Selective Association of TRPC Channel Subunits in Rat Brain Synaptosomes

TRPC genes encode a ubiquitous family of ion channel proteins responsible for Ca\(^{2+}\) influx following stimulation of G-protein-coupled membrane receptors linked to phospholipase C. These channels may be localized to large multimeric signaling complexes via association with PDZ-containing scaffolding proteins. Based on sequence homology, the TRPC channel family can be divided into two major subgroups: TRPC1, -C4, and -C5 and TRPC3, -C6, and -C7. Although TRPC channels are thought to be tetramers, the actual subunit composition remains unknown. To define subunit arrangement, individual TRPC channel pairs were heterologously expressed in Sf9 insect cells and immunoprecipitated using affinity-purified rabbit polyclonal antibodies specific for each channel subtype. Reciprocal co-immunoprecipitations showed that TRPC1, -C4, and -C5 co-associate and that TRPC3, -C6, and -C7 co-associate but that cross-association between the two major subgroups does not occur. Additionally, the interaction between each TRPC channel and the PDZ-containing protein, INAD (protein responsible for the inactivation-no-gating-potential Drosophila mutant), was examined. TRPC1, -C4, and -C5 co-immunoprecipitated with INAD, whereas TRPC3, -C6, and -C7 did not. To define channel subunit interactions in vivo, immunoprecipitations were performed from isolated rat brain synaptosomal preparations. The results revealed that TRPC1, -C4, and -C5 co-associate and that TRPC3, -C6, and -C7 co-associate in both cortex and cerebellum but that cross-association between the two major subgroups does not occur. These results demonstrate that TRPC channels are present in nerve terminals and provide the first direct evidence for selective assembly of channel subunits in vivo.

TRP\(^1\) genes, originally identified as critical components of phototransduction in Drosophila, encode a ubiquitous and heterogeneous family of ion channel proteins that appear to play a fundamental role in cell signaling, cell growth, and cell death (recently reviewed in Refs. 1–3). In the last 7 years, 21 mammalian homologs have been discovered. There are currently seven mammalian TRP siblings designated TRPC1–TRPC7 that exhibit 32–47% overall identity at the amino acid level compared with the Drosophila isoforms. Based on amino acid sequence homologies, the TRPC family can be divided into two major subgroups: TRPC1, -C4, and -C5 and TRPC3, -C6, and -C7. TRPC2, which is a pseudogene in humans (4), is related but is clearly distinct from the two major subgroups. The primary TRPC channels, like the Drosophila versions, appear to be regulated by phospholipase C (PLC)-dependent mechanisms and thus are thought to be intimately involved in receptor-mediated Ca\(^{2+}\) signaling. In many cell types, a specific receptor-activated channel appears to be regulated by the level of Ca\(^{2+}\) within the inositol 1,4,5-trisphosphate-sensitive internal Ca\(^{2+}\) store (5, 6). These so-called store-operated channels are responsible for the Ca\(^{2+}\) release-activated current, \(I_{\text{CRAC}}\) (7). There is evidence from heterologous expression studies (8–12), antisense experiments (13–16), genetic disruption (17, 18), and adenovirus-mediated in vivo overexpression (19) that mammalian TRPC channel subunits are components of store-operated channels, although results in the literature are often conflicting. There is, however, general agreement that TRPC3 and TRPC6, and perhaps TRPC7, comprise channels that can be regulated by diacylglycerol (20, 21).

Based on the known structure of K\(^+\), Na\(^+\), and Ca\(^{2+}\) channels, it is assumed that TRPC channels are composed of four subunits, and there is growing evidence to suggest heteromeric channel assembly (22–25). However, the channel composition in vivo and the rules governing subunit assembly remain to be determined. Likewise, how these tetrameric channels are localized in the plasmalemma is unknown. Selective localization to specific domains of the membrane appears to play an important role in signal transduction (26). In this regard, studies in Drosophila photoreceptor cells have shown that TRP channels are held in a signaling complex (i.e. a signalplex) by a scaffolding protein called INAD (1). INAD contains five tandem PDZ domains that serve as protein binding modules mediating the clustering of proteins involved in the Drosophila phototransduction cascade (e.g. TRP, TRPL, PLC, protein kinase C, and calmodulin). Likewise, PDZ-containing proteins may provide the scaffolding necessary for TRPC channel signalplex formation and localization in mammalian cells. Immunoprecipitation experiments in heterologous expression systems and rat brain have shown that the first PDZ domain of the Na\(^+\)-H\(^+\) exchanger regulatory factor (NHERF) binds TRPC4, TRPC5, PLC\(\beta\)1, and PLC\(\beta\)2 (27). Interestingly, neither NHERF nor INAD appear to bind TRPC3 (27, 28), suggesting that only certain TRPC channel subunits may associate with PDZ-containing proteins and consequently contribute to the signalplex in mammalian cells.

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The abbreviations used are: TRP, transient receptor potential; NHERF, Na\(^+\)-H\(^+\) exchange regulatory factor; PLC, phospholipase C; IP, immunoprecipitation.
In order to determine the subunit arrangement of native TRPC channels, we isolated and affinity-purified rabbit polyclonal antibodies directed against amino acid sequences specific for each channel subtype. Reciprocal co-immunoprecipitations of individual TRPC channel pairs heterologously expressed in Sf9 insect cells using recombinant baculovirus revealed that TRPC1, -C4, and -C5 co-associate and that TRPC2, -C6, and -C7 co-associate but that cross-association between the two major subgroups does not occur. Additionally, we designed peptide sequences to provide greater exposure of the peptide on the PDZ-containing protein, INAD, TRPC1, -C4, and -C5 or immunoprecipitated with INAD, whereas TRPC3, -C6, and -C7 did not. In order to define channel subunit interactions in vivo, synaptosomal preparations were isolated from rat cerebral cortex and cerebellum. Again the immunoprecipitation experiments revealed that TRPC1, -C4, and -C5 co-associate and that TRPC3, -C6, and -C7 co-associate in both cortex and cerebellum but that cross-association between the two major subgroups does not occur. These results not only demonstrate that TRPC channels are present in nerve terminals, but they provide the first direct evidence for selective assembly of channel subunits in vivo. Selective subunit assembly and selective interaction with PDZ-containing proteins may underlie differences in the activation mechanism for the various TRPC channels and help to explain conflicting results with regard to their putative role as store-operated channels.

MATERIALS AND METHODS

Cell Culture—Spodoptera frugiperda (Sf9) cells were obtained from ATCC and cultured as previously described (29, 30) using Grace’s insect medium supplemented with 2% lactalbumin hydrolysate, 2% yeastolate solution was washed with ice-cold column buffer until the absorbance of 280 of the IgG fraction at 0.01. Bound antibody was eluted with 0.1M glycine (pH 2.8) as described above, and the protein fractions were pooled. The INAD CDNAs and antibodies were generous gifts from Drs. Bib-Hwa Shieh (Vanderbilt University). The CDNA encoding the various mammalian TRPC channel proteins and INAD were individually subcloned into baculovirus transfer vector, PVL1393, using standard techniques. Recombinant baculoviruses were produced using the BaculoGold™ transfection kit (Pharmingen) as described in the instructions provided by the manufacturer. Recombinant viruses were plaque-purified and amplified to obtain a high titer viral stock solution. The virus was stored at 4 °C under sterile conditions.

Infected Sf9 Insect Cells with Recombinant Baculovirus—Sf9 cells in Grace’s medium were plated into 100-mm plastic tissue culture dishes or onto glass coverslips (106 cells/cm2). Following incubation for 30 min, an aliquot of viral stock was added (multiplicity of infection = 10), and the cells were maintained at 27 °C in a humidified air atmosphere. Unless otherwise indicated, cells were used at 28 h postinfection.

Isolation of Synaptosomes—Synaptosomes were isolated from rat cerebral cortex and cerebellum exactly as described by Cotman (31). Lysates were prepared as described below immediately following isolation of the final synaptosomal preparation. Lysates were stored frozen (–80 °C) at a protein concentration of 0.5 mg/ml.

Isolation of Sf9 Cell Membrane Fractions—Following infection with recombinant baculovirus, Sf9 cells were harvested, subjected to centrifugation, and recentrifuged. After three such washes, the purified antibody was concentrated and 1% penicillin/streptomycin/neomycin solution (Invitrogen). The supernatants were centrifuged at 42,000 g for 30 min. The microsomal pellets were resuspended in lysis buffer at a protein concentration of 5–10 mg/ml and stored at –80 °C until use.

Immunoprecipitations and Immunoblotso—Sf9 cells infected with baculovirus and expressed in Sf9 insect cells (Pierce). The figures show representative results from at least three independent experiments. For the co-expression experiments in Sf9 cells, this represents at least three independent infections.

RESULTS

Characterization of Anti-TRPC Antibodies—Antibodies were generated in rabbits against peptide sequence specific to each human TRPC channel protein (Table I). The sequences chosen were either identical or had only one or two amino acid differences when compared with the same mouse, rat, and bovine sequences. All of the sequences are in the COOH-terminal
region and are predicted to be cytoplasmic. In preliminary experiments, each antibody was evaluated for its ability to selectively recognize the appropriate TRPC channel protein expressed in Sf9 insect cells using recombinant baculovirus. The proteins examined and their predicted molecular masses were as follows: human TRPC1 (accession number X89066; Mr/H 87.6 kDa) and TRPC3 (U47050; 97.3 kDa); bovine TRPC4 (X99792; 112.5 kDa); and mouse TRPC5 (AF029983; 111.4 kDa), TRPC6 (U49069; 106.7 kDa), and TRPC7 (AF139923; 99.5 kDa). Sf9 cells were infected, and membrane fractions were isolated as described under “Materials and Methods.” Membrane proteins were separated by SDS-PAGE and subjected to Western blot analysis using each of the affinity-purified anti-TRPC antibodies. With the exception of anti-TRPC1 and anti-TRPC6, the results showed that each antibody 1) recognized the appropriate TRPC channel protein, 2) did not recognize other membrane proteins endogenously expressed in Sf9 cells, 3) did not recognize any baculovirus proteins, and 4) exhibited no cross-reactivity with any of the other TRPC channel proteins (see Fig. 1 in Supplementary Material). Anti-TRPC1 also selectively recognized the TRPC1 channel protein, but an additional band at a slightly higher molecular mass was often observed (Fig. 1 in Supplementary Material). Likewise, anti-TRPC6 recognized the TRPC6 channel protein, but an additional band was sometimes observed at or slightly above the dye front (∼30 kDa). These bands were also seen in Sf9
cells expressing each of the TRPC proteins and in Sf9 cells infected with an unrelated baculovirus containing the human B₁α bradykinin receptor cDNA. Thus, these additional bands probably reflect either an endogenous Sf9 cell membrane protein or a baculovirus protein. We next examined the ability of each anti-TRPC antibody to immunoprecipitate the appropriate protein from total Sf9 cell lysates (see Fig. 2 in Supplementary Material). Each anti-TRPC antibody was able to immunoprecipitate the appropriate TRPC channel protein. Two bands were immunoprecipitated using the anti-TRPC1 antibody. As indicated above, the lower molecular mass band reflects TRPC1, whereas the upper band may reflect an endogenous Sf9 cell protein.

**Association of TRPC Channel Proteins in Vitro**—The Sf9 cell-baculovirus system is useful for the co-expression of proteins or co-expression of protein subunits. When used at appropriate multiplicities of infection, individual subunits are properly assembled into a functioning unit in Sf9 cells co-infected with individual recombinant baculoviruses. We took advantage of this system to determine whether the TRPC channel proteins co-assemble. Each TRPC channel was individually co-expressed in channel pairs with the other members of the channel family. Reciprocal co-immunoprecipitation experiments were performed for each pair. The results show that TRPC1, TRPC4, and TRPC5 co-immunoprecipitate from Sf9 cell lysates when expressed in the appropriate combinations

**Fig. 3.** TRPC1, TRPC4, and TRPC5 do not immunoprecipitate from Sf9 cell lysates with TRPC3, TRPC6, or TRPC7. Sf9 cells were co-infected with recombinant baculovirus for expression of individual TRPC channel pairs. Upper panels, TRPC1 co-expressed with TRPC3 (left), TRPC6 (center), or TRPC7 (right); middle panels, TRPC4 co-expressed with TRPC3 (left), TRPC6 (center), or TRPC7 (right); bottom panels, TRPC5 co-expressed with TRPC3 (left), TRPC6 (center), or TRPC7 (right). Each panel shows a Western blot using the indicated anti-TRPC antibody. Lane 1, proteins from total cell lysates; lane 2, proteins immunoprecipitated with the indicated anti-TRPC channel antibody.

**Fig. 4.** INAD co-immunoprecipitates with TRPC1, TRPC4, and TRPC5 but not with TRPC3, TRPC6, and TRPC7 from Sf9 cell lysates. Sf9 cells were co-infected with recombinant baculovirus for expression of the individual TRPC channels and INAD; upper panels, TRPC1, TRPC4, and TRPC5; bottom panels, TRPC3, TRPC6, and TRPC7. Each panel shows a Western blot using anti-INAD antibody. Lane 1, proteins from total cell lysates; lane 2, proteins immunoprecipitated with the indicated anti-TRPC channel antibody.
Likewise, TRPC3, TRPC6, and TRPC7 co-immunoprecipitate (Fig. 2). In contrast, TRPC1, TRPC4, and TRPC5 did not co-precipitate with TRPC3, TRPC6, or TRPC7 (Fig. 3; the reciprocal immunoprecipitations for each channel pair are not shown in Fig. 3 but were also negative). These results suggest that there is association between the members of the two major TRPC channel subgroups but that there is no cross-association between groups.

Interaction of PDZ-containing Protein with TRPC Channels—Previous studies have shown that TRPC4 and TRPC5, but not TRPC3, co-immunoprecipitate with NHERF (27). Likewise, Drosophila TRP and TRPL interact with INAD but not with TRPC3 (28). Together, these results suggest that there may be subgroup-specific interaction of TRPCs with PDZ domains. To begin to examine this question, we co-expressed INAD in Sf9 cells along with the individual TRPC channel proteins. As seen in Fig. 4, INAD co-immunoprecipitated with TRPC1, TRPC4, and TRPC5 but not with TRPC3, TRPC6, or TRPC7.

Interactions of TRPC Channels in Rat Brain—To determine whether the subgroup specificity of interaction is also true of native TRPC channels as they exist in their normal signaling environment, we performed immunoprecipitations from isolated nerve endings (synaptosomes) obtained from rat cerebral cortex and cerebellum. Synaptosomes were isolated from the 6/13% interface of a Ficoll step gradient as previously described (31). In preliminary experiments, TRPC channel proteins were only observed in lysates prepared from material obtained at the 6/13% interface of a Ficoll step gradient; TRPCs were not present at the 4/6% interface. This result is consistent with co-enrichment of TRPC channel proteins with synaptosomes. We next evaluated the ability of each antibody to immunoprecipitate the appropriate channel protein from synaptosomal lysates. With the exception of TRPC4, each of the anti-TRPC antibodies immunoprecipitated a protein of appropriate molecular mass from both cortex and cerebellum, although TRPC7 was difficult to detect in cortex (Fig. 5). We were unable to immunoprecipitate or detect TRPC4 in either cortex or cerebellum, a rather paradoxical result,
given that TRPC4 was detected in immunoprecipitations from Sf9 cells (Fig. 1) and, in previous studies, from rat brain (25, 27). In some gels, additional minor bands were seen. These may reflect glycosylated forms or alternatively spliced versions of the TRPC proteins in brain. We next performed co-immunoprecipitation experiments from cortex and cerebellar synaptosomal lysates. As seen in Fig. 6, TRPC1 co-immunoprecipitated with TRPC5. Much to our surprise, TRPC4 also co-immunoprecipitated with TRPC1 and TRPC5 (Fig. 6), but neither TRPC1 nor TRPC5 immunoprecipitated with TRPC4 (not shown). These results suggest that the epitope on native TRPC4 is not accessible to the anti-TRPC4 antibody. Furthermore, the results suggest that TRPC1, TRPC4, and TRPC5 all co-associate in rat cerebral cortex. The same results were obtained in synaptosomal preparations isolated from cerebellum (not shown).

As seen in Fig. 7, TRPC3, TRPC6, and TRPC7 co-immunoprecipitated from rat cerebellar synaptosomal lysates but did not co-immunoprecipitate with TRPC1, TRPC4, and TRPC5 (Fig. 8; the reciprocal immunoprecipitations for each channel pair are not shown in Fig. 8 but were also negative). Results essentially identical to those shown in Figs. 7 and 8 were also obtained in synaptosomal preparations isolated from cortex (not shown). These results suggest that there is association between the members of the two major TRPC channel subgroups in vivo but that there is no cross-association between groups.

**DISCUSSION**

In order to determine the subunit composition and to begin a proteomic study of the mammalian TRPC channel signalplex, specific immunoprecipitating antibodies are needed. In the present study, we characterized rabbit polyclonal antibodies generated against amino acid sequences specific for each TRPC channel protein expressed in Sf9 insect cells using recombinant baculovirus. Using this system, we found that the affinity-purified TRPC antibodies were specific and selective and recognized the appropriate TRPC channel proteins with high affinity. Furthermore, each TRPC antibody preparation was capable of immunoprecipitating the appropriate TRPC protein from Sf9 cell lysates, suggesting that the epitope recognized by the antibodies was exposed in each homomeric channel. With the exception of TRPC4, each TRPC antibody was also able to immunoprecipitate the appropriate TRPC channel protein from rat brain synaptosomal preparations, again suggesting that the epitope is exposed in the native channel signalplex. However, we were unable to immunoprecipitate TRPC4 from synaptosomes using the TRPC4-specific antibody. This apparently did not reflect the absence of TRPC4 protein in the nerve terminals, since TRPC4 was immunoprecipitated with anti-TRPC1 and anti-TRPC5 antibodies. The simplest explanation, consistent with all of the results, is that the epitope for TRPC4 is not exposed in the native channel signalplex. Thus, amino acids 826–839 of TRPC4 may be partially or totally occluded by other TRPC subunits present in the heteromultimeric channel or may be blocked by some other protein present in the signalplex.

Based on amino acid sequence similarities, the TRPC channel family can be subdivided into two major subgroups: (a) TRPC1, TRPC4, and TRPC5 and (b) TRPC3, TRPC6, and TRPC7. Two lines of evidence suggest that members of each subgroup assemble to form heteromeric channel structures but that cross-association between subgroups does not occur. First, only members of each group co-immunoprecipitate following in
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**vitro** expression of individual subunit pairs. All of the immunoprecipitations were performed in a reciprocal fashion. Thus, association of the individual subunits is not dependent on the antibody used for precipitation. Likewise, reciprocal co-immunoprecipitations of all channel pairings between the two major subgroups were negative. Thus, the interactions found within subgroups were specific and unrelated to technical artifacts such as mutual micellar associations. Second, only members of subgroups were specific and unrelated to technical artifacts subgroups were negative. Thus, the interactions found within antibody used for precipitation. Likewise, reciprocal co-immunoprecipitations were performed in a reciprocal fashion. Thus, expression of individual subunit pairs. All of the immunosays applied to heterologously expressed TRPC channel proteins, Hofmann et al. (24) reported that TRPC1, TRPC4, and TRPC5 co-assemble and that TRPC3, TRPC6, and TRPC7 co-assemble but that there was no detectable interaction between the two major subgroups. The in vitro results of the present study using a different expression system with nonmodified channel proteins and the in vivo results obtained in rat brain synaptosomes clearly support this proposed subunit interaction. One note of caution; splice variants of the TRPC proteins lacking the antibody epitope may exist. If these are expressed in rat brain, they would not be seen in our assays and may exhibit a different interaction profile. Likewise, splice variants with molecular mass similar to IgG heavy or light chains would be hidden.

It is now well established that *Drosophila* TRP and TRPL are held in a large signalplex by a PDZ-containing scaffolding protein called INAD (1). This signalplex appears to be essential for normal signal transduction, and both TRP and INAD are mislocalized when the interaction is disrupted through genetic mutations (36, 37). An analogous signalplex may be necessary for normal signal transduction and localization in mammalian cells, but there is relatively little direct evidence for TRPC signalplex formation. A number of mammalian PDZ-containing proteins with homology to INAD have been identified. These include human INAD-like (hINAD-L) (38), multiple PDZ-containing protein (MUPP1) (39), and the glutamate receptor-interacting protein (GRIP) (40). Their interaction with TRPC channels, however, has not been demonstrated. TRPC4 and TRPC5 have been shown to co-immunoprecipitate with NHERF from brain lysates and from lysates of cells heterologously co-expressing the proteins (27), but the functional consequences of this interaction remain unknown. However, neither NHERF nor INAD bind to TRPC3, suggesting that the interaction of PDZ domains with TRPC channel proteins may be selective. The results of the present study show that TRPC1, TRPC4, and TRPC5 interact with INAD, whereas TRPC3, TRPC6, and TRPC7 do not. This result suggests that TRPC1, TRPC4, and TRPC5 may form heteromultimeric channels that are tethered to specific subcellular sites via interaction with INAD-like PDZ-containing proteins, whereas TRPC3, TRPC6, and TRPC7 may form nontethered heteromultimers. In this regard, Delmas et al. (26) showed that TRPC1 overexpressed in superior cervical ganglion neurons forms store-operated channels localized to a specific microdomain that includes a plasmalemmal bradykinin receptor and an endoplasmic reticulum inositol 1,4,5-trisphosphate receptor. Furthermore, these investigators showed that diacylglycerol-sensitive TRPC6 channels are not localized to specific domains but rather are randomly distributed on the cell surface. In contrast, activation of either TRPC5 or TRPC1-TRPC5 heteromultimers expressed in HEK293-M1 cells is independent of Ca2+ store depletion (25). Thus, the mechanism of TRPC channel activation may depend on subunit composition, localization to specific subcellular domains, and the constellation of accessory proteins present in the signalplex.

There is little information in the literature concerning TRPC protein distribution in the brain. Immunohistochemical staining of rat brain slices showed that TRPC1 immunoreactivity is present in the hippocampus, amygdala, purkinje cell layer of the cerebellum, and neurons of the cortex and striatum (25). In primary cultured hippocampal neurons, TRPC1 immunofluorescence, detected by confocal microscopy, was found in the cell bodies, dendrites, and axons, but TRPC1 was not observed in synapse-like structures identified by the presence of synaphtin. In the present study, all of the TRPCs appear to be present in freshly isolated synaptosomal preparations from rat cerebral cortex and cerebellum, suggesting that native TRPC channels are present in nerve terminals. Preliminary confocal imaging suggests that the TRPC channel proteins are differentially expressed in various regions of rat brain. It will be interesting to determine whether the subgroup-specific association reflects selective localization to specific nerve endings or if all nerve terminals contain both tethered and nontethered forms of these channels.

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REFERENCES

1. Montell, C. (2001) Science 291, 90–95
2. Clapham, D. E., Runnels, L. W., and Strubing, C. (2001) Nat. Rev. Neurosci. 2, 387–396
3. Harteneck, C., Plant, T. D., and Schultz, G. (2001) Trends Neurosci. 23, 159–166
4. Wes, P. D., Chevesich, J., Jeromin, A., Rosenberg, C., and Montell, C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9652–9656
5. Putney, J. W., Jr., and McKay, R. R. (1999) Biosens 21, 38–46
6. Putney, J. W., Jr., Broad, L. M., Braun, F. J., Lievremont, J.-P., and Bird, G. S. (2001) J. Cell Sci. 114, 2223–2229
7. Parekh, A. B., and Penner, R. (1997) Physiol. Rev. 77, 901–930
8. Zitt, C., Zobel, A., Obukhov, A. G., Harteneck, C., Kalkbrenner, F., Luckhoff, A., and Schultz, G. (1996) Neuron 16, 1189–1196
9. Warnat, J., Philippine, S., Zimmer, S., Flochker, V., and Cavalie, A. (1999) J. Physiol. (Lond.) 518, 631–638
10. Philippine, S., Cavaillé, A., Freichels, M., Wiesnack, U., Zimmer, S., Trust, C., Marquart, A., Murakami, M., and Flochker, V. (1996) EMBO J. 15, 6166–6171
11. Philippine, S., Hambrecht, J., Bralavalki, L., Schroth, O., Freichels, M., Murakami, M., Cavaillé, A., and Flochker, V. (1998) EMBO J. 17, 4274–4282
12. Vanzequez, G., Lievremont, J.-P., Bird, G. S. J., and Putney, J. W., Jr. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 11777–11782
13. Wu, X., Babnigg, G., and Villereal, M. L. (2000) Am. J. Physiol. 278, C526–C536
14. Liu, X., Wang, W., Singh, B. B., Lockwood, T., Jadowiec, J., O’Connell, B., Wellner, R., Zhu, M. X., and Ambudkar, I. S. (2000) J. Biol. Chem. 275, 3403–3411
15. Brough, G. H., Wu, S., Cifetti, D., Moore, T. M., Li, M., Dean, N., and Stevens, T. (2001) FASEB J. 15, 1727–1738
16. Philippine, S., Trust, C., Warnat, J., Rautmann, J., Himmerkus, N., Schroth, G.,

² M. C. Buniel, P. Glazebrook, W. P. Schilling, and D. L. Kunze, manuscript in preparation.
Kretz, O., Nastainczyk, W., Cavalié, A., Hoth, M., and Flockerzi, V. (2000) J. Biol. Chem. 275, 23965–23972
17. Freichel, M., Suh, S. H., Pfeifer, A., Schweig, U., Trost, C., Weibgerber, P., Biel, M., Philipp, S., Freise, D., Droogmans, G., Hofmann, F., Flockerzi, V., and Nilius, B. (2001) Nat. Cell Biol. 3, 121–127
18. Mori, Y., Wakamori, M., Miyakawa, T., Hermsosura, M., Hara, Y., Nishida, M., Hirase, K., Mizushima, A., Kurotsuki, M., Mori, E., Gotoh, K., Okada, T., Pfeil, A., Penner, R., Iino, M., and Kurotsuki, T. (2002) J. Exp. Med. 195, 1–10
19. Singh, B. B., Zheng, C., Liu, X., Lockwich, T., Liao, D., Zhu, M. X., Birnbaumer, L., and Ambudkar, I. S. (2001) FASEB J. 15, 1652–1654
20. Hofmann, T., Obukhov, A. G., Schaefer, M., Harteneck, C., Gudermann, T., and Schultz, G. (1999) Nature 397, 259–263
21. Okada, T., Inoue, R., Yamazaki, K., Maeda, A., Kurotsuki, T., Yamakuni, T., Tanaka, I., Shimizu, S., Ikenaka, K., Imoto, K., and Mori, Y. (1999) J. Biol. Chem. 274, 27359–27370
22. Xu, X. Z. S., Li, H. S., Guggino, W. B., and Montell, C. (1997) Cell 89, 1155–1164
23. Lintschinger, B., Balzer-Geldsetzer, M., Baskaran, T., Graier, W. F., Romanin, C., Zhu, M. X., and Groschner, K. (2002) J. Biol. Chem. 277, 27799–27805
24. Hofmann, T., Schaefer, M., Schultz, G., and Gudermann, T. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7461–7466
25. Strubing, C., Krapivinsky, G., Krapivinsky, L., and Clapham, D. E. (2001) Neuron 29, 645–655
26. Delmas, P., Wanaverbecq, N., Abogadie, P. C., Mistry, M., and Brown, D. A. (2002) Neuron 34, 209–220
27. Tang, Y., Tang, J., Chen, Z., Trost, C., Flockerzi, V., Li, M., Ramesh, V., and Zhu, M. X. (2000) J. Biol. Chem. 275, 37559–37564
28. Xu, X. S., Choudhury, A., Li, X., and Montell, C. (1998) J. Cell Biol. 142, 545–555
29. Hu, Y., Rajan, L., and Schilling, W. P. (1994) Am. J. Physiol. 266, C1736–C1743
30. O’Reilly, D. R., Miller, L. K., and Luchow, V. A. (1992) Baculovirus Expression Vectors: A Laboratory Manual, W. H. Freeman and Co., New York
31. Cotman, C. W. (1974) Methods Enzymol. 31, 445–452
32. Puri, T. S., Gerhardtstein, B. L., Zhao, X. L., Ladner, M. B., and Hesey, M. M. (1997) Biochemistry 36, 9605–9615
33. Schwarz, D., Kisselev, P., Honeck, H., Cascarbi, I., Schunck, W.-H., and Routs, I. (2002) Xenobiotica 31, 345–356
34. Loo, T. P., and Clarke, D. M. (1994) J. Biol. Chem. 269, 7750–7755
35. Rao, U. S., Steimle, R. E., and Balachandran, P. (2002) J. Biol. Chem. 277, 4900–4905
36. Shieh, B. H., and Niemeyer, B. (1995) Neuron 14, 201–210
37. Chevesich, J., Kreuz, A. J., and Montell, C. (1997) Neuron 18, 95–105
38. Philipp, S., and Flockerzi, V. (1997) FEBS Lett. 413, 243–248
39. Ullmer, C., Schmuck, K., Figge, A., and Lubbert, H. (1998) FEBS Lett. 424, 63–68
40. Deng, H., O’Brien, R. J., Fung, E. T., Lanahan, A. A., Warley, P. F., and Huganir, R. L. (1997) Nature 386, 279–284