E3-targeted anti-TRPC5 antibody inhibits store-operated calcium entry in freshly isolated pial arterioles

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Arterioles are small precapillary arteries containing a single smooth muscle layer. Because of the small diameter and number of arterioles, they have a major impact on peripheral resistance and provide the primary structure regulating local blood flow according to metabolic needs of surrounding tissue. Knowledge of the specific molecular mechanisms governing the phenotype and behavior of arteriolar smooth muscle cells is quite rudimentary when compared with that of many other cellular systems. Nevertheless, significant progress has been made, particularly toward understanding mechanisms of rhythmicity, myogenic tone, and ion transport (11, 12, 14, 17, 20, 25, 26, 31, 33, 34). An area of particular interest is calcium signaling (10, 15, 26, 38). Calcium has a pivotal role in regulating contraction but also emerging importance as a regulator of gene expression and cellular phenotype (2, 50). A regulator of gene expression and cellular phenotype (2, 50). A regulator of gene expression and cellular phenotype (2, 50). A regulator of gene expression and cellular phenotype (2, 50). A regulator of gene expression and cellular phenotype (2, 50). A regulator of gene expression and cellular phenotype (2, 50). A regulator of gene expression and cellular phenotype (2, 50).

Voltage-gated calcium channels, which open in response to depolarization and link to contraction (23, 34, 43). However, there are also other types of calcium channels. We and others have sought to determine the identity of these channels, as well as their relevance to arteriolar function. Perhaps surprisingly, the potent vasoconstrictor agent endothelin-1 suppresses voltage-gated calcium entry in pial arterioles while switching the cells to a receptor-operated, non-voltage-gated type of calcium entry (23). Passive depletion of intracellular calcium stores also evoke calcium entry in pial arterioles, and this mechanism is pharmacologically distinct from voltage-gated calcium channels (18, 19). Intriguingly, store-operated calcium entry is not directly linked to contractile function in these arterioles (18). The relevance of the mechanism remains to be determined, but one possibility is that it has other noncontractile functions, putatively relating to cell housekeeping, gene expression, cell survival, proliferation, or phenotypic switching (2, 30). In an effort to find the relevance of these calcium entry channels, we sought to know more about their fundamental properties.

Over 25 years ago, calcium-45 tracer studies revealed that depletion of intracellular stores stimulated the rate of calcium uptake from the extracellular solution in vascular smooth muscle (7). The pathway coupled tightly to stores, was resistant to conventional calcium antagonists, and was inhibited by potassium-induced depolarization or manganese. These observations contributed to the concept of calcium entry linked to store depletion, often referred to as store-operated channels (SOCs), ion channels in the plasma membrane that open in response to a signal from depleted calcium stores (36, 37, 41). Many, perhaps all, cells have this type of calcium entry, suggesting fundamental importance and wide-ranging biological significance (3, 32, 47). Outputs from pharmacological and electrophysiological studies suggest that SOCs are not a single entity but a family of channels with potential diversity akin to that of voltage-gated ion channels (3, 19, 32). One type of SOC associated particularly with blood cells is the highly calcium-selective and strongly inwardly rectifying calcium release-activated calcium (CRAC) channel. Another, associated with vascular smooth muscle cells, is a mixed cationic channel with modest voltage dependence and substantial permeability to other ions including sodium (1, 46).

Identification of genes encoding mammalian SOCs has been a major quest of biological research (37, 47). One area of attention has been the homologs of the Drosophila melanogaster transient receptor potential (TRP) gene, which has a...
pivotal role in the phospholipase C-dependent light response in photoreceptors. Depending on classification criteria, there are about 30 mammalian homologs of TRP, all of which translate into cationic channels, often with significant calcium permeability (13, 49). Whether TRP genes encode proteins of the SOC channels has been controversial, but substantial evidence links mixed cationic SOCs to the canonical (or classical) subfamily of TRPC genes (5, 8). Concurrently, a range of other vascular functions of TRP channels has emerged (4, 7, 16).

We previously suggested that TRPC1 is a membrane-spanning protein in arteriolar smooth muscle cells that contributes to SOCs (53). However, overexpression of TRPC1 generates small signals in the hands of some investigators and no signals in the hands of others (5, 8). One explanation is that TRPC1 does not function alone but requires protein partners. A protein known to enable TRPC1 function is TRPC5 (35, 39, 44, 45, 54). We were interested in whether TRPC5 is involved in arteriolar calcium signaling, having detected mRNA encoding TRPC5 in rabbit arterioles (19) and found functional importance in human saphenous vein (54). Because there is rapid phenotypic modulation of arteriolar smooth muscle cells in culture, we were not able to adopt antisense DNA or small interfering RNA approaches. Instead we developed a strategy for making isoform-specific antibodies having acute effects on specific ion channels (56). This is referred to as E3 targeting and enabled us to make an anti-TRPC5 blocking agent (T5E3), characterization of which has been carried out using electrophysiology and calcium measurement techniques (56). Here we show the effect of T5E3 on store-operated calcium entry in arterioles and consider the hypothesis that this type of calcium entry involves TRPC1 and TRPC5.

METHODS

Male Dutch dwarf rabbits (1–1.5 kg) were killed by an intravenous overdose of 70 mg/kg pentobarbital sodium in accordance with the Code of Practice, United Kingdom Animals Scientific Procedures Act 1986 in accordance with ethical procedures at the University of Leeds. Arterioles were isolated as described previously (42). Briefly, the brain was placed in ice-cold oxygenated Hanks’ solution, and fragments of pial membrane were dissected from across the cortical surface and incubated in Hanks’ solution containing 0.032 mg/ml protease and 0.2 mg/ml collagenase (type 1A) for 10 min at 37°C. The mixture was placed at 4°C for 15 min and mechanically agitated to isolate fragments of arterioles. After centrifugation (1,000 rpm) for 5 min, the supernatant was replaced with fresh Hanks’ solution. Arterioles were resuspended and dropped onto polylysine-coated coverslips and stored at 4°C. Experiments were performed at 22–26°C within 12 h. Arteriole fragments used in recordings had an external diameter of <45 μm and lacked visible adventitia or endothelial cells (24). Hanks’ solution contained the following (in mM): NaCl, 137; KCl, 5.4; CaCl2, 0.01 NaH2PO4, 0.34; K2HPO4, 0.44; d-glucose, 8; and HEPES, 5. HEK-293 cells stably expressing mouse TRPC6 have been described (9); the cells were grown in DMEM-F-12 (GIBCO) medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and maintained at 37°C under 95% air and 5% CO2.

For arteriolar diameter measurements, a video edge-detection system (Living Systems Instrumentation) was used. Arterioles were placed on a bath on the stage of an inverted trinocular microscope (Nikon TMS) with a CCD camera (Sony). Signals were captured by an A-D converter (Picolog software; Pico Technology, Cambridge, UK) and stored on a computer. Recordings were made in artificial cerebrospinal fluid containing the following (in mM): NaCl, 125; KCl, 1.72; NaHCO3, 24; MgSO4, 1.74; KH2PO4, 1.17; d-glucose, 5.35; CaCl2, 2.47; and EDTA, 0.023. When the extracellular K+ concentration was raised to 60 mM, the NaCl concentration in the bath solution was reduced by the equimolar amount.

Whole cell patch recording (12, 23, 42, 53) was in a bath solution containing the following (in mM): 135 NaCl, 8 d-glucose, 10 HEPES, 1.2 MgCl2, and 1.5 CaCl2 (pH 7.4). The K+ channel blocker cocktail was included and contained the following (in μM): 1,000 3,4-diaminopyridine, 1 glibenclamide, 0.1 apamin, 0.1 penitrem A, 100 BaCl2, and 4,000 tetraethyllumonium chloride. Niflumic acid (0.1 mM) and methoxyverapamil (D600, 10 μM) were also included to block Ca2+-activated Cl– current and voltage-dependent Ca2+ current, respectively. The patch pipette solution contained the following (in mM): 130 CsCl, 1 EGTA, 2 MgCl2, 10 HEPES, 5 Na2ATP, and 0.5 Na2GTP (pH titrated to 7.4 using CsOH).

The ratio of fura-PE3 (delivered to cells as fura-PE3 AM) fluorescence excited alternately at 340 and 380 nm was monitored (Improvision) (18, 53). Standard bath solution and Ca2+free solution (0.4 mM EGTA) for arteriolar experiments included D600 (10 μM). T5E3 was affinity purified (56) and dialyzed in PBS before dilution and use in experiments. Preincubation of arterioles in 60 μg/ml T5E3, T5E3 plus peptide, or PBS alone was for 8–12 h at 4°C (53, 56). Incubation under test or control conditions began at the same time on separate coverslips. After the incubation period, a recording was made from a test and then control arteriole. In the next paired experiment, the control arteriole was recorded from first, etc. In this manner, paired data sets were accumulated. For the Ca2+ reentry protocol, arterioles were perfused with Ca2+-free solution for 4 min before reapplication of Ca2+.

For immunofluorescence, isolated arterioles adhered to poly-L-lysine-coated slides were fixed in 2% paraformaldehyde (0.5 h) and immersed in −20°C methanol (1 min) and 1% BSA with 0.1% Triton X-100 in PBS for 1 h at room temperature. After incubation in 1% BSA-PBS, arterioles were transferred to primary antibody (rabbit anti-TRPC6; Alomone Labs) for 12 h at 4°C and secondary antibody (mouse anti-rabbit IgG-FITC, 1:160; Sigma) for 1 h at room temperature. Smooth muscle cells were identified by colabeling with anti-smooth muscle α-actin antibody (anti-α-SMA-Cy3, 1:200; Sigma-Genosys). Microscopy images were processed with Openlab software (Improvision).

Averaged data are shown as means ± SE. For Ca2+ imaging, fura-PE3 fluorescence was measured from at least five "regions of interest" in each arteriole, each one primarily sampling the Ca2+ signal from one smooth muscle cell; for this type of analysis, the number of cells and the number of arterioles (i.e., independent experiments) are given. For patch-clamp experiments, n is the number of arterioles. Immunofluorescence data are representative of at least three independent experiments. Statistical analysis was based on n values from independent experiments, and comparisons were made by two-tailed unpaired Student’s t-test [not different (ND; P > 0.05), *P < 0.05, **P < 0.01, and ***P < 0.001].

RESULTS

Short, endothelium-denuded, arteriolar fragments were visually identified and ionic currents recorded from the smooth muscle cells using whole cell patch clamp (Fig. 1A). Arterioles were functional, constricting in response to elevated potassium concentration (Fig. 1B) or agonists (12, 19, 23, 24). Previous studies have shown that cyclopiazonic acid (CPA) or thapsigargin (TG) can be used to evoke passive store depletion and Ca2+ entry in these arterioles (18). In the presence of a cocktail of inhibitors of Ca2+-activated Cl– channels and voltage-gated Ca2+ and K+ channels (11, 12), CPA evoked a slowly developing, sustained, inward current at −60 mV (Fig. 2A). The time course of the response was consistent with activation of
SOCs subsequent to Ca\(^{2+}\) release. Evoked current had a current-voltage relationship (I-V) with mild outward rectification and reversing at 0 mV (Fig. 2B), which is \(\sim 10\) mV positive of the reversal potential for chloride channels (12). The I-V has strong similarity to that of the TRPC1-TRPC5 heteromultimeric channel (Fig. 3). An additional but not unique characteristic expected of channels involving TRPC5 is strontium permeability (48). Ca\(^{2+}\) indicator dyes like fura-PE3 bind strontium and so detect strontium entry. Store depletion of arterioles significantly enhanced strontium entry (Fig. 4).

To test the effect of T5E3 antibody, we used Ca\(^{2+}\) imaging because patch clamping on these arterioles is technically difficult, preventing comparison of a sufficient number of paired arterioles on the same day and thus from the same rabbit. The store-operated signal was studied using the Ca\(^{2+}\) add-back protocol as previously described (18, 19, 53). Return of Ca\(^{2+}\) to the external solution led to a progressive rise in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) as Ca\(^{2+}\) entered the cells (Fig. 5). The amplitude of the response related to the amount of Ca\(^{2+}\) entering, but the rate of rise of the fura-PE3 ratio (Fig. 5) is a more accurate measure, less influenced by counterbalancing Ca\(^{2+}\) extrusion mechanisms. Store depletion by pretreatment with TG at least doubled the amplitude (53) and rate of rise (Fig. 6, A cf. B), consistent with store depletion leading to opening of plasma membrane store-operated Ca\(^{2+}\) channels (18).

Paired experiments were performed to determine the effect of pretreating arterioles with affinity-purified T5E3 antibody

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Fig. 1. Patch clamp on functional arteriolar fragments. A: bright-field image of an arteriole showing the external diameter (i) and the patch-clamp pipette (ii) during recording from the smooth muscle cells. Scale bar = 30 μm. B: time series for the external (Ext.) diameter of an arteriole showing constriction as extracellular potassium was elevated from 5 to 60 mM.

Fig. 2. Ionic current in response to passive store depletion. A: mean current at \(-60\) mV (\(n = 6\)). The superfusate was K\(^+\)-free and contained D600, the K\(^+\) channel blocker cocktail, and 0.1 mM niflumic acid. Inward current occurred in response to bath-applied 10 μM cyclopiazonic acid (CPA). B: for the same recordings as A, but the mean CPA-induced current-voltage relationship (I-V).

Fig. 3. Similarity of the I-V to that of canonical transient receptor potential 1 (TRPC1)-TRPC5. Mean data from Fig. 2B (solid circles) compared with the I-V for heteromeric TRPC1-TRPC5 channels (continuous black line) activated by sphingosine-1-phosphate (Fig. 3d from Ref. 54). Both data sets are normalized (norm.) to their own maximum current at \(+80\) mV.

Fig. 4. Strontium permeability of the store-operated pathway. Intracellular strontium in arteriolar smooth muscle cells is indicated by the change (Δ) in ratio of the fura-PE3 fluorescence (F). Strontium was added at 1.5 mM in Ca\(^{2+}\)-free medium. Strontium entry was enhanced in arterioles store depleted with 1 mM thapsigargin (TG) (\(n = 35–40\) cells, 8–9 independent arterioles/experiments). **\(P < 0.01\).
Antibody inhibited the Ca\(^{2+}\) signal by \(\sim 40\%\), suggesting a significant role for TRPC5. Residual Ca\(^{2+}\) entry is largely independent of store depletion (18). This background Ca\(^{2+}\) entry (in the absence of store depletion) was unaffected by T5E3, suggesting independence of this signal from TRPC5 (Fig. 6B). Involvement of the peptide-recognition site of T5E3 was shown by the lack of effect of T5E3 when preadsorbed to its antigenic peptide (Fig. 6C). Nitric oxide suppresses store-operated Ca\(^{2+}\) entry (51), and we previously showed that endogenous nitric oxide produced by arterioles has a similar effect (24). N\textsuperscript{ω}-nitro-L-arginine methyl ester (L-NAME), which inhibits endogenous nitric oxide production, enhances the Ca\(^{2+}\) signal and was used previously when studying the role of TRPC1 (53). We repeated the T5E3 experiments in L-NAME-treated arterioles. Similarly, Ca\(^{2+}\) entry was suppressed (Fig. 6D).

TRPC6 has been strongly implicated in vascular smooth muscle function (4, 28, 52). Furthermore, RNA encoding TRPC6 is detected in arterioles (19), and immunofluorescence data suggest that the TRPC6 protein is present in the smooth muscle layer (Fig. 7). It was therefore important to test for an effect of T5E3 antibody on TRPC6 function. TRPC6, stably expressed in HEK-293 cells, was studied by Ca\(^{2+}\) imaging (Fig. 8). An activator of TRPC6 is carbachol, a muscarinic receptor agonist that evokes a transient [Ca\(^{2+}\)]\(_i\) rise in TRPC6-expressing cells (Fig. 8A). The decay of the response reflects desensitization of Ca\(^{2+}\) entry and not Ca\(^{2+}\) release, because TG failed to inhibit the response, and there was acute dependence on the presence of extracellular Ca\(^{2+}\) (Fig. 8B). T5E3 had no effect on carbachol-evoked TRPC6 activity (Fig. 8A), and, therefore, its effect on Ca\(^{2+}\) entry in arterioles (Fig. 6) cannot be explained by an effect on TRPC6, further supporting the conclusion that T5E3 is a specific tool to study TRPC5 function (56).

**DISCUSSION**

Here we show that smooth muscle cells within freshly isolated pial arterioles exhibit ionic current and strontium entry in response to passive depletion of Ca\(^{2+}\) stores. These data support previous evidence for the existence of store-operated Ca\(^{2+}\) channels (SOCs) in these cells and are consistent with our hypothesis that SOCs comprise TRPC5 as well as TRPC1 ion channel subunits. Direct testing of TRPC5 contribution comes from E3-targeted T5E3 TRPC5 blocking antibody.
T5E3 had a highly significant and peptide-specific inhibitory effect on Ca\(^{2+}\) entry in store-depleted but not control arterioles. TRPC6, a related protein linked strongly to vascular smooth muscle function and suggested by some to contribute to store-operated Ca\(^{2+}\) entry (4, 57), is expressed in the arterioles but not affected by T5E3. Therefore, inhibition of store-operated Ca\(^{2+}\) entry in arterioles by T5E3 is not explained by TRPC6.

General characteristics of the endogenous vascular smooth muscle store-operated cationic channel are consistent with the involvement of TRPC5. The endogenous pathway is nonselective for cations, passing sodium, barium, and strontium. This is also true for TRPC5 (6, 48). 2-Aminoethoxydiphenyl borate (2-APB) inhibits the arteriolar SOC and TRPC5, whereas both mechanisms are resistant to block by nifedipine (19, 55). Endogenous vascular smooth muscle SOC has a conductance of 3–5 pS (1, 22, 46), which does not compare favorably with the unitary conductance of TRPC5 (40, 54) but is similar to the conductance of the TRPC5-TRPC1 heteromultimer (45). The conclusion that a heteromultimeric arrangement of TRPC1-TRPC5 contributes to the arteriolar SOC is further supported by the similarity of the TRPC1-TRPC5 I-V to that of the SOC (Fig. 3) and immunoprecipitation data suggesting association of TRPC1 and TRPC5 in vascular smooth muscle and other cell types (21, 27, 45, 54).

A potential area of concern may be that activation of TRPC5 by lanthanides is a hallmark feature (29, 44, 55). Therefore, it might be expected that the arteriolar SOC would show a similar property. The most obvious effect of lanthanides on the SOC, however, is inhibition (18, 19). Nevertheless, stimulation of the SOC is also observed, particularly after prolonged application of the lanthanide (19, 56). Furthermore, this stimulatory effect is suppressed by T5E3 (56). While recognizing that we do not yet have a full understanding of the situation, we suggest that the difference in effect of lanthanides on TRPC5 and arteriolar SOC arises as a consequence of heteromultimerization of TRPC5 with other subunits, including TRPC1. Other effects of heteromultimerization include marked changes in the shape of the I-V and unitary conductance of the channel. It should also be recognized that other subunits may be involved in the endogenous channel complex.

Heterogeneity is apparent in SOCs, as it is in the types of ion channel and amounts of ion channel expressed in different blood vessels. Therefore, findings suggesting TRPC5 function in pial arterioles (this study) and human saphenous vein (54) do...
not necessarily mean that all SOCs in vascular smooth muscle contain TRPC5. Indeed some investigators have been unable to detect mRNA encoding TRPC5 in vascular smooth muscle from other blood vessels, as reviewed (7).

In conclusion, we provide evidence for involvement of an additional TRPC protein, TRPC5, in the calcium entry evoked by passive store depletion in pial arterioles. Taking this evidence with that from previous studies, we suggest that TRPC5 operates in a partnership with TRPC1, but it would be premature to exclude involvement of other proteins, even as part of the ion permeation pathway. The emerging molecular components of the SOCs should aid understanding of the role of these channels in arterioles. Although, in some blood vessels, calcium entry through SOCs is linked to contraction, in pial arterioles we could find no such connection (18). Instead we envisage roles related to other properties of the smooth muscle cells, for example, in determination of cell migration and phenotype (54).

GRANTS

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