td>1:500</td>
<td>NE</td>
<td>Yes</td>
<td>Sigma (P1124)</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Kcα 1.1 (BK-β₁)</td>
<td>Rat AA 2–17</td>
<td>1:500</td>
<td>NE</td>
<td>Yes</td>
<td>Alomone (APC-036)</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Kcα 1.1 (BK-β₁)</td>
<td>Hu N*‡</td>
<td>1:500</td>
<td>NE</td>
<td>Yes</td>
<td>Santa Cruz (SC-14749)</td>
<td>Goat</td>
</tr>
<tr>
<td>Kcα 1.1 (BK-β₁)</td>
<td>AA 61–75</td>
<td>1:2000</td>
<td>NE</td>
<td>Yes</td>
<td>Merck (Garcia, NJ)</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Kcα 1.1 (BK-β₁)</td>
<td>AA 118–132</td>
<td>1:2000</td>
<td>1:400</td>
<td>Yes</td>
<td>Merck</td>
<td>Rabbit</td>
</tr>
</tbody>
</table>

Data from whole adult SD cremaster arteriole and middle-cerebral artery (69) and rat aorta (Grayson and Sandow, unpublished results). Msc, mouse; AA, amino acid; NE, not examined; Hu, human. *Numbering for these positions relates to 2 different splice variants. †This polyclonal antibody was not tested because it is raised against a large chimaeric immunogen that includes a GST fusion protein, with a thus increased likelihood of the presence of cross-reactive epitopes and therefore the risk of nonspecific staining; 2) the BK-α portion of the immunogen contains a substantial region (70 of 108 residues) that represents a splice variant which may not be expressed in all tissues (a critical point for tissues or disease states for which the precise nature of BKCa splicing is not known); and 3) the conserved (unspliced) region of BK-α included in the immunogen is relatively small (38 out of 108 residues) compared with the full-length immunogen of GST + 108 residues of BK-α. In such a polyclonal antibody preparation, this would reduce the proportion of antibody recognizing this unspliced region and further reduce the effectiveness with which the antibody would detect all possible BK-α splice variants. This is important in tissues or disease states for which the precise nature of BKCa splicing is not known. §Sequence of antigen is not identified by manufacturer.

Table 1. In addition, in some vascular beds, there is evidence for particular BKCa constituents, such as mRNA and/or of a particular BK subunit, whereas a functional BKCa response is absent (Table 1). For example, BKCa-α is present in porcine aortic endothelium (48), whereas a similar (likely) BKCa-α current is also present in freshly isolated rabbit aortic endothelial cells (51; and Table 1), although function is absent. The absence of BKCa-β subunits, which regulate calcium sensitivity, is likely causative with the lack of function (48, 51). Interestingly, at nonphysiological potentials, a BKCa current is detected in freshly isolated porcine coronary artery endothelial cells (9), consistent with the possibility that the machinery for BKCa may be present and activated under certain conditions such as in disease. BKCa are also reported in cultured human umbilical vein endothelial cell lines where their expression increases with passaging (10, 13, 17, 19, 32, 61, 67, for example), as well as in cultured bovine and human aortic, bovine pulmonary, and porcine coronary artery endothelial cells after multiple passaging (4, 28, 31, 66; and Table 1). In contrast, BKCa protein is absent in the endothelium of most intact arteries and in freshly isolated and cultured endothelial cells (Table 1). This absence corresponds with a lack of a functional role for BKCa in intact and freshly isolated bovine coronary and freshly isolated porcine coronary artery endothelium (9, 18), as well as in cultured commercial bovine pulmonary artery endothelial cells BKCa (30; and Table 1).

Unfortunately, studies directly comparing vascular endothelial BKCa properties are limited (Table 1). However, when examined systematically, these data allude to a relationship between experimental conditions and altered BKCa expression and activity (Table 1, items in boldface). Such a relationship is present in porcine coronary, bovine pulmonary artery, bovine aorta and human capillary endothelial cells, as well as in human umbilical vein endothelial cells (Table 1, items in boldface). For example, in cultured commercial human capillary endothelial cells, a BKCa current is absent, whereas after conditioning of the media and altering cell density, such a current is present (29). Furthermore, under basal conditions in cultured bovine aortic endothelial cells, functional BKCa are absent, although BKCa-α mRNA and protein are present, and treatment to reduce membrane cholesterol redistributes BKCa, enabling functional BKCa activity (66). Such cholesterol redistribution and associated caveolin-caveolae activity, which plays a significant role in vascular disease (16), is consistent with a potential relationship with vascular disease, thus being consistent with the hypothesis of this review, where it is suggested that endothelial BKCa may be upregulated and functional in at least some forms of vascular disease.

Taken at face value, these studies suggest that specific conditions of cell isolation and passaging may induce a synthesis of BKCa protein, being consistent with BKCa potentially having a functional role in isolated, cultured vascular endothelial cells where the phenotype is distinct from that of most intact vessels. Demonstrating a dramatic culture-induced change, ~40% of proteins in intact pulmonary artery endothelium are not expressed in such endothelial cells in culture (12). Reflecting this observation, the individual endothelial cell population in an intact artery, as well as in cultured and serially passaged endothelium from different sources (3, 8), is phenotypically heterogeneous (1, 3, 46, 58, 62), whereas cultures derived from limited populations of endothelial cells are usually not. This latter point implies that the relevance of data from such cultured cells to overall artery function is questionable, since isolated cell data do not generally represent a phenotypically diverse endothelial cell population, as is present in intact arteries (1–3, 12, 46, 62, 63).

Although data obtained from cell isolation and culture suggest that endothelial cells may express BKCa, primarily due to methodology (29), questions remain regarding the circumstances/stimuli and local environments where such channels may be expressed in intact (real) tissue and whether disease, development, and aging represent such circumstances. Indeed, BKCa are expressed and have a functional role in intact vascular endothelium in some disease states, such as in third-order (150–200 μm) femoral arteries in a rat chronic hypoxia model (25) and in human mesenteric artery from patients with colon cancer, where it is absent in vessels from patients without cancer (35). Furthermore, in isolated rat aortic endothelial cells under chronic hypoxia conditions, functional BKCa are de-
lected, despite the apparent absence of functional BKCa in control and immunohistochemical data, suggesting the presence of BKCa in both states (49).

In addition to BKCa, when cultured and freshly isolated cell and intact tissue data are compared, other aspects of endothelial channel and receptor activity differ (see Table 2, for example). For example, Na:K:2Cl membrane cotransporter expression decreases with successive passaging in cultured bovine corneal endothelium (6), a study which concludes that "expression levels in cell culture may not be reflective of those in these freshly isolated tissues." Indeed, few studies highlight the marked differences in vascular endothelial cell phenotype from data derived from freshly isolated and cultured cells compared with intact tissue (particularly in tissue from which the isolated cells were originally derived or are purported to represent), although there are studies that attempt to allude to this issue (see Table 2, for example). This is not surprising, given the well-characterized functional heterogeneity within and between vascular beds, species, aging, and disease (3, 21, 58), as well as in cultured endothelium of different origin (8, 62). In a similar manner, marked differences in the channel and receptor phenotype of vascular smooth muscle cells occur in freshly isolated compared with cultured cells compared with intact arteries (20). For example, in rat aortic vascular smooth muscle cells (44), a low-voltage- and two L-type-related high-voltage calcium-activated currents show differential activity in freshly isolated compared with cultured cells, while in rat caudal artery smooth muscle cells after 1 day of culture a voltage sensitive potassium current predominates, whereas in freshly dissociated such cells, a KCa current predominates (61).

Significant differences in culture compared with intact tissue are also present in other models of vascular function. For example, in coculture of mouse aortic endothelial and smooth muscle cells, myoendothelial gap junctions are prevalent (26), whereas such junctions are absent in intact adult mouse aorta (52), these junctions being critical for the control of tone in many vascular beds. Indeed, these junctions are present in the aorta of juvenile and diseased apolipoprotein E−/− mice (52), thus suggesting that the coculture model may reflect such developing and diseased states. In a similar manner, as suggested above, altered channel and receptor expression and function in isolated and cultured vascular cells potentially reflect the expression changes in disease states of intact vessels.

In a similar manner, data from tissues in organ culture differ compared with intact vessels and also show characteristics akin to diseased vessels (see Table 2, for example). Relative to intact fresh porcine coronary artery, porcine coronary organ culture dilatory endothelial endothelin-B receptors are downregulated (47), this being potentially associated with an upregulation of constrictor smooth muscle endothelin-B receptors, as found in rat coronary artery organ culture (27). Indeed, similar changes are present in smooth muscle, such as of rat cerebral artery organ culture, where the activity and expression of transient receptor potential cation channel 1 (TRPC1) and TRPC6 are increased and TRPC3 decreased, corresponding with an overall increase in store-operated calcium entry as the culture time is increased (5). Furthermore, in rat mesenteric artery organ culture smooth muscle, 5-hydroxytryptamine type 2A receptors are upregulated and angiotensin-1 receptors are downregulated compared with intact fresh tissue (40). Encour-

agingly, that study concludes that this altered activity may provide clues to channel function in cardiovascular disease, rather than imply that the data are related to normal intact vessel function. Unfortunately, in some studies, claims that culture data are directly relevant to intact vessel function are made. For example, bovine aortic endothelial cell culture data are suggested to provide "new insights not only on the regulation of BK channels in endothelial cells, but also on the fundamental mechanisms that regulate endothelial and vascular function" (66). However, as outlined above, predominant evidence shows that BKCa are not expressed in healthy intact arterial endothelial cells, and thus, when applied to intact vessels, such statements are misleading.

Although data derived from freshly isolated cells and cell and organ culture have broad and fundamental implications for systems and cell biology (1, 3, 20, 45, 46), their limitations need to be considered, in that the data obtained from such preparations may not directly relate to function in an intact vessel (3), a factor that requires objective recognition. This is particularly the case for vascular endothelial potassium channels (1) and specifically plasmalemmal BKCa, where there are marked characterized and often ignored differences in the data derived from isolated and cultured cells and those derived from intact vessels. Predominant evidence suggests that BKCa are not present in the endothelium of nondiseased arteries. Thus, referring to data gathered from isolated and cultured cells and organs, Lygate (41) states that "where the entire message is dependent on several key assumptions, it is imperative that more consideration be given to whether these assumptions are indeed valid." It is essential that data gathered from freshly isolated cells and cultured cells and tissues be examined in comparison with intact vessel data and that unwarranted conclusions based overwhelmingly on data gathered from cultured cells and tissues be avoided (3).

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