Northern and western blots. A northern blot was hybridized with a 32P-labelled 1,074-bp SPH cDNA fragment. The polyclonal antibody FP-44K, directed against an SPH fusion protein (amino acids 662–767), was purified from rabbit serum by affinity chromatography. Sperm flagella were isolated from the head as described. Purified membranes from flagella, heads and HEK293 cells were homogenized in solubilization buffer containing (in mM): 150 NaCl, 1 MgCl2, 20 HEPES-NaOH at pH 7.5 and 0.1 EDTA, with 0.5% Triton X-100. Proteins were dephosphorylated with 1 unit of alkaline phosphatase for 30 min at 30°C. Immunoreactivity in western blots was visualized with the electrochemiluminescence detection kit (Amersham). Immunocytochemical experiments were as described.

Electrophysiology. SPH was expressed in HEK293 cells as described. Currents were recorded with a patch clamp in the whole-cell configuration and from excised membrane patches. When necessary, leak currents were subtracted off-line using steady-state currents between +10 and +40 mV. Experiments with caged cAMP or caged cGMP were performed as described.

In the whole-cell configuration, the bath contained (in mM): 135 NaCl, 5 KCl, 1.8 CaCl2, 2.8 MgCl2, and 5 HEPES-NaOH at pH 7.4; the pipette solution contained (in mM): 126 KCl; 10 HEPES-KOH and 10 EDTA at pH 7.4. In the excised-patch configuration, this pipette solution, with the indicated additions, was used on both sides of the patch. Ionic selectivity was determined in inside-out patches by stepping Vm from −70 mV to test values between −30 mV and +30 mV in 5 mV increments. Pipette solution (in mM): 150 KCl, 10 HEPES-NMDG and 10 EDTA at pH 7.4; bath solution (in mM): 50 KCl, 100 XC1, 10 HEPES-NMDG, 10 EDTA at pH 7.4 and 0.1 CAMP. Relative ion permeabilities Pi/PK were calculated according to the equation Pi/PK = ([K+]i − [K+]o)exp(ΔFV/RT))/([K+]i − [K+]o)exp(ΔFV/RT) − [K+]i exp(ΔFV/RT)). Vm was corrected for liquid junction potentials.

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permeability of these channels may differ from that of classic K⁺ channels.

The voltage-sensing S4 segment of HAC1–3 has a unique structure, consisting of two sequences, each of which contains five positively charged residues; a charged residue is found at every third position. These two sequences are separated by an ‘in-frame’ serine residue. Taken together, HAC1–3 define a new branch of the voltage-gated cation-channel superfamily. HAC1–3 have the highest overall similarity to the CNG channel α-subunits and the eag K⁺ channel (28% and 27% sequence identity, respectively; Fig. 1c) of this family. A sequence (BCNG-1) that is identical to HAC2, except that it has an arginine instead of a glycine at position 42, has been isolated from mouse brain. This cDNA could not be expressed and therefore a functional characterization was not possible.

Using a probe specific for HAC1, we detected a messenger RNA species of 3.4 kilobases in mouse and human brain and heart (Fig. 2a, b). The size of the mRNA concurs with the length of the cloned cDNA. We detected mRNA species of similar size in mouse brain by using HAC2- and HAC3-specific probes (Fig. 2c, d). There was no expression of HAC2 and HAC3 in the heart. The HAC2-specific probe recognized other transcripts of 4.6 kb, 6.2 kb and 8.2 kb. In situ hybridization indicated that HAC1 mRNA is highly expressed nearly ubiquitously in brain, with most prominent signals being seen in the hippocampus, thalamus and brain stem (Fig. 2e). Expression of HAC2 is restricted to specific regions of the brain. Strong signals were detected in the hippocampal CA1 region, superior colliculus, cerebral cortex and cerebellum (Fig. 2f). HAC1 mRNA could also be detected in sections of mouse heart ventricles (Fig. 2g).

Among all three HAC channels identified, only HAC1 was expressed in both heart and brain. Therefore we chose this channel for heterologous expression. We transfected human embryonic kidney (HEK)293 cells with a eukaryotic expression vector containing the HAC1 coding sequence. Under whole-cell voltage-clamp conditions, hyperpolarizing voltages caused a slow inward current whose amplitude and rate of activation increased with increasing hyperpolarization (Fig. 3a, c). The current did not decay during voltage steps of longer than 1.5 seconds, indicating very little, if any, inactivation. In HEK293 cells (n = 20) transfected with empty expression vector, a hyperpolarization-activated current was never detected (Fig. 3b). The rate of current activation was well fitted by a
single exponential with a time constant of 241 ± 12 ms at −140 mV (n = 11). The activation of HAC1 revealed a steep voltage dependence, reaching a half-maximal value (V_{1/2}) at about −100 mV (Fig. 3d).

We determined the ion selectivity of HAC1 by measuring the current/voltage (I/V) relationship of the fully activated channel (Fig. 3e–h). We determined reversal potentials (E_{rev}) of −32 ± 2 mV (n = 7) and −19 ± 1 mV (n = 14) at 5.4 mM and 30 mM extracellular K⁺, respectively. These reversal potentials were significantly more positive than would be predicted for a pure potassium conductance (−81 mV and −37 mV at 5.4 mM and 30 mM extracellular K⁺, respectively) indicating that HAC1, like the native I_{K}, passes both Na⁺ and K⁺. The relative permeability ratio for Na⁺ and K⁺ (P_{Na}/P_{K}), as determined by the Goldman–Hodgkin–Katz equation^8, was 0.24 and 0.31 at 5.4 mM and 30 mM extracellular K⁺, respectively. Thus, the kinetic and voltage dependence of current activation and the ion selectivity of HAC1 are compatible with the properties of I_{K} in a variety of neurons^1,11 and heart cells^4,19,32.

We next investigated whether the HAC1 current, like native I_{K}, is enhanced by direct interaction with cAMP and cGMP10,12,22. Both cyclic nucleotides increased the whole-cell current amplitude by shifting the activation curve by up to 12–14 mV towards positive voltages (Fig. 4a). The V_{1/2} values were −103 ± 1 mV (n = 11) in phosphorimager plates was 6 h for a–c and 36 h for d. Molecular mass standards (in kb) are indicated on the left for a and on the right for b–d. e, f, In situ hybridization analysis of mouse brain sagittal sections labelled with riboprobes directed against HAC1 (e) and HAC2 (f). g, Frontal section through mouse heart ventricles hybridized with a HAC1 riboprobe.

Figure 2 Expression of HAC1–3 mRNA. a, Northern blot of human heart (He) and brain (Br) mRNA labelled with an HAC1-specific probe. b–d, Northern blot analysis of mRNA from mouse heart (He), brain (Br), spleen (Sp), lung (Lu), liver (Li), skeletal muscle (Sk), kidney (Ki) and testis (Te). The blot was labelled with a probe directed against HAC1 (b), HAC2 (c), or HAC3 (d). Exposure time on

Figure 3 Electrophysiological properties of the expressed HAC1 channel measured in whole-cell voltage clamp. a–d, Determination of the voltage dependence of HAC1 activation. a, Cells were voltage-clamped from a holding potential of −40 mV to the various voltages (ranging from −140 mV to 0 mV, in 10-mV increments); this was followed by a step to −140 mV. b, c, Current traces measured in a HEK293 cell transfected with empty expression vector (b) and the HAC1-expression vector (c). d, Tail current analysis of HAC1 activation. The amplitude of tail-current traces measured immediately after the voltage step to −140 mV (indicated by the arrow in Fig. 3c) was plotted as function of the preceding membrane potential. The data were fitted by the Boltzmann equation with A_{1} = −0.06 nA, A_{2} = −1.82 nA, V_{1/2} = −103 mV and k = −0.12 (see Methods). The leak current was digitally subtracted. e–g, Determination of the I/V relationship for fully activated HAC1. e, Voltage protocol. Steps to test voltages ranging from −100 mV to +40 mV, in 10-mV increments, were either delivered after HAC1 was fully activated for 1.5 s by a step from −40 mV to −140 mV (protocol A) or after a step from −40 mV to −20 mV (protocol B). f, Current traces obtained using protocol B. g, Current traces obtained using protocol A. h, I/V relationship for the fully activated channel obtained by subtracting the tail-current amplitudes of the two sets of tail currents. The tail current amplitudes were measured immediately after the voltage clamp to the indicated test voltages was settled (arrow in Fig. 3g). I/V relationships were determined at 5.4 mM (filled circles) and 30 mM (open circles) extracellular K⁺, respectively.
the absence of cyclic nucleotides, and $-90 \pm 3$ mV ($n = 10$) and $-89 \pm 3$ mV ($n = 7$) in the presence of 1 mM cAMP and 1 mM cGMP, respectively. The steepness of the activation curve was not significantly affected by cAMP and cGMP. cAMP and cGMP did not further increase the current when it was fully activated by hyperpolarization. To determine the cAMP sensitivity of HAC1, we measured cAMP-induced shifts of the activation curve in excised inside-out patches for different cAMP concentrations (Fig. 4b). Fitting the data to a Hill equation yielded values of 0.5 $\mu$M for the half-maximal cAMP concentration ($K_a$) and 0.8 for the Hill coefficient ($n$). Both values are in good agreement with properties of native $I_h$ (ref. 10).

cAMP altered not only the voltage dependence of current activation, but also significantly accelerated the kinetics of channel opening (Fig. 4c inset). The potentiation of the current by cAMP was readily reversible by washing out the cyclic nucleotide from the patch, and persisted in the presence of the nonspecific protein kinase inhibitor H-89 (Fig. 4c). Thus cAMP affects HAC1 by directly interacting with the CNBD of the channel protein. Like cAMP, cGMP also enhanced both current amplitude and rate of activation in excised inside-out patches (Fig. 4c). The apparent affinity and the Hill coefficient for cGMP were $K_a = 6$ $\mu$M and $n = 1.2$ ($n = 8$), respectively.

As expected for a channel that mediates $I_h$ (refs 1,4,13,21), the current measured in whole-cell voltage clamp was almost completely abolished by 2 mM extracellular Cs+, but was not sensitive to 20 mM extracellular tetraethylammonium (TEA) or 1 mM 4-aminopyridine. Similarly, 2 mM Ba2+ only slightly reduced the HAC1 current (Fig. 4d, e).

Our results indicate that HAC1 is the first member of a class of channels that are dually gated by hyperpolarization of the membrane and by direct binding of cyclic nucleotides. The functional properties of the HAC1 current, that is, the voltage-dependence of activation, ion selectivity, pharmacological profile and modulation by cyclic nucleotides, concur with the general criteria that characterize $I_h$ in several neuronal and non-neuronal cells14. The expression pattern of HAC1 indicates that it may mediate the current that is involved in control of pacemaker activity in both central nervous system and cardiac cells. The identification of two other, brain-specific, members of the HAC family, HAC2 and HAC3, is consistent with the diversity of $I_h$ currents detected in different types of neurons1.

**Methods**

**Molecular cloning of HAC1–3.** A 217-base-pair fragment (nucleotides 11–228 of the EST MMA23393) was amplified by reverse transcription with polymerase chain reaction from mouse brain mRNA. We used the 3P-labelled fragment to screen 1 x 106 colonies of an oligo(dt)-primed cDNA library from mouse brain. The library was constructed using pCDNAII vector (Invitrogen). From a total of 120 positive clones, 36 were randomly chosen and analysed by restriction mapping and partial sequencing. The clones fell into three separate classes, designated HAC1–3. We sequenced both strands of the two longest clones from each class.

**Northern blot.** Northern blots (Clontech) containing 2 $\mu$g mRNA were hybridized under high stringency with 3P-labelled probes. A human multiple tissue blot was hybridized with a fragment corresponding to amino acids 575–647 of HAC1. The mouse tissue blot was hybridized sequentially with probes corresponding to amino acids 98–209 of HAC1, 636–722 of HAC2 and 131–197 of HAC3.

**In situ hybridization.** In situ hybridization was done as described21. Cryostat sections from BALB/c mice brains and hearts were hybridized with riboprobes labelled with [35S]UTP or [32P]UTP, respectively. Slides were exposed to film for four days. RNA probes were transcribed from fragments corresponding to amino acids 98–209 (5’ probe) and 653–741 (3’ probe) of HAC1, and amino acids 7–101 (5’ probe) and 636–722 (3’ probe) of HAC2. Both 5’ probes (Fig. 1e, f) and 3’ probes of each HAC subtype gave identical results.

**Expression and electrophysiological analysis of HAC1.** The complete coding region of HAC1 was cloned into the pcDNA3 expression vector (Invitrogen). Transfection of HEK293 cells was described21. Currents were measured 2–4 days after transfection, either in whole-cell mode or in excised inside-out patches at room temperature. For all measurements, except in part of the experiment shown in Fig. 3b, the following solutions were used: extracellular solution (bath in whole-cell mode, pipette in inside-out mode), 110 mM NaCl, 0.5 mM MgCl2, 1.8 mM CaCl2, 5 mM HEPES, 30 mM KCl, adjusted to pH 7.4 with NaOH; intracellular solution (pipette in whole-cell mode, bath in inside-out mode), 130 mM KCl, 10 mM NaCl, 0.5 mM MgCl2, 1 mM EGTA, 5 mM HEPES, adjusted to pH 7.4 with KOH. For the determination
Determination of cAMP sensitivity of HAC1. The sensitivity of HAC1 to cAMP was investigated in excised inside-out patches by determining the dose–response relationship for the shift of the activation curve as a function of the cAMP concentration. IV relationships for HAC1 activation were determined with cAMP concentrations in the bath solution ranging from 0.01 µM to 100 µM. The voltage protocol described in Fig. 3a was used, the only difference being that the hyperpolarizing voltage steps were extended to ~150 mV and that the tail currents were measured after stepping to ~150 mV. This extension to more negative voltages was needed to fully activate the channel, as in inside-out patches, V1/2 was shifted by about 20 mV to more hyperpolarizing voltages with respect to the V1/2 measured in whole-cell mode. The shift in V1/2 did not affect the kinetics of current activation or the steepness of voltage dependence. A similar phenomenon has been described for the wild-type I(K) current of sinoatrial node cells and may be due to channel run-down or the loss of an intracellular factor that regulates voltage dependence of activation. Amplitudes of tail currents obtained immediately after stepping from the holding potential to test potentials were used to determine the shift in V1/2 measured in the absence of cAMP was plotted against the respective cAMP concentration.

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The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract

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Vascularization of organs generally occurs by remodelling of the preexisting vascular system during their differentiation and growth to enable them to perform their specific functions during development. The molecules required by early vascular systems, many of which are receptor tyrosine kinases and their ligands, have been defined by analysis of mutant mice1–3. As most of these mice die during early gestation before many of their organs have developed, the molecules responsible for vascularization during organogenesis have not been identified. The cell-surface receptor CXCR4 (refs 4–6) is a seven-transmembrane-spanning, G-protein-coupled receptor for the CXC chemokine PBL/SDF-1 (for pre-B-cell growth-stimulating factor/stromal-cell-derived factor), which is responsible for B-cell lymphopoiesis, bone-marrow myelopoiesis and cardiac ventricular septum formation7. CXCR4 also functions as a co-receptor for T-cell-line tropic human immunodeficiency virus HIV-1 (ref 8). Here we report that CXCR4 is expressed in developing vascular endothelial cells, and that mice lacking CXCR4 or PBSF/SDF-1 have defective formation of the large vessels supplying the gastrointestinal tract. In addition, mice lacking CXCR4 die in utero and are defective in vascular development, haematopoiesis and cardogenesis, like mice lacking PBSF/SDF-1, indicating that CXCR4 is a primary physiological receptor for PBSF/SDF-1. We conclude that PBSF/ SDF-1 and CXCR4 define a new signalling system for organ vascularization.

CXCR4 was identified by screening chemokine receptor-like ‘orphans’ for their ability to respond to PBSF/SDF-1 (refs 4–6), but it remains unresolved whether CXCR4 is the only receptor for PBSF/SDF-1. Moreover, most chemokine receptors bind more than one ligand9, so there may be additional ligands for CXCR4. To elucidate the physiological functions of CXCR4, we generated mice lacking CXCR4 expression. We made a vector to target the