Functional expression of the epithelial Ca²⁺ channels (TRPV5 and TRPV6) requires association of the S100A10–annexin 2 complex

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TRPV5 and TRPV6 constitute the Ca²⁺ influx pathway in a variety of epithelial cells. Here, we identified S100A10 as the first auxiliary protein of these epithelial Ca²⁺ channels using yeast two-hybrid and GST pull-down assays. This S100 protein forms a heterotetrameric complex with annexin 2 and associates specifically with the conserved sequence VATTV located in the C-terminal tail of TRPV5 and TRPV6. Of these five amino acids, the first threonine plays a crucial role since the corresponding mutants (TRPV5 T599A and TRPV6 T600A) exhibited a diminished capacity to bind S100A10, were redistributed to a subplasma membrane area and did not display channel activity. Using GST pull-down and co-immunoprecipitation assays we demonstrated that annexin 2 is part of the TRPV5–S100A10 complex. Furthermore, the S100A10–annexin 2 pair colocalizes with the Ca²⁺ channels in TRPV5-expressing renal tubules and TRPV6-expressing duodenal cells. Importantly, down-regulation of annexin 2 using annexin 2-specific small interfering RNA inhibited TRPV5 and TRPV6-mediated currents in transfected HEK293 cells. In conclusion, the S100A10–annexin 2 complex plays a crucial role in routing of TRPV5 and TRPV6 to plasma membrane.

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Introduction

The epithelial Ca²⁺ channels TRPV5 and TRPV6 (recently renamed after ECaC1 and ECaC2, respectively) belong to the superfamily of transient receptor potential (TRP) channels (Montell et al., 2002a,b). The physiological function of this group of non-selective cation channels is diverse and ranges from involvement in phototransduction, olfaction, nociception, sexual behavior, heat and cold sensation, to epithelial Ca²⁺ transport (Montell et al., 2002a). Moreover, these channels display a plurality of ion selectivities and activation mechanisms, some of which represent previously unrecognized modes of channel regulation (Montell et al., 2002a).

TRPV5 and TRPV6 are by far the most Ca²⁺-selective channels of the TRP superfamily and constitute the rate-limiting influx step in active Ca²⁺ (re)absorption that takes place in kidney, proximal intestine and placenta (Hoenderop et al., 2001b, 2002b). A delicate regulation of their activity is of utmost importance to maintain the extracellular Ca²⁺ balance. Several lines of evidence demonstrate that these Ca²⁺ channels are, indeed, controlled by various mechanisms (Hoenderop et al., 2002b). First, TRPV5 and TRPV6 display a Ca²⁺-dependent feedback regulation of channel activity. The intracellular Ca²⁺ concentration in close proximity to the channel mouth exerts an inhibitory effect on channel activity. Several domains in the channel protein sequence have been implicated in this inhibitory mechanism (Nilius et al., 2002). Secondly, the recovery from the Ca²⁺-dependent inactivation renders another plausible site of regulation. This recovery is a relatively slow process that may reflect an intrinsic property of TRPV5 and TRPV6 or could result from (re)insertion of channels from an intracellular pool (Vennekens et al., 2000; Nilius et al., 2001). A significant subset of TRPV5 channels is localized subapically in distal tubules of the kidney, hinting at a shuttling mechanism of these channels to the plasma membrane (Loffing et al., 2001). Thirdly, vitamin D, estrogen and dietary Ca²⁺ content have been shown to regulate the abundance of these Ca²⁺ channels, resulting in normalization of hypocalcemia in 25-hydroxyvitamin D₃-1α-hydroxylase (1α-OHase) knockout mice (Hoenderop et al., 2001a, 2002a; Van Cremput et al., 2001; Van Abel et al., 2002). Fourthly, the amino acid sequence of TRPV5 and TRPV6 contains conserved motifs for putative regulatory activities including protein kinase C phosphorylation sites, ankyrin repeats and PDZ motifs (Hoenderop et al., 2001b).

To date, little information is available concerning the molecular players responsible for these processes regulating the activity of TRPV5 and TRPV6. A number of regulatory proteins have recently been described that modify the biophysical, pharmacological and expression properties of ion channels and transporters by direct interactions (Li and Montell, 2000; Liedtke et al., 2000). These newly identified associated proteins have facilitated the elucidation of important molecular pathways modulating transport activity.

The aim of the present study was, therefore, to identify auxiliary proteins interacting specifically with TRPV5 and TRPV6. To this end, we have used a yeast two-hybrid screen to identify proteins associated with the epithelial Ca²⁺ channels. The functional interaction between TRPV5
and TRPV6 and the identified interacting protein was further substantiated by pull-down assays, immunohistochemical studies, RNA interference (RNAi) and electrophysiological analysis of the regulatory effect of the newly identified protein ligands on TRPV5 and TRPV6 activity.

**Results**

**Identification of S100A10 as TRPV5-associated protein**

To identify proteins that interact with TRPV5, the C-terminal tail of TRPV5 was used to screen a mouse kidney cDNA library using the yeast two-hybrid technique. One of the positive clones encoded S100A10, a distinct member of the EF-hand-containing S100 protein family. S100A10 is also known as calpactin light chain, p11 or annexin 2 light chain (Rety et al., 1999). To confirm this interaction, the full-length S100A10 coding sequence was analyzed with the C-terminal tail of TRPV5 as bait using the yeast two-hybrid system (Figure 1A). As a negative control the γ subunit of the epithelial Na+ channel, γENaC, was used. S100A10 strongly interacted with TRPV5, whereas no binding was observed with γENaC, indicating the specificity of the S100A10–TRPV5 interaction (Figure 1B). In addition, β-galactosidase activity was not detectable in the absence of prey, or after co-transformation of the bait with the empty pACT2 (prey) vector (data not shown).

**S100A10 interacts with TRPV5 and TRPV6**

To further establish the interaction between TRPV5 and S100A10, GST pull-down binding assays were performed. *Xenopus laevis* oocytes were injected with S100A10 cRNA and homogenized after 3 days. The S100A10-containing homogenate was incubated with GST or GST–TRPV5 fusion proteins immobilized on glutathione–Sepharose 4B beads. S100A10 bound specifically to the C-terminal tail of TRPV5, since no interaction was observed with GST alone (Figure 1C). Subsequently, this binding was investigated in the presence of 1 mM Ca2+ or 2 mM EDTA as shown in Figure 1D. S100A10 interacted with the C-terminal tail of TRPV5 in a Ca2+-independent manner. In addition, [35S]methionine-labeled full-length TRPV5 protein bound to GST–S100A10, whereas GST alone was negative (Figure 1E). Interestingly, the interaction with S100A10 was not restricted to TRPV5, since S100A10 also interacted with the C-terminal tail of TRPV6 (Figure 1C). Likewise, [35S]methionine-labeled full-length TRPV6 bound to S100A10 immobilized on glutathione–Sepharose 4B beads, confirming the TRPV6–S100A10 interaction (Figure 1E).

**TRPV5 interacts with the S100A10–annexin 2 complex**

S100A10 forms a heterotetramer with annexin 2, which is a member of the Ca2+ and phospholipid binding proteins (Gerke and Moss, 2002). This heterotrmer consists of a S100A10 dimer binding to two annexin 2 molecules in a highly symmetrical manner (Rety et al., 1999). In *X. laevis* oocytes S100A10 also forms a dimer, as was demonstrated by chemical cross-linking using dimethyl-3,3′,3′-dithiobispropionimidate-2HCl (DTBP) in S100A10 cRNA injected oocytes (Figure 2A). To demonstrate the presence of annexin 2 in the S100A10–TRPV5 complex, GST pull-down assays and co-immunoprecipitations of these proteins in their native form were performed. To this end, annexin 2 and a vesicular stomatitis virus glycoprotein (VSV)-tagged S100A10 cRNA were co-injected in *X. laevis* oocytes. Subsequently, these oocytes were metabolically labeled with [35S]methionine and homogenized in pull-down buffer containing 2 mM EDTA. Solubilized proteins were immunoprecipitated using anti-VSV antibodies. Annexin 2 could be co-immunoprecipitated with S100A10, demonstrating the presence of an S100A10–annexin 2 complex in these oocytes (Figure 2A). In addition, annexin 2 was precipitated from homogenates of S100A10–annexin 2 cRNA co-injected oocytes using GST–TRPV5 C-terminal tail-loaded glutathione–Sepharose 4B beads (Figure 2B), demonstrating a physical interaction between TRPV5, S100A10 and annexin 2.
investigated by RT–PCR. Using two specific primer sets a 239 and 360 bp product was amplified in kidney and duodenum, corresponding to the expected sizes of the S100A10 and annexin 2 fragments amplified. Subsequently, the cellular localization of these proteins was studied in kidney and duodenal sections by immunohistochemistry. In kidney, immunopositive staining for TRPV5 was predominantly present at the apical membrane of distal convoluted and connecting tubules (Figure 3A). S100A10 and annexin 2 co-localized with TRPV5, since an immunopositive staining for these proteins was observed along the apical domain of the TRPV5-expressing distal tubular cells. Importantly, the same observations were made for the localization of these proteins in duodenum, where TRPV6, S100A10 and annexin 2 were observed along the brush-border membrane (Figure 3B; for color images of this figure see the Supplementary data available at The EMBO Journal Online).

Mapping of the S100A10 binding site in TRPV5
To identify the S100A10 binding site in TRPV5, a series of deletion mutants of the C-terminal tail of TRPV5 was constructed as shown in Figure 4. Truncated forms of TRPV5 were expressed as GST fusion proteins, and pull-down experiments were performed as described above. The interaction between S100A10 and TRPV5 was lost when TRPV5 was truncated at position 598, whereas truncations at positions 680 up to 603 had no effect on the interaction with S100A10. Subsequently, the identified S100A10 binding region between amino acids 598 and 603 (VATTV) was mutated into glycines, and the binding of S100A10 was analyzed by a GST pull-down assay. The binding between S100A10 and TRPV5 was virtually abolished when this putative binding region was mutated. Mutation of the first threonine in this motif into an alanine (TRPV5 T600A) also prevented the association of this protein, indicating the crucial role of this amino acid for binding of S100A10.

S100A10 binding to TRPV5 or TRPV6 is critical for channel activity
The effect of S100A10 on TRPV5 and TRPV6 activity was determined by whole-cell patch-clamp analysis in transiently transfected human embryonic kidney (HEK293) cells, as shown in Figure 5A and B. HEK293 cells heterologously expressing wild-type TRPV5 displayed Ca\textsuperscript{2+} and Na\textsuperscript{+} currents in line with our previous studies, including a high Ca\textsuperscript{2+} selectivity over Na\textsuperscript{+}, the presence of large Na\textsuperscript{+} currents in the absence of extracellular divalent ions and strong inward rectification (Vennekens et al., 2000). The T600A mutant of TRPV5 failed to produce significant Na\textsuperscript{+} and Ca\textsuperscript{2+} currents in HEK293 cells in the absence and presence of extracellular Ca\textsuperscript{2+}, respectively, while the channel was readily detectable by immunoblotting. Likewise, the corresponding mutant in TRPV6 (T599A) expressed in HEK293 cells was functionally inactive. Notably, HEK293 cells endogenously expressed S100A10 and annexin 2 as confirmed by RT–PCR analysis (data not shown). Thus, these findings suggested that the binding of S100A10 to TRPV5 and TRPV6 is crucial to obtain channel activity.

However, the TRPV5–annexin 2 interaction could only be observed when S100A10 was co-expressed with annexin 2, indicating that S100A10 bridges annexin 2 to TRPV5. Finally, co-immunoprecipitations were performed using in vitro-translated TRPV5, S100A10 and annexin 2. S100A10 and annexin 2 co-immunoprecipitated with TRPV5, but not in a control reaction carried out with an unrelated antibody, confirming the specificity of the procedure (Figure 2C).

Co-localization of TRPV5, S100A10 and annexin 2 in Ca\textsuperscript{2+}-transporting epithelia
Co-expression of S100A10 and annexin 2 in kidney and small intestine, which express TRPV5 and TRPV6 (Hoenderop et al., 1999a; Peng et al., 1999), was first
**S100A10 is essential for trafficking of TRPV5 and TRPV6**

The role of S100A10 in the routing of TRPV5 and TRPV6 was investigated using *X.laevis* oocytes, as shown in Figure 5C. Immunocytochemical analysis of TRPV5 or TRPV6 cRNA-injected oocytes demonstrated a strong immunopositive labeling of the plasma membrane for both channels, whereas the cytoplasm was only faintly stained. This finding suggests that wild-type TRPV5 and TRPV6 are targeted efficiently to the plasma membrane. In contrast, the subcellular localization of the TRPV5 T600A and TRPV6 T599A mutants was seriously disturbed. The plasma membrane was virtually devoid of immunopositive staining for these mutant channels, but instead a strong immunopositive signal accumulated in an area just below the plasma membrane. Notably, *X.laevis* oocytes endogenously express S100A10 and annexin 2 as demonstrated by RT–PCR analysis. Taken together, these results imply that the association of S100A10 is necessary for correct trafficking (i.e. targeting or retention) of TRPV5 and TRPV6 to the plasma membrane.

**Gene silencing of annexin 2 using RNAi**

The use of small interfering RNAs (siRNAs) has become a powerful tool to knock down specific gene expression in mammalian cell lines including HEK293 cells (Caplen *et al.*, 2001; Elbashir *et al.*, 2001). We have, therefore, applied this novel technique to further substantiate the role of annexin 2 in the regulation of Ca^{2+}-channel activity. First, HEK293 cells were transfected with annexin 2-specific siRNAs, subsequently with the pIRES-TRPV5 or pIRES-TRPV6 vector and finally analyzed by immunoblotting, immunofluorescence and patch–clamp as shown in Figure 6. Annexin 2 expression was significantly downregulated by annexin 2-specific siRNA transfection (Figure 6A), while the expression of TRPV5 and TRPV6
averaged over the total population of cells a significant decrease in the Na⁺ current was apparent for both TRPV5 and TRPV6 (Figure 6l).

**Hormonal regulation of S100A10**

Recently, we have shown that the TRPV5 and TRPV6 expression levels in kidney and duodenum are positively regulated by 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] in vitamin D receptor and 1α-OHase knockout mice, animal models for pseudovitamin D-deficiency rickets (Van Cromphaut et al., 2001; Hoenderop et al., 2002a). The renal expression level of S100A10 was, therefore, analyzed under identical circumstances. Treatment with 1,25(OH)₂D₃ significantly upregulated the mRNA abundance of S100A10 (158 ± 16% compared with control levels, P < 0.05). Thus, in kidney, S100A10 is, like TRPV5, upregulated by 1,25(OH)₂D₃.

**Discussion**

The present study identified the S100A10–annexin 2 pair as the first auxiliary protein complex for the newly identified epithelial Ca²⁺ channels, TRPV5 and TRPV6. S100A10 forms a well-defined heteromeric complex with annexin 2 and associates specifically with the conserved sequence VATTV located in the C-terminal tail of TRPV5 and TRPV6. Of these five amino acids, the first threonine residue plays a crucial role since the corresponding channel mutants preclude S100A10 binding and accumulate below the plasma membrane preventing the facilitation of Ca²⁺ inward currents.

**S100A10–annexin 2 complex associates with TRPV5 and TRPV6**

S100A10 was initially identified as a TRPV5-interacting protein by a yeast two-hybrid screening, and the binding between both proteins was subsequently confirmed by GST pull-down assays. The binding was not restricted to TRPV5 since TRPV6 was also shown to bind S100A10, indicating a mutual mechanism in the regulation of these Ca²⁺ channels. Considering the high degree of homology and the similarities in electrophysiological behavior between both channels (Hoenderop et al., 2001b), it is indeed likely that TRPV5 and TRPV6 have common regulatory factors such as associated regulatory proteins. S100A10 is a distinct member of the S100 family, since its two EF hands carry mutations that render it Ca²⁺ insensitive (Gerke and Weber, 1985), explaining the Ca²⁺-independent association with TRPV5. Furthermore, we could show by co-immunoprecipitations and GST pull-down experiments that the membrane-associated and microfilament-binding protein, annexin 2, is part of the channel–S100A10 complex. Employing lysates from cells co-expressing TRPV5 and S100A10/annexin 2, we were not able to immunoprecipitate S100A10 and annexin 2 together with TRPV5 using anti-TRPV5 antibodies (data not shown). The physical association of the S100A10–annexin 2 complex with TRPV5 could be too weak to resist detergent solubilization, or could be spatially restricted to the plasma membrane, only locally regulating TRPV5 and TRPV6. Both scenarios would preclude an efficient co-immunoprecipitation. Interestingly, an association of annexin 2 with TRPV5 could be shown only in the
Fig. 5. Activity and subcellular localization of TRPV5 and TRPV6 with a mutated S100A10 binding site. HEK293 cells were transiently transfected with TRPV5 (wild type or T600A) or TRPV6 (wild type or T599A) and analyzed using the whole-cell patch-clamp configuration and immunoblotting. (A) Currents were measured during voltage ramps from -150 to +100 mV (400 ms) in the absence of permeable cations (all substituted by NMDG+), in the absence of divalent cations only, and in the presence of 1 mM Ca2+. The *I/V* curves showed inward rectification typical for TRPV5 and TRPV6. In the TRPV5 T600A and TRPV6 T599A mutants Na+ and Ca2+ currents were virtually abolished, while the channel was readily detectable. NT, not transfected. (B) The average currents measured in the absence of divalent cations and in the presence of 1 mM Ca2+ of three independent transfections for wild-type and mutant TRPV5 and TRPV6 are depicted. (C) Immunocytochemistry was performed on *X. laevis* oocytes injected with 5 ng of HA-tagged TRPV5 (wild type or T600A) or Flag-tagged TRPV6 (wild type or T599A) cRNA. Oocytes injected with wild-type channels showed predominant immunopositive staining at the plasma membrane, whereas the channels containing a mutation in the S100A10 binding site accumulated in an area just below the plasma membrane. Representative images of three independent experiments are shown.

Presence of S100A10, demonstrating that annexin binds indirectly to the TRPV5 C-terminal tail, with S100A10 most likely operating as a bridge between TRPV5 and annexin 2. Annexin 2 forms a heterotetrameric complex with S100A10, consisting of a S100A10 dimer associated with the N-terminal tails of two annexin 2 molecules in a highly symmetric manner (Rety et al., 1999). Furthermore, we have recently demonstrated that TRPV5 and TRPV6 form homo- and heterotetrameric channel complexes, which possibly create four S100A10 binding sites per functional channel (Hoenderop et al., 2003). Taken together, TRPV5 and TRPV6 interact with the S100A10– annexin 2 heteromultimer possibly with four available interaction sites.

**Co-localization of S100A10– annexin 2 complex and the epithelial Ca2+ channels in kidney and duodenum**

In kidney, TRPV5 is primarily expressed along the apical membrane of distal convoluted and connecting tubules (Hoenderop et al., 2000; Loffing et al., 2001). Importantly, S100A10 and annexin 2 were consistently detected in these TRPV5-expressing tubules, where they concentrated along the apical membrane. Furthermore, the S100A10– annexin 2 complex was present along the brush-border membrane of duodenum, which is in agreement with the TRPV6 localization. S100A10 and annexin 2 were previously identified in placenta and pancreas, sites with a prominent TRPV5 and TRPV6 expression (Kaczan-Bourgois et al., 1996; Dreier et al., 1998; Massey-Harroche et al., 1998; Hoenderop et al., 1999a; Wissenbach et al., 2001; Janssen et al., 2002). Generally, the S100A10– annexin 2 complex is localized to the cytoskeleton underlying the plasma membrane (Thiel et al., 1992; Zobiack et al., 2001) and it has been proposed to participate in membrane trafficking and/or organization in this area of the cell (Gerke and Moss, 2002). Thus, the observed apical localization of the S100A10– annexin 2 complex in TRPV5- and TRPV6-expressing epithelia is in line with a regulatory role in the plasma membrane localization of the Ca2+ channels and thus their function in the process of Ca2+ (re)absorption.
S100A10 interaction with TRPV5 and TRPV6 is essential for trafficking

The association of S100A10 with TRPV5 and TRPV6 was restricted to a short peptide sequence VATTV located in the C-terminal tail of these channels. This stretch is completely conserved among all identified species of TRPV5 and TRPV6 (Hoenderop et al., 2001b). Interestingly, the TTV sequence in the S100A10 binding motif resembles an internal type I PDZ consensus binding sequence, which is S/TTXV (Songyang et al., 1997). However, S100A10 does not contain PDZ domains, indicating that the TRPV5–S100A10 interaction has a different nature. The first threonine of the S100A10 interaction motif was identified as a crucial determinant for binding. Furthermore, the activity of TRPV5 and TRPV6 was abolished when this particular threonine was mutated, demonstrating that this motif is essential for channel function. Malfunctioning of these mutant channels was accompanied by a major disturbance in their subcellular localization. These findings suggest a role for the S100A10–annexin 2 heterotramer in the trafficking process of TRPV5 and TRPV6 to the plasma membrane. Whether this involves facilitated translocation of TRPV5 and TRPV6 channels to the plasma membrane or enhancement of channel retention remains to be elucidated.

Recently, S100A10 was demonstrated to interact with the background K⁺ channel TASK-1, controlling the membrane trafficking and, therefore, the functionality of this K⁺ channel (Girard et al., 2002). TASK-1 associated with S100A10 via its C-terminal sequence SSV. This sequence resembles the binding motif in TRPV5 and TRPV6 identified in the present study, suggesting a shared structural S100A10 binding pocket. However, this motif is absent in the tetrodotoxin-insensitive voltage-gated Na⁺ channel (Na,1.8), which has been shown to bind S100A10 via its N-terminal tail (Okuse et al., 2002). This S100A10 interaction promoted the translocating of Na,1.8 to the plasma membrane producing functional Na⁺ channels.

Role of annexin 2 in Ca²⁺-channel activity

S100A10 is predominantly present as a heterotetrameric complex with annexin 2, which has been implicated in numerous biological processes including endocytosis, exocytosis and membrane–cytoskeleton interactions (Ali et al., 1989; Gerke and Moss, 2002). Annexin 2 is postulated to bind to the cytoplasmic face of membrane rafts to stabilize these domains, thereby providing a link to the actin cytoskeleton. The present study demonstrated the presence of annexin 2 in the S100A10–channel complex, indicating a possible function in regulating
channel localization and/or activity. Importantly, down-regulation of annexin 2 using annexin 2-specific siRNAs significantly inhibited the currents through TRPV5, indicating that annexin 2 in conjunction with S100A10 is crucial for TRPV5 activity. In line with the cortical localization of annexin 2 and its postulated function in organizing certain plasma membrane domains, our findings provided the first functional evidence for a regulatory role of annexin 2 controlling Ca2+ channel trafficking. A role of annexin 2 was neither assessed for TASK-1 nor Na1.8 (Girard et al., 2002; Okuse et al., 2002). It remains, therefore, to be determined whether annexin 2 also mediates the regulatory role of S100A10 for these ion channels. Importantly, annexin 2 has been shown to be able to modulate the activity of volume-activated Cl- channels in vascular endothelial cells (Nilius et al., 1996). Disruption of the S100A10–annexin 2 complex resulted in a gradual decrease of Cl- current, possibly indicating a decrease of functional channels at the plasma membrane. These findings, together with our data, clearly show that the S100A10–annexin 2 complex is a significant component for the trafficking of different ion channels to the plasma membrane and is thus a major regulator of ion homeostasis.

**Regulation of S100A10**

Vitamin D is an important calcitropic hormone determining the Ca2+ balance in the body. An increase in functional expression of TRPV5 and TRPV6 has been implicated as one of the major determinants of long-term vitamin D efficacy (Hoenderop et al., 2001a, 2002a; Van Cromphout et al., 2001). Here, we indicated that S100A10 abundance, analogous to TRPV5 and TRPV6, is regulated by 1,25(OH)2D3. In addition, annexin 2 expression levels have been shown to increase upon prolonged incubation with 1,25(OH)2D3 (Menaa et al., 1999). Together, our observations imply that 1,25(OH)2D3 exerts a major calcitropic effect by concomitantly increasing the S100A10, annexin 2 and epithelial Ca2+-channel expression and thus efficiently stimulating Ca2+-channel recruitment during Ca2+ demand.

In conclusion, our data provided the first evidence of a regulatory role for the S100A10–annexin 2 heterotetramer in vitamin D-mediated Ca2+ (re)absorption in general and in particular in TRPV5 and TRPV6 routing. The elucidated molecular mechanism involves tethering of the S100A10–annexin 2 complex to the Ca2+ channel, resulting in functional plasma membrane localization. This mechanism is likely applicable to other ion transporters given the wide tissue distribution of S100A10 and the associated annexin 2 protein.

**Yeast two-hybrid system**

The Y153 yeast strain (Durfee et al., 1993) was first transformed to Tp prototrophy with pAS1-1 containing the TRPV5 C-terminal tail. Expression of the Gal4–TRPV5 hybrid protein was confirmed by immunoblotting using monoclonal antibodies against the HA epitope (Sigma). Yeast was transformed with a mouse kidney cDNA library (Clontech) present in the pACT2 vector, containing a leucine selection marker. Subsequently, yeast cells were plated onto Trp–Leu–His selective medium supplemented with 25 mM 3-aminotriazole. Positive colonies were assayed for β-galactosidase activity as described previously (Vojtek et al., 1993). Yeast DNA of positive colonies was isolated (Hoffman and Winston, 1987), and prey plasmids were rescued by transformation into K8 cells, which carry trpC, leuB and histB mutations (Clontech). Yeast two-hybrid results were confirmed using purified library plasmids and negative controls were performed by replacing a binding partner with either a pAS1-1 construct containing the N-terminal tail (amino acids 1–53) of rat aEaNAC (Firsov et al., 1999) or the empty pACT2 vector.

**GST–TRPV5 fusion protein and interaction assays**

pGEX6p-2 constructs were transformed in *Escherichia coli* BL21, and GST fusion proteins were expressed and purified according to the manufacturer’s protocol (Amersham Pharmacia Biotech). Xenopus laevis oocytes were injected with 20 ng of S100A10 cRNA, 20 ng of annexin 2 cRNA, or co-injected with 10 ng of S100A10 cRNA and 10 ng of annexin 2 cRNA as described elsewhere (Hoenderop et al., 1999a). After 48–72 h, oocytes were homogenized in pull-down buffer [20 mM Tris–HCl pH 7.4, 140 mM NaCl, 1 mM CaCl2, 0.2% (v/v) Triton X-100 and 0.2% (v/v) NP-40] and centrifuged twice for 10 min at 16 000 g. Oocyte supernatants were added to GST or GST–TRPV5 fusion proteins immobilized on glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech). [35S]methionine-labeled full-length TRPV5 protein was prepared using a Retiulocyte lysate system in the presence of canine microsomal membranes (Promega) and added to GST or GST–S100A10 immobilized on glutathione–Sepharose 4B beads. After 2 h incubation at room temperature, the beads were washed extensively with pull-down buffer. Bound proteins were eluted with SDS–PAGE loading buffer, separated on SDS–PAGE gels and visualized either by autoradiography (for TRPV5 and TRPV6) or immunoblotting using monoclonal anti-VSV (1:10 000 clone PS54; Sigma) (for S100A10) or monoclonal anti-annexin 2 (1:5000; Transduction Laboratories).

**Co-immunoprecipitation**

For chemical cross-linking experiments, 20 ng of S100A10 cRNA injected oocytes were lysed in 20 mM HEPES pH 7.2, 5 mM KCl, 130 mM NaCl, 5 mM EDTA and 10% (v/v) glycerol containing 2 mM
DTBP (Pierce) incubated on ice for 30 min, and 100 mM Tris–HCl pH 7.4 was added. The lysates were centrifuged for 30 min at 16 000 g and supernatant was analyzed by immunoblot as described above. For co-immunoprecipitation experiments, S100A10 and annexin 2 cRNA co-injected oocytes were labeled with 1[35S]methionine for 48 h and homogenized in pull-down buffer containing 2 mM EDTA instead of 1 mM CaCl₂ to allow for annexin 2 solubilization. Oocyte homogenates were centrifuged twice for 10 min at 16 000 g. Supernatants were incubated with anti-BSA antibodies immobilized on protein A-agarose beads (Kem-En-Tec A/S, Copenhagen, Denmark) for 2 h at room temperature. After three washing steps with pull-down buffer, immunoprecipitated proteins were eluted with SDS–PAGE loading buffer, separated on SDS–PAGE gel and visualized by autoradiography.

RT–PCR analysis
Total RNA from mouse kidney, mouse duodenum, HEK293 and X.laevis oocytes was isolated using Trizol (Gibco BRL). Total RNA (2 µg) was reverse transcribed and PCR reactions for S100A10 and annexin 2 were performed (S100A10 forward primer 5′-GGTCAAGACACCATGATG-3′ and reverse primer 5′-AAATAGTCTATGATGCAATG-3′; annexin 2 forward primer 5′-ATGCTACTGTCACGAAATC-3′ and reverse primer 5′-CCCTTGATCACGTGAATTC-3′). For PCR analysis of oocyte mRNA different primers were used (S100A10 forward 5′-GATGTTGGCCGCCCTCGA-3′ and reverse primer 5′-CCCAAGATTAGTGTTAAGAT-3′; annexin 2 forward primer 5′-GCTGATAGCTTGGATGATG-3′ and reverse primer 5′-CCATGCTGT-3′). Real-time quantitative PCR using the ABI Prism 7700 Sequence Detection System (PE Biosystems, Rotkreuz, Switzerland) as described previously (Hoenderop et al., 2002a). S100A10 mRNA expression levels in kidney were quantified by real-time quantitative PCR using the ABI Prism 7700 Sequence Detection System (PE Biosystems, Rotkreuz, Switzerland) as described previously (Hoenderop et al., 2002a). (forward primer 5′-GGTCAAGACACCATGATG-3′, reverse primer 5′-TCGCTCTGAAAACCTTGA-3′ and probe 5′-GCCGAGGCACAAGTTGATAGTAC-3′). The expression level of mouse hepatocyte growth factor mRNA (HPRT) was detected with the forward primer 5′-TTACACTGATGAGAGGACTCTC-3′, reverse primer 5′-TTACACTGTCCTACTTCTTACCAAT-3′ and probe 5′-TGAGGATGATCCTCCCAACTAATT-3′, used as an endogenous control to normalize variations in RNA extractions, the degree of RNA degradation and variations in RT efficiencies.

Immunohistochemistry
Immunohistochemistry was performed as described previously (Hoenderop et al., 2001a). Briefly, rat and rabbit duodenum sections were incubated for 16 h at 4°C with rabbit antisera against TRPV6 (1:100) kindly provided by Dr. M.Suzuki (Jichi Medical School, Tochigi, Japan), monoclonal anti-S100A10 (1:50; Swant, Bellinzona, Switzerland) or monoclonal anti-annexin 2 (1:50; Transduction Laboratories). Rabbit kidney sections were incubated for 16 h at 4°C with affinity purified guinea pig antisera against TRPV5 (1:50; Hoenderop et al., 2000) and monoclonal anti-S100A10 (1:100) or monoclonal anti-annexin 2 (1:50). To visualize TRPV5 and TRPV6, a goat anti-guinea pig Alexa 488 conjugated antibody (1:300) or a goat anti-rabbit Alexa 488 conjugated antibody (1:300) was used. To visualize S100A10 and annexin 2, sections were incubated with a goat anti-mouse Alexa 594 conjugated antibody (1:300; Molecular Probes) was used. To visualize S100A10 and annexin 2, sections were incubated with a goat anti-mouse Alexa 594 conjugated antibody (1:300; Molecular Probes). Xenopus laevis oocytes were injected with 5 ng of HA-tagged TRPV5 (wild type or T660A) or Flag-tagged TRPV6 (wild type or T599A) cRNA. Three days after injection immunohistochemistry was performed as described previously (Hoenderop et al., 1999b) using monoclonal anti-HA (1:400; Sigma) or monoclonal anti-Flag (1:400; Sigma). Images were taken with a Bio-Rad MRC 100 confocal laser scanning microscope. All negative controls, including sections incubated with pre-immune serum or conjugated antibodies alone, were devoid of any staining.

Electrophysiology
The full-length cDNA encoding TRPV5 and TRPV6 was cloned into the pIRE5 vector and transfected in HEK293 as described previously (Vennekens et al., 2000; Hoenderop et al., 2001b). Currents using the whole-cell configuration were measured with an EPC-9 (HEKA Elektronik, Lambrecht, Germany; 8-Pole Bessel filter 10 kHz). Electrode resistances were between 2 and 5 MΩ, capacitance and series resistance were compensated, and access resistance was monitored continuously. Current–voltage (IV) relations were measured by linear 400 ms voltage ramps, which were applied every 5 s from a holding potential of +20 or +70 mV with a sampling interval of 0.8 ms. The step protocol consisted of 3 s voltage steps to −100 mV from a holding potential of +470 mV. The standard extracellular solution contained 150 mM NaCl, 1 mM CaCl₂, 6 mM MgCl₂, 1 mM MgCl₂, 10 mM glucose and 10 mM HEPES/Ca(OH)₂ pH 7.4. Monovalent cation currents were measured in nominally Ca²⁺- and Mg²⁺-free solution (free Ca²⁺ concentration is 10 mM), and Ca²⁺ currents in 1 mM CaCl₂ but Mg²⁺-free solutions. Monovalent cation currents were inhibited by replacing 150 mM NaCl with an equimolar amount of N-methyl-D-glucamine (NMDG)-Cl. The standard internal (pipette) solution contained: 20 mM CsCl, 100 mM Cs aspartate, 1 mM MgCl₂, 10 mM BAPTA, 4 mM Na₂ATP and 10 mM HEPES/Ca(OH)₂ pH 7.2. Cells were kept in a nominally Ca²⁺-free medium to prevent Ca²⁺ overload, and exposed for a maximum of 5 min to a Krebs solution containing 1.5 mM Ca²⁺ before sealing the patch pipette to the cell. In the siRNA experiments, 0.5 mM EGTA was used for intracellular Ca²⁺ buffering. All experiments were performed at room temperature (20–22°C).

RNAs
RNAs employed a 21 nucleotide RNA duplex corresponding in sequence to nucleotides 94–113 of the human annexin 2 mRNA with a two nucleotide dT 3’ overhang. Cells grown on coverslips were transfected with 100 nM of the annealed RNA duplex using oligofectamine according to the manufacturer’s instructions (Invitrogen). After 24 h these siRNA-transfected HEK293 cells were transfected with pIRE5-TRPV5 or pIRE5-TRPV6 vector and analyzed in a patch-clamp setup 24 h following the last transfection. Expression of TRPV5 and TRPV6 was determined using immunoblotting with affinity-purified guinea pig (TRPV5) or rabbit (TRPV6) antibodies as described elsewhere (Hoenderop et al., 2003).

Statistical analysis
In all experiments, the data are expressed as mean ± SEM. Overall statistical significance was determined by analysis of variance (ANOVA). In the case of significance (P < 0.05), individual groups were compared by Student’s t-test.

Supplementary data
Supplementary data are available at The EMBO Journal Online.

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