Functional TRPM7 Channels Accumulate at the Plasma Membrane in Response to Fluid Flow

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Abstract—Many cells are constantly exposed to fluid mechanical forces generated by flowing blood, and wall shear stresses modulate aspects of their structure and function. However, the mechanisms for mechanotransduction of flow are not well understood. Here we report that TRPM7, which is both an ion channel and a functional kinase, is translocated within cells in response to laminar flow. After exposure of cells to physiological values of laminar fluid flow, the number of TRPM7 molecules localized at or near the plasma membrane increased up to 2-fold, in less than 100 seconds. This increase in membrane-localized GFP-TRPM7, as seen by total internal reflection fluorescence microscopy, closely correlated with increases in TRPM7 current. Both endogenous and heterologously expressed TRPM7 was found in tubulovesicular structures that were translocated to the region of the plasma membrane on induction of shear stress. In vascular smooth muscle cells, but not in several types of endothelial cells, fluid flow increased endogenous native TRPM7 current amplitude. We hypothesize that TRPM7 plays a role in pathological response to vessel wall injury. (Circ Res. 2006;98:0-0.)

Key Words: TRP ion channels • TRPM7 • shear force • total internal reflection fluorescence (TIRF) microscopy • vascular smooth muscle cells

TRPM7 channels may be involved in anoxia-induced cell death in brain6 and has been reported to be required for cell viability in TRPM7-null avian DT40 cells.7 Cells sense shear stress–induced mechanical stimulation and convert it into a biochemical response that impacts normal and abnormal tissue development, including growth, differentiation, migration, gene expression, protein synthesis, and apoptosis.8 The response of cells to fluid flow depends on the cell type and the magnitude and characteristics of the shear stress applied under physiological or pathological conditions. Endothelial cells are constantly subjected to blood flow that regulates physiological blood vessel responses as well as pathological arterial wall responses. In large arteries, the endothelium is exposed to shear stress values in the range 10 to 40 dyne/cm.8 The vascular smooth muscle cells are protected from shear stress by endothelial cell lining under physiological conditions but become exposed to shear stress after endothelial injury.

TRPM7 is highly expressed endogenously in vascular smooth muscle cells that respond to shear stress under pathological conditions. The endogenous TRPM7 and the expressed GFP-TRPM7 share the same highly dynamic subcellular distribution of “vesicles on tracks.” Moreover, the endogenous TRPM7-like current increases significantly in response to fluid flow. Our results show that intracellular TRPM7 channels are found in tubulovesicular structures that
are delivered to the plasma membrane in response to fluid flow.

Materials and Methods

cDNA Constructs

Green fluorescent protein (GFP) was amplified by PCR and inserted in the NotI site between the FLAG tag and the N terminus of mTRPM7. FLAG-mTRPM7 in pcDNA4/TO was provided by Dr. Andrew Scharenberg (University of Washington). GFP-TRPC5 was obtained by fusing GFP to the C terminus of TRPC5 into the pCI vector. GFP-TRPV5 was cloned in the eGFP/C1 vector by fusing TRPV5 to the C terminus of GFP and was provided by Dr. Rene Bindels (University of Nijmegen). pEYFP-Mem, pEYFP-ER and cDNA constructs were purchased from Clontech.

Cell Culture

HEK293T cells were cultured in medium containing DMEM/Ham’s F12, 10% FBS, and 1:1 glycerine and HT (Gibco). Cells were cultured on glass coverslips for the imaging experiments and on plastic Petri dishes for the electrophysiology experiments. In some imaging experiments, the glass coverslips were coated with Poly-L-Lysine (Sigma). A7R5 cells were cultured in DMEM supplemented with 10% FBS. Cells were tested periodically for a smooth muscle phenotype by assessing spontaneous calcium spikes mediated with 10% FBS. Cells were transfected using Lipofectamine 2000 (Invitrogen) and used at 50% to 70% density. Thirty-five-millimeter Superfect (Qiagen). Cells were plated at least 24 hours before the transfection and used at 50% to 70% density. Thirty-five-millimeter dishes were transfected with 20 μL of Superfect and 2 μg of DNA mixed with 100 μL of OPTI-MEM and added to 500 μL of medium.

DNA Transfection

HEK293T cells were transiently transfected using Lipofectamine 2000 (Invitrogen). Cells were incubated with the transfection agent for 2 to 3 hours and then cultured on glass or plastic 24 to 48 hours before the experiment. A7R5 cells were transiently transfected with Superfect (Qiagen). Cells were plated at least 24 hours before the transfection and used at least 24 hours after transfection. Cells were split before becoming confluent (70% to 80% density) to a density >20% and the media changed every 2 to 3 days.

Imaging and Image Analysis

All live imaging experiments were performed at ~25°C with cells grown on glass coverslips and placed in a custom chamber with standard extracellular buffer. Extracellular buffer contained (in mmol/L): 135 NaCl, 5 KCl, 1.5 CaCl2, 1.5 MgCl2, 2.0 HEPEs, pH 7.4. Scanning confocal microscopy was used to image the distribution of GFP-TRPM7 in different cell types. Cells expressing GFP-TRPM7 or immunostained with anti-TRPM7 primary antibody were excited using confocal microscopy. Images were collected using an Excaliber and MetaMorph (Universal Imaging, Westchester, Pa.) Flow Setup and Calculation

A syringe pump (Harvard Apparatus, Harvard, Mass) connected through thin tubing to a 250-μm ID plastic pipette provided fluid flow and flow values set between 0.123 and 1.23 mL/min. The pipette was positioned ~150 μm from the cell. The magnitude of the shear stress generated by the fluid flow was calculated using the formula:

\[ \tau = \frac{pV}{2} \frac{0.644}{R_x} \]

where \( p = 1025 \text{ kg/m}^3 \) is the density of water and \( V \) is the fluid velocity, calculated by \( V = Q/A \), where \( A = \pi d^2/4 = 0.785 \times 10^{-6} \text{ m}^2 \) for a pipette diameter of \( d = 250 \mu \text{m} \); \( Q \) is the flow generated by the syringe pump measured in m/s. \( R_x \) is the Reynolds number, calculated as \( R_x = Vd/\nu \), where \( \nu = 1.139 \times 10^{-6} \text{ m}^2/\text{s} \) is the kinematic viscosity of the water. \( R_x \) varied from 5.5 to 27.5, depending on flow rate.

To determine whether flow was laminar, we also used the Reynolds number defined as \( R_x = Vd/\nu \), where \( h \) is the fluid level in the dish (3 mm), was kept constant; \( V \) and \( \nu \) are the kinematic viscosity of the water and the fluid velocity, respectively.

For the highest value of the flow used in our experiments the Reynolds numbers were \( R_x > 27.5 \) and \( R_x = 627. \) Laminar flow occurs for \( R_x < 500.00 \) and \( R_x < 3.900.16 \)

Results

GFP-TRPM7 Forms a Functional Channel and Reveals a Dynamic Tubulovesicular Subcellular Distribution

To follow the intracellular distribution of TRPM7, GFP was fused to the N terminus of the channel via a (GA) linker (Figure 1A). We first tested the functionality of the GFP–tagged channel by transfected GFP-TRPM7 into HEK-293 cells and measuring the TRPM7 current in response to a voltage ramp (Figure 1B). GFP-TRPM7 retained all the characteristics of native and recombinant TRPM7 current, including a gradual increase in outward rectification or “run-up”17,18 (Figure 1C).

The intracellular distribution of GFP-TRPM7 was initially studied using confocal microscopy. GFP-TRPM7 in HEK-293 cells is expressed in a reticular pattern throughout the
cytoplasm (Figure 1D). Although the cells have functional TRPM7 current, the plasma membrane localized GFP-TRPM7 is relatively less abundant compared with its intracellular distribution and thus not apparent in these images.

Because of the limited resolution of the confocal images, we used evanescent field objective-based (or TIRF) microscopy to visualize GFP-TRPM7 in HEK-293 cells. The evanescent field selectively illuminates fluorophores within ~250 nm of the plasma membrane-glass cover-slip interface.19 Evanescent light decreases exponentially in the axial direction, such that fluorescence emission is brighter as the fluorophore approaches the plane of the interface. Although TIRF axial resolution is superior to confocal microscopy, it is insufficient by itself to determine whether a protein is truly in the ~4-nm plasma membrane.

TIRF imaging of HEK-293 cells expressing GFP-TRPM7 revealed the GFP-labeled channels at or near the plasma membrane (Figure 1E). The proteins appear punctate, which we assume to be vesicular structures, and in tubular structures with diameters <100 nm. Most cells contain a mix of the 2 types of structures (first cell in Figure 1E), with some cells containing predominantly tubular structures (second cell in Figure 1E) or predominantly vesicular structures (third cell in Figure 1E). This distribution pattern is markedly different from TIRF images of TRPC5-GFP15 but similar to that of GFP-TRPM6 and GFP-TRPV5 channels (data not shown).

We recorded series of 100 images at 1- or 2-s intervals, under steady-state conditions at 22°C. The time series show that both the tubular and the vesicular structures are highly mobile (Movie 1 in the online data supplement available at http://circres.ahajournals.org). Most strikingly, linear tracks or trajectories form in seconds, and vesicles begin to travel on them; then just as quickly, they disappear (Figure 1F). The same type of movement is not observed by stepping the axial (z) position of the objective in fixed cells (data not shown), suggesting that the observed motility is attributable to move-
ment in the x-y plane and not to movement in and out of the z-plane.

Increased GFP-TRPM7 at the Plasma Membrane in Response to Shear Force

To determine the effects of shear stress on the distribution and functional current of GFP-TRPM7 channels, individual cells were exposed to flow as shown in Figure 2A (see Materials and Methods). When sequential TIRF images were recorded before and after the application of flow, we observed a gradual increase in the fluorescence at and near the plasma membrane (Figure 2C and supplemental Movie II). Higher resolution TIRF imaging (Figure 2D) shows that the global increase in fluorescence is attributable to both the appearance of new vesicles in the evanescent field and an increase in the background fluorescence of the cell, most likely attributable to new tubulovesicular structures approaching the plasma membrane.

The formula in Figure 2B quantified the flow-induced change in fluorescence (ΔF) for a region of interest in each cell. The change in fluorescence in response to flow for the cell shown in Figure 2C is graphed in Figure 2E and compared with a cell recorded under the same conditions in the absence of flow. When flow was repetitively applied (Figure 2F), the fluorescence signal increased additively. Flow termination resulted in a variable decrease in the fluorescence signal, attributable either to retrieval of GFP-TRPM7 from the plasma membrane or to reversal of mechanical deformation.

The flow-induced change in fluorescence observed for GFP-TRPM7 (Figure 2G) was not observed for TRP proteins representative of 2 other subfamilies. GFP-TRPC5 is found in large (∼300 nm) vesicles and is inserted in the plasma membrane in response to epidermal growth factor15 but not in response to flow (supplemental Movie IV). GFP-TRPV5 displayed a tubulovesicular distribution similar to that of...
GFP-TRPM7, but no change in its distribution was observed in response to flow (supplemental Movie V).

To determine whether GFP-TRPM7 fluorescence changes resulted from plasma membrane motion or to mechanical deformation of the cells under flow, experiments were repeated using a fluorescent plasma membrane marker (PMem-EYFP), as well as a fluorescent ER marker (pEYFP-ER). We did not detect a global change in fluorescence in response to flow with either of these probes (supplemental Movie VI). These findings suggest that the increase in GFP-TRPM7 fluorescence is attributable to the transport of new molecules to the region of the plasma membrane.

To test whether application of flow leads to an increase in the number of functional GFP-TRPM7 channels in the plasma membrane, we used the perforated-patch clamp technique to monitor whole-cell GFP-TRPM7 current in response to a voltage ramp (Figure 3A). Before flow application, the mean current amplitude at +100 mV increased at a rate of ~1 pA/s, as is commonly observed with TRPM7 recordings under our conditions. On application of flow (shear force of ~20 dyne/cm²), the gradual increase in the maximal current was accelerated by 3- to 4-fold and reached a plateau after persistent flow (Figure 3B). In response to successive applications of flow, the GFP-TRPM7 current increased repetitively, each time with a similar rate (Figure 3C and 3D).

Was the shear force-induced run-up a new component of TRPM7 current or a separate, endogenous current? The use of low chloride concentrations (~4 mmol/L) should have eliminated any significant anionic conductances arising in response to shear force. TRPM7 channels, also known as magnesium-inhibitable channels (MICs), are blocked by millimolar concentrations of Mg²⁺ (via entry and block from the inside of the channel). Current amplitude at +100 mV decreased to ~5% of the initial value within a few seconds of application.

Figure 3. Increased I_{GFP-TRPM7} in response to fluid flow. A, GFP-TRPM7 current amplitude (I_{GFP-TRPM7}) in response to a voltage ramp. On application of ~20 dyne/cm² fluid flow at 60 s, the rate of increase of current amplitude increased 3- to 4-fold. B, I_{GFP-TRPM7} at 100 mV increased with a T_1/2 = 40 s and plateaued with persistent flow. C, Response of I_{GFP-TRPM7} to successive applications of flow. Flow was applied from 40 s to 120 s, 160 s to 260 s, and 330 s to 380 s. D, I_{GFP-TRPM7} at +100 mV in the same cell as C. E, I_{GFP-TRPM7} increased in response to flow; both the initial and flow-induced currents were blocked by external 10 mmol/L Mg²⁺. Flow was applied after 25 s, and Mg²⁺ after 150 s, from the beginning of the recording. F, External Mg²⁺ application blocked 95% of the maximal amplitude of I_{GFP-TRPM7} after flow application. The maximal amplitude of I_{GFP-TRPM7} at +100 mV after the application of flow was assigned a value of 100%.
adding 10 mmol/L extracellular Mg$^{2+}$ (Figure 3E; inset in Figure 3F). These results show that Mg$^{2+}$/H1101 similarly blocked both TRPM7 and the flow-induced current. Mg$^{2+}$ blocked the total current after flow application to the same extent as TRPM7 channels in the absence of flow (95±1%; Figure 3F), suggesting that TRPM7 channels carried the flow-induced current.

**GFP-TRPM7 Fluorescence and I$_{TRPM7}$ Response to Increasing Rates of Flow**

The change in fluorescence and I$_{TRPM7}$ amplitude were measured over a range of flow rates (Figure 4 and Materials and Methods) and plotted as a function of applied shear force. Flow rates varied between 1.2 mL/min and 0.03 mL/min, generating shear stress values between 30 and 0.2 dyne/cm$^2$.

The response of the cells for the same flow rate was variable, both in amplitude (Figure 4B and 4E) and time course. The data show that increasing amounts of shear stress increase both GFP-TRPM7 fluorescence at and near the plasma membrane, and I$_{TRPM7}$. More importantly, the 2 measured responses have a similar dependence on flow rate; the responses are minimal for Q = 15 dyn/cm$^2$ and reach a maximum at 15 dyn/cm$^2$. Responses to shear stress greater than 30 dyn/cm$^2$ detached the cells from the surface.

These results suggest that the 2 types of response to shear stress describe the same cellular process. The observed increase in current amplitude on flow application in perforated patch (~6 to 8 pA/s) corresponds to the incorporation of ~1 to 2 channels/s (τ$_{TRPM}$ at 100 mV = 105 pS$^{17}$). The flow-induced increase in current may result from an increase in the rate of TRPM7-containing vesicle fusion, increased channel gating, or both. To further characterize the mechanism mediating the response of TRPM7 to fluid flow, we tested a signaling pathway that we had previously found to regulate TRPC5.$^{15}$ Both the increase in fluorescence measured by TIRF, and the increase in current amplitude, were unchanged in the presence of phosphatidylinositol 3-kinase (PI3K) inhibitors (50 nmol/L wortmannin; supplemental Figure I; or 100 μmol/L LY294002). The PLC inhibitor, U73122, also had no effect on the response of TRPM7 to flow. Thus the flow enhancement of TRPM7 does not appear to require PI3K or PLC, and in this respect is different from the mechanism mediating growth factor-induced TRPC5 incorporation into the plasma membrane.$^{15}$

TRPM7 contains both functional channel and kinase domains. A kinase inactive mutant of the channel (GFP-TRPM7-kDa; containing the K1645A mutation) showed the same current amplitude and fluorescence response to flow as wt TRPM7 (supplemental Figure I). We conclude that the kinase domain of TRPM7 is not required in the immediate

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**Figure 4.** Dose response of GFP-TRPM7 fluorescence and I$_{TRPM7}$ in response to increasing values of fluid flow. A, Maximal change in fluorescence, %ΔFmax, after application of flow. B, Distribution of %ΔFmax in individual cells for flow between 0 to 20 dyn/cm$^2$. C, Bar graph representing the average %ΔFmax (±SEM) for the cells shown in B (for each value of the flow between 10 and 16 cells from independent experiments were averaged). *P<0.05. D, Calculation of the flow-induced change in current amplitude, ΔImax, in response to a given flow rate. ΔImax the current amplitude at +100 mV was plotted for each cell. E, Distribution of ΔImax (pA) of individual cells for flow values between 0 and 30 dyn/cm$^2$ (for each value of flow n = 6 to 10 cells). *P<0.05. F, Bar graph representing the average ΔImax (pA±SEM) for the cells represented in E.
response to flow but cannot rule out the possibility that it mediates downstream effects of shear stress either by phosphorylating protein targets or by acting as a binding and/or anchoring protein for downstream effectors.

**Distribution and Mobility of GFP-TRPM7 in a Vascular Smooth Muscle Cell Line**

Several cells from tissues that are exposed to flow-induced shear stress values in the 10 to 20 dyne/cm² range were examined. Among these, endothelial cells (HUVECs, HAECs, and HPAECs) had very low levels of TRPM7-like current, and showed no significant response to flow. However, TRPM7 is present in vascular smooth muscle cells, which are exposed to blood flow under pathological conditions. In A7R5 aortic smooth muscle cells expressing GFP-TRPM7, the distribution of the channel was markedly different from that in HEK-293 cells (Figure 5A versus Figure 1D), attributable in part to the particular morphology of vascular smooth muscle cells. A7R5 cells are very thin (maximum height = 3 to 5 μm in the region of the nucleus), so that the distribution pattern of GFP-TRPM7 is more readily apparent, even under the lower z-axis resolution of confocal microscopy (=0.5 to 1 μm). In A7R5 cells, GFP-TRPM7 appears mostly vesicular and these vesicles are restricted to linear filamentous structures (Figure 5B). A similar distribution is observed for the endogenous TRPM7 protein when A7R5 cells were immunostained with an antibody against the C terminus of TRPM7 (Figure 5C and 5D). Endogenous TRPM7 was also detected in A7R5 cells by Western blot using the same antibody (data not shown).

Under TIRF microscopy, the same type and size of vesicular structures were seen in A7R5 cells expressing GFP-TrpM7 (Figure 5E) and in A7R5 cells immunostained for native TRPM7 (Figure 5G). When a series of 100 images of A7R5 cells expressing GFP-TRPM7 were recorded, the highly dynamic nature of the vesicles became apparent (supplemental Movie III). The vesicles moved along linear trajectories with variable speeds in the 1 μm/s range (Figure 5F). Flow did not induce any significant increase in the fluorescence at or near the plasma membrane in A7R5 cells. This may be attributable to their flat morphology; the pool of vesicles above the evanescent field may be too small to compensate for photobleaching. To determine whether the paths along which the vesicles moved were related to actin or tubulin cytoskeletal elements, fixed A7R5 expressing GFP-TRPM7 were immunostained with Alexa 568 labeled phalloidin or anti-α-tubulin antibody. Confocal images did not reveal any significant correlation between actin or α-tubulin and the distribution of GFP. In HEK-293 cells expressing GFP-TRPM7 and Red-ER (endoplasmic reticulum) marker, colocalization was inconclusive because both molecules appear diffusely localized throughout the cytosol. In A7R5 cells, however, the distributions were distinct, with the Red-ER marker homogeneously distributed throughout the cell, whereas GFP-TRPM7 displayed the typical “vesicles on tracks” distribution.

**Increase in Endogenous TRPM7-Like Current in Response to Fluid Flow**

TRPM7-like currents were recorded in A7R5 cells, but the initial current amplitude at +100 mV varied greatly (from <50 pA to >1000 pA; Figure 6C). When shear force corresponding to 15-dyne/cm² was applied to the cells, the maximal amplitude of the current increased significantly (Figure 6A). The amplitude of the increase in TRPM7-like current at +100 mV (ΔImax, calculated as described in Materials and Methods) was 148±15 pA, with a half-time to maximum current (T½) of 42±1.7 s (Figure 6B). The amplitude and time course of the response to flow was in the
same range for all the measured cells, despite highly variable initial values of the TRPM7-like current. This observation suggests that the number of channels incorporated or activated in response to flow is not dependent on the number of functional channels already in the membrane.

The flow-induced component of the TRPM7-like current was blocked by 10 mmol/L extracellular Mg2+ (Figure 6). In the absence of flow, 10 mmol/L extracellular Mg2+ blocked 93 ± 2% of the initial TRPM7-like current, whereas in the presence of flow the same concentration of Mg2+ blocked 90 ± 2.5% of the TRPM7-like current and the flow-induced component of the current. We conclude that the observed response to flow in A7R5 cells, and in our recombinant expression system, involves the same channel and uses a similar mechanism.

Discussion

We have shown that exposure of cells to shear stress leads to a significant accumulation of functional TRPM7 channels at the plasma membrane in <2 minutes. Shear force increased both GFP-TRPM7 and endogenous TRPM7-like current and GFP-TRPM7 fluorescence at and near the plasma membrane. The rapid time course for this response suggests that TRPM7 is among the first molecules to respond to shear stress. Most cellular events induced by shear stress, such as cytoskeletal remodeling22,23 and changes in gene expression pattern,24,25 occur in the time scale of minutes to hours.

Under steady-state conditions, TRPM7 channels exhibit a gradual increase in outward current amplitude (run-up), both in perforated and ruptured whole-cell voltage-clamp. Among the TRP channel family, this effect is characteristic for TRPM7 and its close relative, TRPM6. This may represent gradual insertion of channels into the plasma membrane or an alteration in baseline channel gating. By either mechanism, this increase is accelerated in response to physiological levels of shear stress. The observed translocation of vesicular and tubulovesicular GFP-TRPM7 (in HEK-293, CHO-K1, COS, MEF, and A7R5 cells) suggests that flow leads to an increase in the fusion rate of the TRPM7-containing vesicles with the plasma membrane. We did not detect vesicle fusion followed by diffusion of the fluorescent molecules in the plasma membrane as described for post-Golgi carriers by Schmoranzer et al26 and Toomre et al.27 Insufficient temporal imaging resolution, or increased fluorescence background generated by the reticular structures overlapping with the vesicles, could have prevented such observations in our system.

Potential Mechanisms for the Response of TRPM7 to Fluid Flow

We examined several general hypotheses of how flow is translated into TRPM7 translocation. The most readily testable hypothesis involved the TRPM7 kinase domain, activation of PI3K or PLC pathways, and stretch sensing via the
cytoskeleton. Unlike the mechanism mediating growth factor–induced TRPC5 incorporation into the plasma membrane, flow-induced TRPM7 enhancement is not likely to involve PI3K or PLC. Similarly, the kinase activity of TRPM7 is not involved in the immediate response to flow. The role of the cytoskeleton in translocation could not be adequately tested and remains an unexplored area for research. Thus, we do not know the steps that lead to the increase in plasma membrane TRPM7 in response to flow. Our hypothesis is that shear stress, first sensed by the extracellular matrix interaction molecules of the cell, signals to cytoskeletal proteins, which in turn lead to activation of motor proteins that transport TRPM7 toward the plasma membrane. The cytoskeletal elements and the motor proteins involved in this process remain to be determined.

Endogenous TRPM7 As an Acute Responder to Shear Stress

Unlike endothelial cells, vascular smooth muscle cells (VSMCs) derived from aortic smooth muscle had high levels of TRPM7–like current and TRPM7 protein, and a significant and consistent response to flow. VSMCs are in direct contact with the endothelium cells via myoendothelial bridges. Under normal physiological conditions, they are subject to low levels of flow but are directly exposed to shear stress at sites of vascular damage caused by the rupture of atherosclerotic lesions or invasive techniques, such as angioplasty. Such exposure of these cells to shear stress may elicit an acute response to fluid flow. Cells that are permanently exposed to higher levels of shear stress, like endothelial cells, may downregulate their responses to adapt to steady-state conditions. In contrast, cells like VSMCs, which are exposed to such levels of shear stress only under extreme conditions, may respond quickly, therefore expressing higher levels of TRPM7 and other molecules that may be involved in the immediate response. In response to TRPM7 activation, intracellular calcium increases. Intracellular calcium is known to induce smooth muscle proliferation at lower levels and cell death at high levels.

Acknowledgments

The Howard Hughes Medical Institute supported this work. We thank members of the Clapham laboratory for experimental support and insightful discussions and Drs Andrew Scharenberg, T. Lambers, and Rene Bindels for constructs.

References

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Circ Res. published online December 15, 2005;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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