Phospholipase Cγ1 Is Required for Activation of Store-Operated Channels in Human Keratinocytes

Chia-Ling Tu, Wenhan Chang, and Daniel D. Bikle
Endocrine Unit, Veteran Affairs Medical Center and Department of Medicine, University of California, San Francisco, California, USA

Store-operated calcium entry depicts the movement of extracellular Ca^{2+} into cells through plasma membrane Ca^{2+} channels activated by depletion of intracellular Ca^{2+} stores. The members of the canonical subfamily of transient receptor potential channels (TRPC) have been implicated as the molecular bases for store-operated channels (SOC). Here we investigate the role of phospholipase C (PLC) in regulation of native SOC and the expression of endogenous TRPC in human epidermal keratinocytes. Calcium entry in response to store depletion with thapsigargin was reversibly blocked by 2-aminoethoxydiphenyl borane, an effective SOC inhibitor, and suppressed by the diacylglycerol analoge, 1-oleoyl-2-acetyl-*sn*-glycerol. Inhibition of PLC with U73122 or transfection of a PLCγ1 antisense cDNA construct completely blocked SOC activity, indicating a requirement for PLC, especially PLCγ1, in the activation of SOC. RT-PCR and immunoblotting analyses showed that TRPC1, TRPC3, TRPC4, TRPC5, and TRPC6 are expressed in keratinocytes. Knockdown of the level of endogenous TRPC1 or TRPC4 inhibited store-operated calcium entry, indicating they are part of the native SOC. Co-immunoprecipitation studies demonstrated that TRPC1, but not TRPC4, interacts with PLCγ1 and the inositol 1,4,5-trisphosphate receptor (IP_3R). The association of TRPC1 with PLCγ1 and IP_3R decreased in keratinocytes with higher intracellular Ca^{2+}, coinciding with a downregulation in SOC activity. Our results indicate that the activation of SOC in keratinocytes depends, at least partly, on the interaction of TRPC with PLCγ1 and IP_3R.

Key words: IP_3R/keratinocyte/PLCγ1/SOC/TRPC
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In most non-excitable cells, release of calcium from intracellular stores triggers entry of extracellular calcium through channels in the plasma membrane, a process known as capacitative calcium entry (CCE) (Putney *et al*, 2001). CCE provides an important means for mediating long-term Ca^{2+} signals and replenishment of Ca^{2+} stores (Parekh and Penner, 1997; Venkatachalam *et al*, 2002). Activation of CCE can occur through various mechanisms (Putney *et al*, 2001; Venkatachalam *et al*, 2002). Binding of ligands to plasma membrane receptors activates phopholipase C (PLC), which catalyzes the hydrolysis of phosphatidyl inositol 4,5-bisphosphate to generate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG has been known for its ability to directly activate ion channels (Vazquez *et al*, 2001; Venkatachalam *et al*, 2001) as well as protein kinase C (PKC), whereas IP_3 triggers Ca^{2+} release from intracellular Ca^{2+} stores, e.g., the endoplasmic reticulum (ER), through binding to IP_3 receptors (IP_3R) (Berridge, 1993). The resulting reduction of Ca^{2+} content in the ER lumen activates Ca^{2+} entry through ion channels in the plasma membrane (Putney, 1986). The term "agonist-induced Ca^{2+} entry" was used to refer to the Ca^{2+} entry mechanism induced by receptor activation (Patterson *et al*, 2002). In addition, agents such as the Ca^{2+} pump inhibitor thapsigargin (TG) and the calcium ionophore ionomycin, which deplete calcium stores independently of receptor activation or IP_3 generation, also activate Ca^{2+} entry (Putney, 1999). Therefore, the CCE channels can be loosely classified as receptor-operated channels (ROC) and store-operated channels (SOC), referring to Ca^{2+} entry channels activated in response to activation of PLC-coupled receptors and to Ca^{2+} store depletion, respectively (Berridge, 1997). It is, however, difficult to clearly define Ca^{2+} entry channels as either ROC or SOC, since both mechanisms converge on Ca^{2+} release from internal stores. The canonical subfamily of transient receptor potential (TRPC) channels has long been considered the candidates for ROC and/or SOC (Clapham *et al*, 2001; Montell, 2001). TRPC channels can be activated by a number of mediators involved in the PLC signaling cascade, including Ca^{2+} (Zitt *et al*, 1997), DAG (Kiselyov and Muallem, 1999; Venkatachalam *et al*, 2003), and activated IP_3R (Kiselyov *et al*, 1998; Boulay *et al*, 1999). Mammalian TRPC channels include seven members: TRPC1, TRPC2, the DAG-responsive subgroup of TRPC3/6/7 (Trebak *et al*, 2003), and the DAG-insensitive subgroup of TRPC4/5

Abbreviations: 2-APB, 2-aminoethoxydiphenylborane; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; CCE, capacitative calcium entry; DAG, diacylglycerol; EGTA, ethylene glycol-bis (β-amnioethyl ether)-N,N,N′,N′-tetracetic acid; ER, endoplasmic reticulum; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PKC, protein kinase C; PLC, phospholipase C; ROC, receptor-operated channel; SH3, Src homology 3 domain; SOC, store-operated channel; TG, thapsigargin; TRPC, canonical transient receptor potential channel

(Kiselyov and Muallem, 1999; Venkatachalam *et al*, 2003). Many studies demonstrate that mammalian TRPC channels can be activated in response to store depletion with calcium pump blockers or ionophores (Clapham *et al*, 2001), even though studies on overexpressed mammalian TRPC channels suggest that receptor-mediated stimulation of PLC is the major activation mechanism for TRPC (Hofmann *et al*, 2000). Nevertheless, gene knockdown (Philipp *et al*, 2000; Bough *et al*, 2001) or knockout (Freichel *et al*, 2001; Mori *et al*, 2002) studies indicate that endogenously expressed TRPC channels contribute to native SOC. In particular, TRPC1, TRPC3, and TRPC4 have been shown to be part of endogenous SOC in human submandibular gland cells (Liu *et al*, 2000), neurons (Li *et al*, 1999), and adrenal cortex cells (Philipp *et al*, 2000), respectively.

Though the underlying mechanisms of agonist-induced and store-operated Ca^{2+} entry are unclear, the favored conformational coupling model (Irvine, 1990; Putney, 1999) proposes that a reduction in ER luminal calcium induces a conformational change of IP_3R, which directly activates SOC in the plasma membrane through direct interaction. Studies of overexpressed TRPC demonstrated that IP_3R interacts with TRPC1 (Lockwich *et al*, 2000; Rosado and Sage, 2000a), TRPC3 (Kiselyov *et al*, 1998; Kiselyov *et al*, 1999), TRPC4 (Tang *et al*, 2001), and TRPC6 (Boulay *et al*, 1999). Overexpression of TRPC peptides containing the IP_3R-interacting domain reduced the activity of endogenous SOC, suggesting that native SOC is activated via IP_3R (Boulay *et al*, 1999). In several cell types store-depletion-induced Ca^{2+} entry was blocked when PLC activity was inhibited or polyphosphoinositides were depleted, indicating that besides activated IP_3 receptors, activation of SOC requires functional PLC activity (Rosado and Sage, 2000b; Broad *et al*, 2001). It has been suggested that the IP_3R–SOC complex may locate in close proximity to a PLC to respond to low levels of IP_3 required for channel activation (Putney, 1999; Broad *et al*, 2001). Recently, Patterson *et al* (2002) demonstrated that PLCγ plays a structural role in regulating agonist-induced calcium entry, since the action of PLCγ is dependent on its Src homology 3 (SH3) domain but independent of its lipase activity. It is unclear whether PLCγ regulates the function of SOC, although overexpression of PLCγ1 has been shown to augment both agonist-induced and store-operated Ca^{2+} entry (Patterson *et al*, 2002).

In human keratinocytes, emptying the intracellular calcium store has been shown to activate SOC (Csernoch *et al*, 2000; Karvonen *et al*, 2000). In this study, we investigated the role of PLCγ1 in regulation of store-operated Ca^{2+} entry and the molecular identities of endogenous SOC in normal epidermal keratinocytes. We demonstrate that SOC function is sensitive to disruption of basal PLC activity, and that activation of SOC requires PLCγ1. Our results indicate that TRPC1 and TRPC4 are part of the native SOC subunits in keratinocytes and that TRPC1 physically associates with PLCγ1 and IP_3R. In addition, we demonstrate that the inhibition of SOC activity by calcium parallels the attenuated interactions of TRPC1 with PLCγ1 and IP_3R. Our findings indicate that the coupling of native SOC to signaling molecules in the PLC/IP_3 pathway provides a gating mechanism for activation of calcium entry by store depletion.

Results

SOC in keratinocytes requires PLC activity To activate the SOC in human epidermal keratinocytes, we treated preconfluent cells grown in 0.03 mM Ca^{2+} with a Ca^{2+}-ATPase inhibitor TG, and monitored SOC activity by measuring the $[Ca^{2+}]_i$. In the presence of 0.1 mM EGTA, 1 μM TG elicited an initial increase in $[Ca^{2+}]_i$ due to passive release from ER Ca^{2+} stores. Subsequent addition of 2 mM extracellular Ca^{2+} induced a second phase of increased $[Ca^{2+}]_i$ (from 83 ± 10 to 256 ± 32 nM, mean $\pm$ SD; $n = 50$) resulting from calcium influx through SOC (Fig 1*a*). As shown in Fig 1*b*, this store-operated Ca^{2+} entry is blocked by application of 75 μM 2-aminoethoxydiphenyl borane (2-APB) (Fig 1*b*), an effective SOC inhibitor independent from its action on IP_3R (Bootman *et al*, 2002; Ma *et al*, 2002). This inhibition of SOC activity was reversible. After 2-APB induced blockade of SOC, removal of 2-APB resulted in a slow return of SOC activity over several minutes ($[Ca^{2+}]_i$

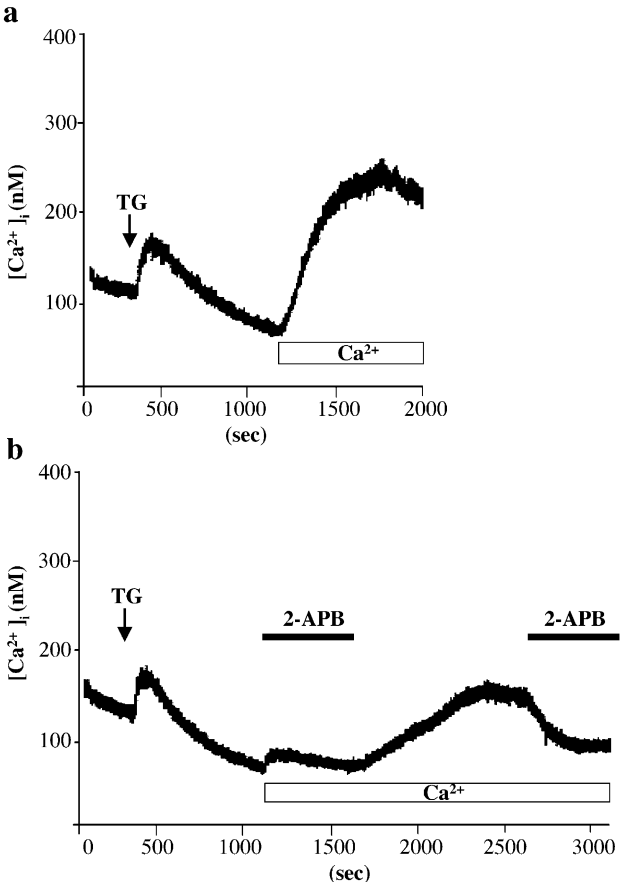

Figure 1
The store-operated channel (SOC) inhibitor 2-aminoethoxydiphenylborane (2-APB) blocked store-operated Ca^{2+} entry. Fura-2-loaded epidermal keratinocytes on glass coverslips were treated with 1 μM thapsigargin (TG) to deplete intracellular calcium stores in the presence of 0.1 mM EGTA. After cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) returned to baseline, 2 mM extracellular Ca^{2+} was applied to induce Ca^{2+} entry (*a*). 75 μM 2-APB was added after application of TG for the time indicated by the bars (*b*). The trace shown in the figure represents the average $[Ca^{2+}]_i$ of 42–50 individual keratinocytes during recording. 2-APB abolished the rise in $[Ca^{2+}]_i$ in response to 2 mM extracellular Ca^{2+} after store depletion. Calcium entry resumed after 2-APB was removed and was blocked by re-addition of 2-APB. The results are representative of three experiments.

increased from 86 ± 5 to 154 ± 23 nM; n = 42). Subsequent re-addition of 2-APB, while SOC-mediated Ca^{2+} entry was maximally active resulted in rapid termination of SOC activity ($[Ca^{2+}]_i$ decreased to 94 ± 11 nM) (Fig 1*b*).

To investigate the role of PLC in the activation of SOC in keratinocytes, we blocked the activation of PLC with a membrane-permeable PLC inhibitor, U73122, and examined its impact on SOC activity. We previously found that 15 min pre-treatment of keratinocytes with 10 μM U73122 fully prevented the PLC-mediated cellular response to extracellular calcium (Xie and Bikle, 1999). A 15 min pre-incubation of cells with 10 μM U73122 had no effect on the ability of TG to release intracellular calcium stores but fully prevented the rise in $[Ca^{2+}]_i$ upon addition of extracellular Ca^{2+} ($[Ca^{2+}]_i$ remained at 103 ± 10 nM; n = 37) (Fig 2*a*). On the contrary, pre-treatment of cells with 10 μM U73343, an inactive analog of U73122, had no effect on TG-induced SOC activity ($[Ca^{2+}]_i$ increased from 100 ± 5 to 284 ± 31 nM; n = 46) (Fig 2*a*). The inhibitory effect of U73122 on Ca^{2+} entry was, however, not due to a direct action on SOC channels. Unlike the SOC inhibitor 2-APB, application of 10 μM U73122 after induction of the Ca^{2+} entry by TG and subsequent addition of calcium did not affect the SOC activity. As shown in Fig 2*b*, 7 min after application of 10 μM U73122, $[Ca^{2+}]_i$ remained at 252 ± 15 nM (n = 29), compared with cells treated with vehicle (273 ± 25 nM; n = 34). Therefore, the specific blocking effect of U73122 on PLC action prevents store-deletion-induced Ca^{2+} entry, indicating that PLC is essential for the activation but not for the continuance of SOC activity.

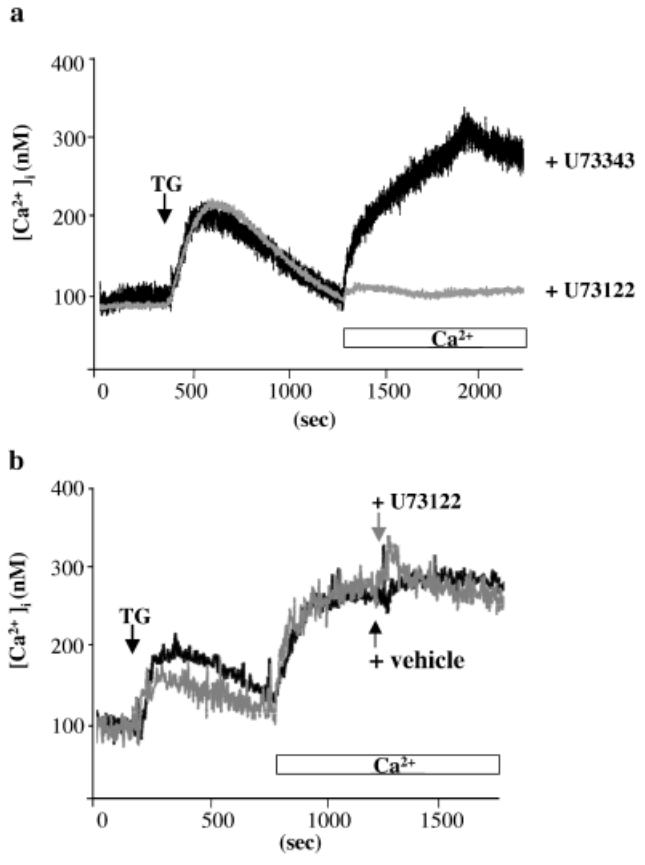

Figure 2
The phospholipase C inhibitor U73122 blocked the activation of store-operated Ca^{2+} entry. Keratinocytes, loaded with Fura-2, were treated with 1 μM thapsigargin (TG) to deplete intracellular calcium stores and to activate store-operated channel (SOC). Nominally calcium-free bath was replaced with buffer containing 2 mM calcium as indicated. (*a*) Cells were pre-treated with a PLC inhibitor U73122 (10 μM, n = 37) or its inactive analog U73343 (10 μM, n = 46) for 15 min before cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) measurement. U73122 abolished the activation of SOC by store depletion. (*b*) Seven minutes after application of 2 mM calcium, 0.5% ethanol (vehicle, n = 34) or U73122 (10 μM, n = 29) was added to cells. The trace shown in the figure represents the average $[Ca^{2+}]_i$ of 29–46 individual keratinocytes during recording. The results are representative of three experiments.

Activation of SOC is prevented by transfection of human PLCγ1 antisense cDNA Our previous studies had demonstrated that PLCγ1 is a critical mediator in the regulation of calcium signaling in keratinocytes. Knockdown of PLCγ1 expression blocked the rise in $[Ca^{2+}]_i$ in response to extracellular Ca^{2+} (Xie and Bikle, 1999) or 1,25-dihydroxyvitamin D3 (Xie and Bikle, 2001). To elucidate the role played by PLCγ1 in SOC function, we blocked the expression of endogenous PLCγ1 by transfecting pre-confluent keratinocytes with a full-length human PLCγ1 antisense cDNA construct. The transfectants were selected with the neomycin analog G418 in KGM containing 0.03 mM calcium for 2 d to enrich transfected cells. Protein extracts were prepared and subjected to immunoblotting analyses to examine the level of endogenous PLCγ1 protein. The expression of PLCγ1 in the keratinocytes transfected with antisense PLCγ1 cDNA was fully blocked compared with that in the cells transfected with the control vector (Fig 3*a*). In contrast, the protein level of PLCβ1 was not affected by the antisense PLCγ1 cDNA (Fig 3*a*), confirming the specificity of the PLCγ1 antisense construct. We next evaluated the effects of the PLCγ1 knockdown on SOC activity by comparing the Ca^{2+} entry evoked by TG-induced store emptying in the keratinocytes transfected with antisense PLCγ1 cDNA and in cells transfected with control vector. As shown in Fig 3*b*, the ability of TG to release intracellular calcium stores was not affected, but the rise in $[Ca^{2+}]_i$ upon the addition of extracellular Ca^{2+} ($[Ca^{2+}]_i$ increased from 107 ± 7 to 203 ± 12 nM in cells transfected with vector, n = 21) was completely blocked by transfection of the PLCγ1 antisense cDNA ($[Ca^{2+}]_i$ decreased from 104 ± 3 to 92 ± 5 nM; n = 12). Our results indicate that PLCγ1 is involved in the activation of SOC.

PLC activation generates IP_3 and DAG. The latter has been reported to directly activate calcium entry mediated by certain TRPC channels (Kiselyov and Muallem, 1999). Therefore, the requirement for PLC in SOC function may signal a need for DAG in addition to an activated IP_3R. To assess the involvement of DAG, we tested the effect of the membrane-permeable DAG analog, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), on SOC activity in keratinocytes. After TG induced store depletion, subsequent addition of 1 mM extracellular Ca^{2+} induced a moderate SOC activity that was not affected by the addition of 0.1% dimethylsulfoxide (DMSO), the vehicle for OAG (Fig 4*a*). 7 min after application of DMSO $[Ca^{2+}]_i$ remained at 210 ± 20 nM (n = 50). The store depletion-induced Ca^{2+} entry was, however, gradually blocked by the application of 100 μM OAG ($[Ca^{2+}]_i$ decreased from 185 ± 8 to 101 ± 6 nM; n = 50) (Fig 4*b*). Similar results were obtained with 100 μM 1,2-dioctanoyl-

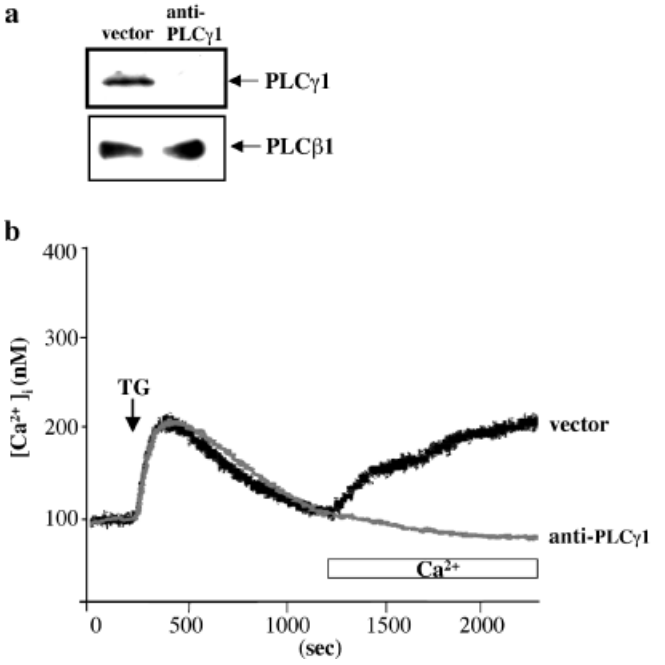

Figure 3
Transfection of a phospholipase C (PLC)γ1 antisense cDNA construct decreased the expression of endogenous PLCγ1 and prevented store-operated channel (SOC) activation. Keratinocytes were cultured, transfected with a full-length PLCγ1 antisense cDNA construct (anti-PLCγ1) or pcDNA3.1 (vector), and selected by G418 as described under "Experimental Procedures". Total proteins were isolated from transfected cells and analyzed by immunoblotting (*a*). The expression of PLCγ1, but not PLCβ1, was decreased by the PLCγ1 antisense construct (anti-PLCγ1). Transfected cells were loaded with Fura-2, and calcium entry activated by thapsigargin (TG)-induced store emptying was measured (*b*). The trace shown represents the average cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of 12–21 individual keratinocytes during recording. Transfection of PLCγ1 antisense construct (anti-PLCγ1) abolished the activation of calcium entry by store depletion. Data are representative of three different experiments.

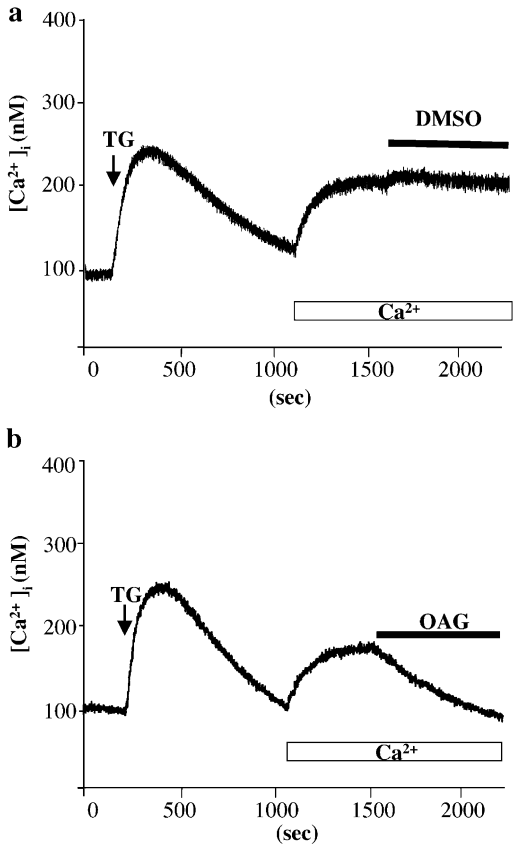

Figure 4
Inhibition of store-operated channel (SOC) by the diacylglycerol analog 1-oleoyl-2-acetyl-*sn*-glycerol (OAG). Calcium entry was activated by thapsigargin (TG)-induced store emptying. Subsequent addition of 1 mM extracellular Ca^{2+} was not affected by application of 0.1% dimethylsulfoxide (DMSO) (vehicle, n = 50) (*a*), but was blunted by application of 100 μM of OAG (n = 50) (*b*).

sn-glycerol (DOG), another DAG analog (data not shown). These results indicate that DAG does not promote but rather inhibits the coupling of SOC activity to PLC function.

Expression of TRPC channels in keratinocytes Substantial evidence has shown that TRPC channels mediate SOC activity (Philipp *et al*, 2000; Clapham *et al*, 2001). To investigate the molecular identities of the native SOC, we first examined the expression of TRPC in keratinocytes by RT-PCR and immunoblotting. Total RNA isolated from preconfluent keratinocytes cultured in KGM containing 0.03 mM Ca^{2+} was reverse transcribed into cDNA and then used as templates. The expression of the TRPC messages was determined by RT-PCR using sets of primers that spanned the pore-forming region of the channels. The expected size of the PCR product was 374 bp for the TRPC1, 323 bp for TRPC3, 415 bp for TRPC4, 341 bp for TRPC5, and 328 bp for TRPC6. No template (no RT) controls were run for all experiments (Fig 5*a*). Our results showed that TRPC1, TRPC3, TRPC4, TRPC5, and TRPC6 were expressed in pre-confluent keratinocytes (Fig 5*a*), whereas no TRPC2 or TRPC7 messages were detected (data not shown). The sequence of each amplified TRPC cDNA was verified by double-stranded DNA sequencing. The primers for TRPC3 also amplified a 450 bp fragment, the identity of which has not been established. To examine the expression of TRPC proteins, membrane proteins were prepared from keratinocytes. Immunoblotting analyses using antibodies recognizing various TRPC proteins detected endogenous TRPC1, TRPC3, TRPC4, TRPC5, and TRPC6 proteins (Fig 5*b*). Their estimated molecular weights were 91 kDa for TRPC1, 135 kDa for TRPC3, 96 kDa for TRPC4, 150 kDa for TRPC5, 125 and 195 kDa for the major and minor band of TRPC6, respectively. No immunoreactive bands were detected when the blots were incubated with the same antibodies pre-absorbed with the specific peptides against which they were raised (Fig 5*b*).

Role of TRPC1 and TRPC4 in SOC activity In addition to store-depletion and receptor activation, DAG has been shown to directly activate members of the TRPC3/6/7 subgroup, but has no effect on other TRPC, and sometimes prevents the activation of members of TRPC4/5 subgroup (Kiselyov and Muallem, 1999; Venkatachalam *et al*, 2003). Our results showed that the endogenous SOC activity in keratinocytes could not be enhanced by the application of the DAG analog (Fig 4), suggesting that the members of the

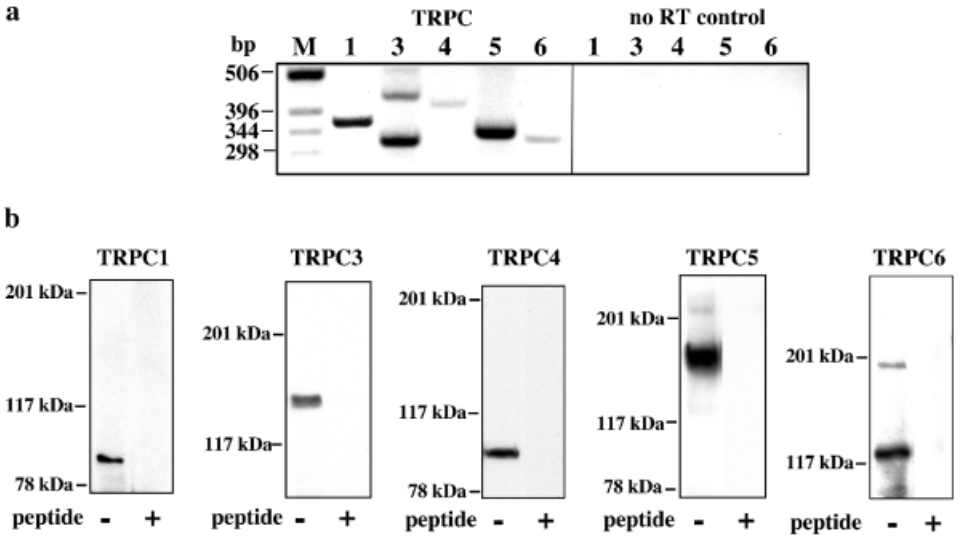

Figure 5
Expression of cononical transient receptor potential channels (TRPC) in human keratinocytes. RT-PCR (*a*) and immunoblotting (*b*) analyses were performed to detect TRPC transcripts and proteins, respectively. Total RNA was collected from pre-confluent human keratinocytes grown in medium containing 0.03 mM, reverse-transcribed into cDNA and used as template in PCR amplification (*a*). Specific primer pairs were used to amplify TRPC. No template control (no RT) was run for all experiments. To examine TRPC protein expression, the membrane proteins were isolated from keratinocytes grown in medium containing 0.03 mM Ca^{2+} and subjected to immunoblotting analyses (*b*). The expression of TRPC proteins was detected by specific antibody against each TRPC as indicated. The specificity of the immunoreactions was confirmed by incubation of the blots with the antibodies pre-absorbed with the specific peptides against which they were raised.

TRP3/6/7 subgroup are not involved in mediating SOC activity. Furthermore, we have found that a decrease in SOC activity with time in culture coincided with a sharp decline in the protein levels of TRPC1 and TRPC4 but not in that of TRPC3, 5, and 6 (data not shown). Therefore, we turned our attention to TRPC1 and TRPC4 channels as the likely mediators of store-operated Ca^{2+} entry. We performed fluorescence immunostaining to detect and localize endogenous TRPC1 and TRPC4 proteins in pre-confluent keratinocytes cultured in 0.03 mM calcium. Examination by confocal microscopy detected extensive fluorescence signal of TRPC1 and TRPC4 at the plasma membrane and the submembrane area. A substantial amount of TRPC protein, however, also localized within an intracellular compartment(s) near the perinuclear region (Fig 6). Minimal fluorescence signal was detected when the antibodies were pre-absorbed with the antigenic peptides, confirming the specificity of the immunoreaction (Fig 6).

To determine whether TRPC1 and TRPC4 mediate SOC activity in keratinocytes, we knocked down the expression of endogenous TRPC1 and TRPC4 by transfection with antisense cDNA constructs, and examined the impact on SOC activity. Pre-confluent keratinocytes were transfected with antisense cDNA and then selected with hygromycin in KGM containing 0.03 mM calcium for 24 h to enrich transfected cells. Protein extracts were prepared and subjected to immunoblotting analyses to examine the level of the endogenous TRPC channels. The level of TRPC1 and TRPC4 proteins was greatly reduced in the keratinocytes transfected with antisense cDNA for TRPC1 (anti-TRPC1) and TRPC4 (anti-TRPC4), respectively, as compared with that in the cells transfected with the control vector (Fig 7*a*). In contrast, transfection of antisense TRPC1 or antisense TRPC4 cDNA had minimal effect on the expression of other TRPC proteins (Fig 7*a*). The Ca^{2+} entry in response to TG-induced store emptying was reduced as a result of the hygromycin selection of transfected cells (Fig 7*b*, as compared with the Ca^{2+} entry in untransfected cells shown in Fig 1*a*). Nevertheless, knockdown of either TRPC1 or TRPC4 further inhibited SOC activity. As shown in Fig 7*b*, 6 min after application of 2 mM extracellular Ca^{2+}, $[Ca^{2+}]_i$ increased from 103 ± 2 nM (mean $\pm$ SD) to 154 ± 5 nM in the cells transfected with vector ($n = 14$). Transfection of antisense cDNA for TRPC1 and TRPC4 resulted in reduction in SOC activity to $\sim$26% ($[Ca^{2+}]_i$ increased from 112 ± 2 to 125 ± 3 nM; $n = 11$) and $\sim$40% ($[Ca^{2+}]_i$ increased from 112 ± 6 to 132 ± 5 nM; $n = 10$), respectively, of that in cells transfected with vector. Knockdown of both channels led to a complete blockade of SOC activity ($[Ca^{2+}]_i$ remained unchanged; $n = 13$) (Fig 7*b*). On the other

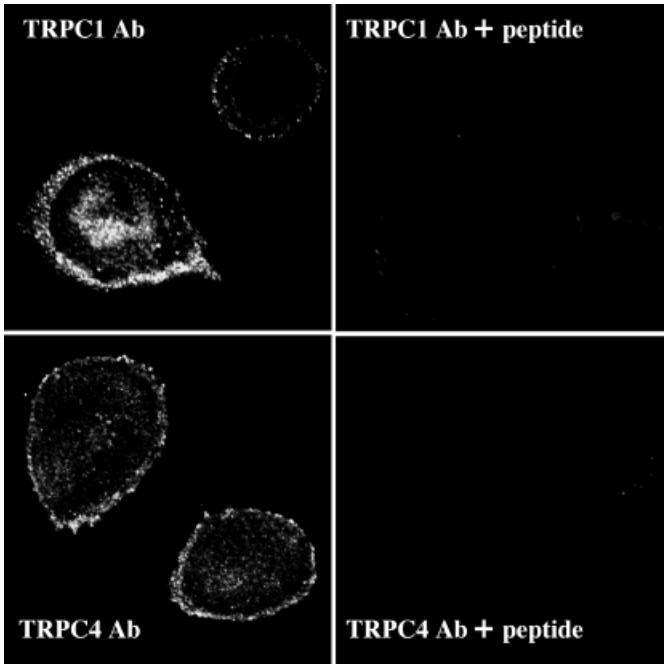

Figure 6
Fluorescence immunolocalization of the canonical transient receptor potential channel (TRPC)1 and TRPC4 protein endogenously expressed in human keratinocytes. Keratinocytes were fixed, permeablized, and incubated with polyclonal antibody against TRPC1 or TRPC4, followed by fluorescein-conjugated anti-rabbit IgG. Fluorescent signals were detected with a confocal microscope. TRPC1 and TRPC4 proteins were detected on the plasma membrane as well as within the cytoplasm in the perinuclear region. Data are representative of five independent experiments.

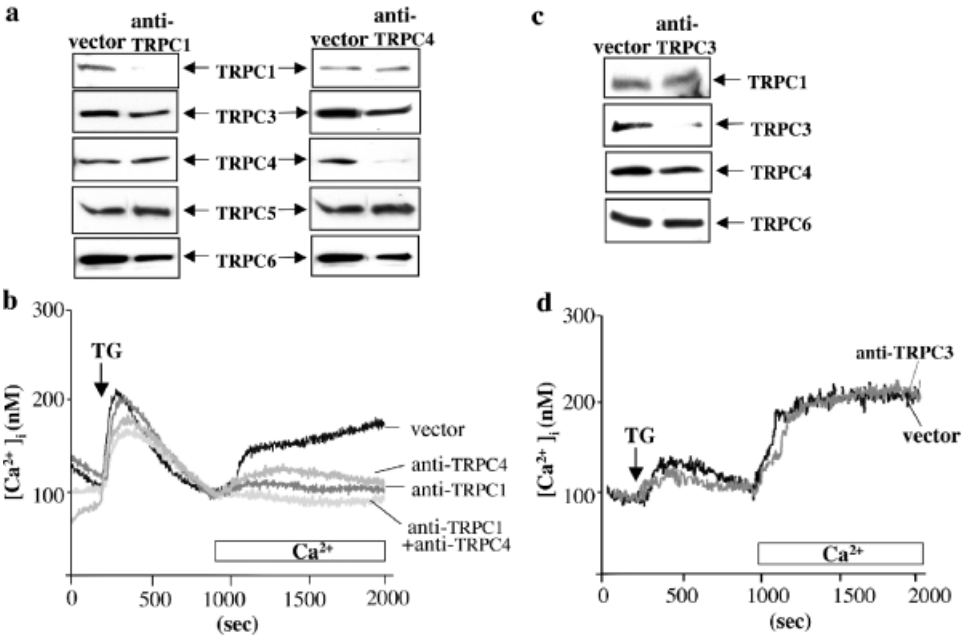

Figure 7
Transfection of antisense cDNA constructs for canonical transient receptor potential channel (TRPC)1 and TRPC4 inhibited store-operated channel (SOC) activity in keratinocytes. Keratinocytes were cultured, transfected with the control vector or the antisense cDNA constructs for TRPC1 (anti-TRPC1), TRPC4 (anti-TRPC4) (*a*, *b*) and TRPC3 (anti-TRPC3) (*c*, *d*), and selected by hygromycin as described under "Experimental Procedures". Total proteins were isolated from transfected cells and analyzed by immunoblotting (*a*, *c*). Note that the expression of endogenous TRPC1, TRPC4 and TRPC3 was reduced by their respective antisense constructs, whereas the levels of other TRPC proteins were minimally affected. Transfected cells were loaded with Fura-2, and thapsigargin-induced store-operated calcium entry was measured (*b*, *d*). The trace shown represents the average cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of 12–14 individual keratinocytes during recording. Knockdown of either TRPC1 or TRPC4 partially suppressed, whereas knockdown of both channels completely abrogated the activation of calcium entry by store depletion. Knockdown of TRPC3 had no effect on SOC activity. The results are representative of three experiments.

hand, transfection of antisense TRPC3 cDNA decreased the endogenous level of TRPC3 (Fig 7*c*) but had no effect on the store-depletion-induced Ca^{2+} entry (Fig 7*d*). After application of 2 mM extracellular Ca^{2+}, $[Ca^{2+}]_i$ increased from 95 ± 5 to 206 ± 10 nM in cells transfected with TRPC3 antisense cDNA ($n = 12$), which is comparable with that in cells transfected with vector (92 ± 3 to 210 ± 21 nM, $n = 14$). Our results indicate that TRPC1 and TRPC4, but not TRPC3, are the major mediators of SOC activity in these cells.

TRPC1, but not TRPC4, forms protein complexes with PLCγ1 and IP_3R Several studies have shown that TRPC proteins can interact with IP_3R through binding to its ligand-binding domain (Boulay *et al*, 1999; Lockwich *et al*, 2000; Tang *et al*, 2001). Furthermore, a recent report demonstrated binding of PLCγ to TRPC3 and TRPC4 (Patterson *et al*, 2002). In order to investigate possible direct interactions of endogenous TRPC channels with PLCγ and IP_3R, we performed co-immunoprecipitation experiments to test whether antibodies against IP_3R and PLCγ1 could also bring down TRPC1 and TRPC4. Protein extracts were prepared from pre-confluent keratinocytes cultured in 0.03 mM Ca^{2+}, incubated with monoclonal antibodies against PLCγ1 or IP_3R, and precipitated with protein G-conjugated beads. Immunoprecipitants were collected and analyzed by immunoblotting to detect the presence of TRPC1 and TRPC4. Both anti-PLCγ1 and anti-IP_3R antibodies co-immunoprecipitated TRPC1, but not TRPC4 (Fig 8*a*). In addition, an agarose-conjugated peptide containing the Src homology domains (SH2SH2SH3) of PLCγ1 was sufficient to bind TRPC1 (Fig 8*a*), as well as TRPC3 (data not shown), indicating that PLCγ1 interacts with TRPC through its SH2SH2SH3 domain. No band was detected when the blots were incubated with the same antibodies pre-absorbed with their respective antigenic peptides, confirming the specificity of the immunoreactions (Fig 8*a*).

We then performed fluorescence immunolocalization of endogenous TRPC1, PLCγ1, and IP_3R proteins to assess the associations among these calcium regulatory molecules. Examination by confocal microscopy confirmed the association of TRPC1 with PLCγ1 and IP_3R. As shown in Fig 8*b*, substantial amounts of PLCγ1 and IP_3R co-localized with TRPC1 near the plasma membrane. Interestingly, TRPC1 protein exhibited little interaction with either IP_3R or PLCγ1 in the perinuclear compartment, even though all three molecules were present in this region (Fig 8*b*). Our findings indicate that these calcium regulators form a signaling complex at or near the plasma membrane to regulate the SOC activity.

Ca^{2+} has been known to be a factor regulating TRPC function (Zitt *et al*, 1997) in part by regulating the interaction of TRP channels and IP_3R (Tang *et al*, 2001; Zhang *et al*, 2001; Singh *et al*, 2002). To assess this possibility we compared the interaction of TRPC1 with PLCγ1 and IP_3R in keratinocytes grown in 0.03 or 1.2 mM extracellular Ca^{2+}. As shown in Fig 9*a*, the interactions between TRPC1 and PLCγ1 or IP_3R were attenuated in the cells treated with 1.2 mM Ca^{2+} for 24 h as compared with cells maintained in 0.03 mM Ca^{2+}. This reduction in association among these molecules was not due to a decrease in the levels of TRPC1 (Fig 9*a*), PLCγ1 or IP_3R (Fig 9*b*). In fact, the level of PLCγ1 was increased by extracellular calcium (Fig 9*b*), consistent with previous studies (Xie and Bikle, 1999). We next compared the thapsigargin-activated SOC activity in these cells. As shown in Fig 9*c*, the basal $[Ca^{2+}]_i$ was higher in cells cultured in 1.2 mM Ca^{2+} for 24h (149 ± 11 nM; $n = 36$) than the cells grown in 0.03 mM Ca^{2+} (101 ± 7 nM; $n = 28$). Keratinocytes cultured in 0.03 mM Ca^{2+} responded to store depletion and subsequent Ca^{2+} application with an active calcium entry ($[Ca^{2+}]_i$ increased from 105 ± 10 to 236 ± 25 nM; $n = 36$), whereas the SOC was markedly down regulated in the cells grown in 1.2 mM Ca^{2+} ($[Ca^{2+}]_i$ increased

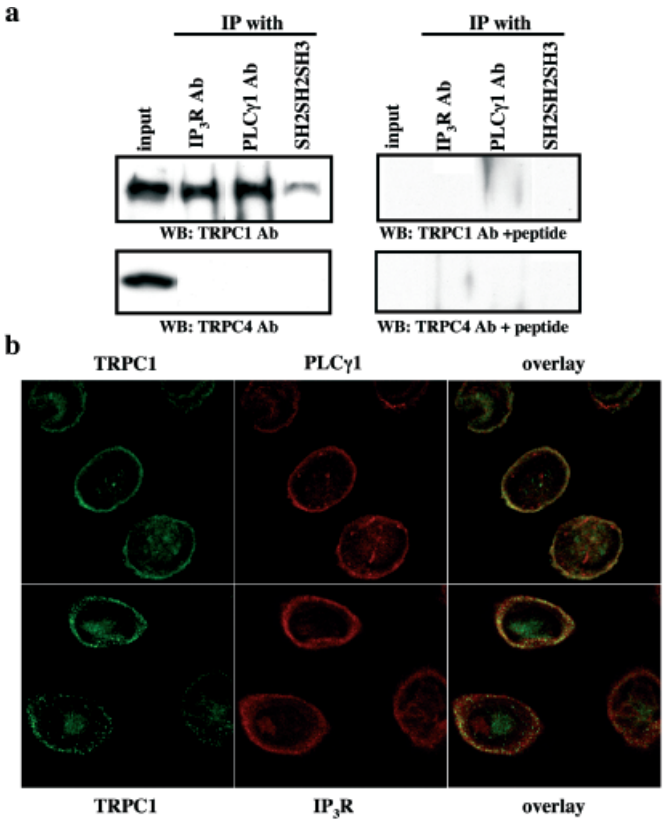

Figure 8
Canonical transient receptor potential channel (TRPC)1 forms a protein complex with phospholipase C (PLC)γ1 and inositol 1,4,5-triphosphate receptor (IP_3R). Co-immunoprecipitation (*a*) revealed direct interaction of TRPC1 with PLCγ1 and IP_3R. Protein extracts collected from pre-confluent keratinocytes grown in 0.03 mM Ca^{2+} were immunoprecipitated (IP) with monoclonal antibodies against PLCγ1 or IP_3R, followed by sepharose-conjugated protein G, or with an agarose-conjugated peptide containing the SH2SH2SH3 domain of PLCγ1. Immunoprecipitants were then analyzed by immunoblotting using polyclonal antibodies against TRPC1 and TRPC4. One-fifth of the amount of protein used in immunoprecipitations was included in the blots as a control (input). The same antibodies pre-absorbed with their specific antigenic peptides were used to confirm the specificity of the immunoreactivities of these bands. Fluorescence immunolocalization (*b*) demonstrated co-localization of TRPC1 with PLCγ1 and IP_3R. Pre-confluent keratinocytes cultured in 0.03 mM Ca^{2+} were incubated with a polyclonal antibody for TRPC1 and a monoclonal antibody against PLCγ1 or IP_3R, followed by fluorescein-conjugated anti-rabbit IgG and Texas Red-conjugated anti-mouse IgG, respectively. Fluorescent signals were detected with a confocal microscope. TRPC1 co-localized with PLCγ1 and IP_3R near the plasma membrane. TRPC1, PLCγ1, and IP_3R were also detected in perinuclear locations, but little interaction was found among these molecules in this region. Data are representative of three different experiments.

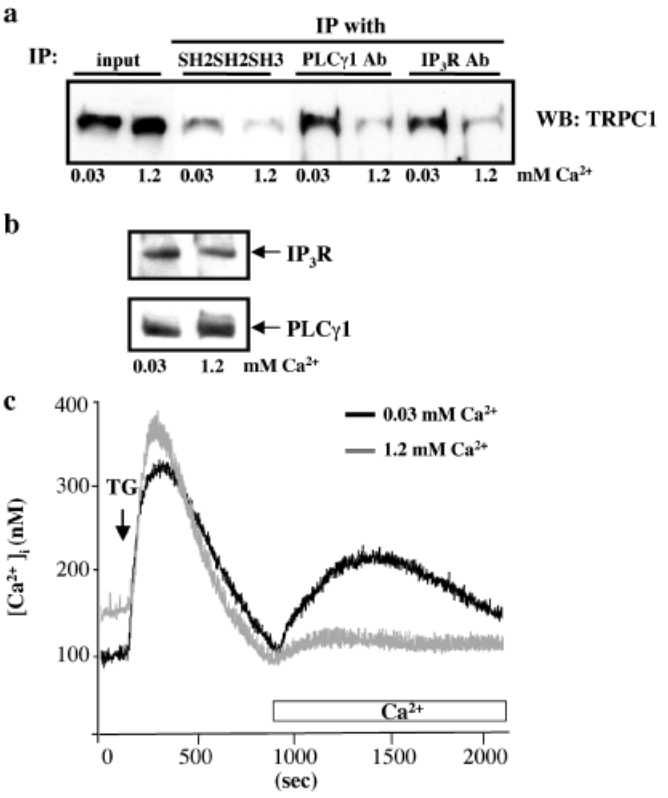

Figure 9
Calcium decreased store-operated channel (SOC) activity and complex formation of canonical transient receptor potential channel (TRPC)1, phospholipase C (PLC)γ1, and inositol 1,4,5-triphosphate receptor (IP_3R). Pre-confluent keratinocytes were cultured in 1.2 mM Ca^{2+} or maintained in 0.03 mM Ca^{2+} for 24 h. Protein extracts were collected and immunoprecipitated as described in the Fig 8 legend. Immunoprecipitants were analyzed by immunoblotting for TRPC1 (*a*). One-fifth of the amount of protein used in the immunoprecipitations was included in the blots as a control (input). The levels of endogenous PLCγ1 and IP_3R were also examined by immunoblotting of protein extracts from these cells (*b*). The interactions of TRPC1 with PLCγ1 and IP_3R were markedly reduced in cells treated with 1.2 mM Ca^{2+}, even though the levels of TRPC1, PLCγ1, and IP_3R were not decreased in these cells. Thapsigargin (TG)-induced SOC was measured in keratinocytes cultured in 1.2 mM Ca^{2+} (n = 28) or in 0.03 mM Ca^{2+} (n = 36) for 24 h (*c*). Results are representative of two independent experiments.

from 99 ± 10 to 127 ± 19 nM; n = 28) (Fig 9*c*). These results support the conclusion that Ca^{2+} inhibits SOC channel function by down regulation of the interactions between TRPC1, PLCγ1, and IP_3R, although the mechanism for this remains under investigation.

Discussion

As found for most non-excitable cells, emptying of intracellular calcium stores in human keratinocytes activates calcium influx across the plasma membrane through the operationally defined SOC (Csernoch *et al*, 2000; Karvonen *et al*, 2000). In this study, we investigated the molecular basis for the native SOC activity in these cells. We placed our attention on TRPC1 and TRPC4 because their expression patterns correspond to a strong SOC activity in the proliferating keratinocytes (data not shown), though TRPC3, TRPC5, and TRPC6 channels are also expressed in these cells. TRPC1 plays a role in the store-operated Ca^{2+} entry pathway and is ubiquitously expressed in many cell types. Numerous studies have demonstrated that store-operated Ca^{2+} entry was inhibited when the expression of TRPC1 was attenuated or prevented (for an overview, see Beech *et al*, 2003). The abolition of a store-operated current in adrenal cortical cells by antisense TRPC4 (Philipp *et al*, 2000) and the lack of store-operated Ca^{2+} entry in endothelial cells from TRPC4 knockout mice (Freichel *et al*, 2001) indicate that TRPC4 is also one of the SOC components. Similarly, inhibiting the expression of either TRPC1 or TRPC4 attenuated the store-operated Ca^{2+} entry in

keratinocytes (Fig 7*a*, *b*), whereas knockdown of the expression of TRPC3 had no effect on SOC function (Fig 7*c*, *d*), indicating TRPC1 and TRPC4, but not TRPC3, are the subunits of the endogenous SOC in these cells. TRPC1 may not act alone, since it can heteromultimerize with TRPC4 or TRPC5 (but not TRPC3, TRPC6, or TRPC7) in brain, vascular smooth muscle and overexpression systems (Goel *et al*, 2002; Hofmann *et al*, 2002). The interaction between TRPC1 and TRPC4 or other TRPC in keratinocytes, however, needs further investigation.

The conformational coupling model (Irvine, 1990; Putney, 1999) for store-operated Ca^{2+} entry hypothesizes that the communication between the ER and plasma membrane involves a direct protein–protein interaction. Kiselyov *et al* (1998, 1999) demonstrated that the C-terminus of TRPC3 directly interacts with IP_3R, and identified the N-terminal IP_3-binding domain of IP_3R as the minimal portion required to activate TRPC3. Our studies have shown that TRPC1 physically associates with IP_3R (Fig 8). These data support the notion that the direct relay of a signal from depleted intracellular calcium stores to calcium channels in the plasma membrane is mediated through protein–protein interactions.

The finding that SOC activation requires both IP_3 and IP_3R (Kiselyov *et al*, 1998; Zubov *et al*, 1999) seems counterintuitive to the observation that store depletion alone, in the absence of IP_3 generation, is able to activate SOC. Putney *et al* (2001), however, proposed that the SOC–IP_3R complex is located in close proximity to a PLC molecule, perhaps through an organized signaling complex, and the latter produces basal levels of IP_3 sufficient to fulfill the requirement for IP_3 in SOC activation. In epithelial cells and in the mast cell line BRL, inhibition of PLC with the PLC inhibitor U73122 or depletion of phosphatidylinositol-4-phosphate (PIP) with wortmannin abrogated both agonist-induced and store-depletion-induced Ca^{2+} entry (Rosado and Sage, 2000b; Broad *et al*, 2001). Likewise in keratinocytes, when U73122 was administrated at the same concentration reported to inhibit PLC-dependent signaling (Xie and Bikle, 1999), this drug but not the inactive isomer U73343, completely blocked TG-induced Ca^{2+} entry (Fig 2*a*). A direct inhibitory effect on SOC channels is unlikely, since U73122 failed to block Ca^{2+} entry after the activation of SOC by store depletion (Fig 2*b*). These results indicate that basal PLC activity is necessary for the activation but not for the maintenance of SOC. Although our findings implicate a role for PLC in SOC activation, it appears that this is not due to a requirement for DAG, since application of the DAG analogs, OAG and DOG, inhibited SOC in keratinocytes (Fig 3). Although other studies on TRPC channels indicate that the inhibitory action of DAG on TRPC4- and TRPC5-mediated calcium entry is PKC-dependent (Venkatachalam *et al*, 2003), it is unclear whether the inhibitory effect of DAG on SOC activity in keratinocytes is mediated by endogenous PKC.

Recently, Patterson *et al* (2002) reported a structural role for PLCγ in mediating agonist-induced calcium entry. Overexpression of PLCγ1 augmented calcium entry induced either by a G protein-coupled receptor agonist or by TG. This action of PLCγ1 is independent of its lipase activity but dependent on its Src homology 3 (SH3) domain (Patterson *et al*, 2002), indicating that PLCγ functions through its ability to associate with other signaling molecules. Yeast two-hybrid analyses and co-immunoprecipitation experiments show that the PLCγ SH3 domain is able to interact with the N-terminus of overexpressed TRPC3 and TRPC4 (Patterson *et al*, 2002). In this study, co-immunoprecipitation results showed that in keratinocytes the endogenous TRPC1 physically interacts with PLCγ1 as well as IP_3R (Fig 8). Consistent with the finding that the SH3 domain of PLCγ1 mediates the interaction with SOC, a peptide containing the SH2SH2SH3 domain of PLCγ1 was able to interact with endogenous TRPC1 (Fig 8). The association of native SOC with PLCγ1 and IP_3R is in agreement with the notion that the SOC–IP_3R complex and PLCγ are located in close proximity via an organized signaling complex. Fluorescence immunolocalization of endogenous TRPC1, PLCγ1, and IP_3R proteins verified that co-localization of TRPC1 with PLCγ1 and IP_3R was restricted to the plasma membrane, whereas these proteins showed little interaction in other intracellular locations. In PC12 cells, HEK293 cells and DT40 lymphocytes, knockdown or knockout of PLCγ expression diminished or abrogated agonist-induced calcium entry, whereas store-depletion-activated calcium entry was not affected (Patterson *et al*, 2002). On the contrary, knockdown of PLCγ1 expression in keratinocytes by antisense transfection completely abolished store-depletion-activated calcium entry (Fig 4), indicating a requirement for PLCγ1 in coupling store emptying to activation of SOC. The explanation for these cell-specific differences is unclear. Although PLCγ1 is capable of directly interacting with SOC, we must consider the possibility that an intermediary target may mediate the actions of PLCγ1. The N-terminus of TRPC proteins contains several ankyrin repeats, a putative interaction site with cytoskeletal proteins (Montell, 2001). PLCγ isoforms have been known to associate with the actin cytoskeleton (Rebecchi and Pentyala, 2000; Rhee, 2001) and have close structural and functional relations to the rho/rac family of GTPases (Hong-Geller and Cerione, 2000; Zeng *et al*, 2000), which are involved in cytoskeletal reassembly and trafficking (Arrieumerlou *et al*, 2000; Zeng *et al*, 2000). Since the activation of SOC is sensitive to cytoskeletal rearrangement (Patterson *et al*, 1999; Ma *et al*, 2000; Rosado *et al*, 2000), PLCγ may activate SOC by altering the cytoskeletal-dependent organization of ER and plasma membrane.

Although both TRPC1 and TRPC4 contributed to SOC activity, only TRPC1 directly interacted with PLCγ1 and IP_3R (Fig 8). Many studies support the idea that TRPC1 functions within a signaling complex (for an overview, see Beech *et al*, 2003). TRPC1 associates with PLC-related signaling proteins such as $G_{\alpha q/11}$, IP_3R, PLC, plasma membrane Ca^{2+}-ATPase, caveolin-1 (Lockwich *et al*, 2000), and calmodulin (Singh *et al*, 2002). Since TRPC1 has the ability to heteromultimerize with TRPC4, it may also function as a regulator for TRPC4. It is possible that TRPC4 activity is gated by PLCγ1 and IP_3R via TRPC1. This hypothesis would explain the complete abolition of store-operated Ca^{2+} entry by antisense PLCγ1 (Fig 4), and the observation that antisense TRPC1 inhibited store-operated Ca^{2+} entry more effectively than antisense TRPC4 (Fig 7*b*). In this study we also demonstrated that TRPC3, which is strongly expressed

in keratinocytes, is not involved in mediating store-depletion-activated calcium entry (Fig 7*d*), even though a weak interaction of TRPC3 with a peptide containing the PLCγ1 SH2SH2SH3 domain was detected (data not shown). The physiologic significance of this observation in keratinocytes is unclear but is consistent with the findings of Patterson *et al* (2002) in other cells showing an interaction between PLCγ and TRPC3.

Many studies have demonstrated the high sensitivity of SOC to inhibition by $[Ca^{2+}]_i$, a negative feedback mechanism for controlling $[Ca^{2+}]_i$ (Parekh and Penner, 1997; Krause *et al*, 1999). Activation of SOC occurs maximally when $[Ca^{2+}]_i$ is buffered to a low level, and reduction in $[Ca^{2+}]_i$ is sufficient to activate the channels (Kerschbaum and Cahalan, 1999; Krause *et al*, 1999). In this study we also observed an inhibition of SOC activity by elevated $[Ca^{2+}]_i$ in keratinocytes and demonstrated that the effect of $[Ca^{2+}]_i$ on SOC was due to decreased association of TRPC1 with PLCγ1 and IP_3R (Fig 9). Although the mechanism(s) by which $[Ca^{2+}]_i$ alters these interactions remains unclear, our findings support the conclusion that the activity of SOC depends on the interactions of TRPC channels with PLCγ and IP_3R.

Materials and Methods

Materials Ionomycin, TG, and 2-APB were purchased from Calbiochem-Novabiochem (La Jolla, California). Membrane permeable DAG analogs, OAG and DOG and the PLC inhibitor U-73122, and its inactive analog U-73343 were purchased from Sigma Chemicals (St Louis, Missouri). Stock solutions of these compounds were prepared in DMSO or ethanol. All DNA constructs used in the transfection were prepared using Qiagen Maxi-prep columns (Chatsworth, California) according to the manufacturer's protocol.

Cell Culture Normal human keratinocytes (NHK) were isolated from newborn foreskin and cultured as described (Gibson *et al*, 1996). Cells were maintained in serum-free keratinocyte growth medium (KGM; Clonetics, San Diego, California) supplemented with 0.03 mM $CaCl_2$.

Measurement of cytosolic Ca^{2+} Keratinocytes cultured on glass coverslips were loaded with 7.5 μM Fura-2/AM (Molecular Probes, Eugene, Oregon) in 0.1% Pluronic F127 in buffer A (20 mM Hepes, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mg per mL sodium pyruvate, 1 mg per mL glucose) containing 0.07 mM calcium. Cells were then washed with Ca^{2+}-free buffer A before Ca^{2+} measurements were made. The fluorescence of Fura2-loaded cells was monitored using a Dual-wavelength Fluorescence Imaging System (Intracellular Imaging, Cincinnati, Ohio). The cells were alternately illuminated with 340 and 380 nm light, and the fluorescence at emission wavelength 510 nm was recorded. Ca^{2+} stores were depleted by 1 μM TG in 0.1 mM EGTA, and Ca^{2+} entry in Fura2-loaded keratinocytes was determined in the presence of 2 mM calcium, unless indicated otherwise. All experiments were performed at room temperature. The signals from 30 to 50 single cells for each measurement were recorded. Each sample was calibrated by the addition of 20 μM ionomycin (R_{max}) followed by 20 mM EGTA/Tris, pH 8.3 (R_{min}). Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated from the ratio of emission at the two excitation wavelengths based on the formula $[Ca^{2+}]_i = K_d Q(R-R_{min})/(R_{max}-R)$, $R = F_{340}/F_{380}$, $Q = F_{min}/F_{max}$ at 380 nm, and K_d for Fura-2 for Ca^{2+} is 224 nM.

RT-PCR analysis of TRPC transcripts The expression of the TRPC channel messages was determined by RT-PCR using sets of primers that spanned the pore-forming region of the TRPC channels. Total RNA isolated from pre-confluent keratinocytes cultured in KGM containing 0.03 mM Ca^{2+} was reverse transcribed into cDNA by reverse transcriptase (Superscript II, Life Science Technology, Goat Island, New York), using an oligo-dT primer. Those cDNAs were then used as templates in PCR amplification by Expand Long Template PCR system (Roche Molecular Biochemicals, Indianapolis, Indiana). The primer pairs used in PCR were: TRPC1 forward primer 5′-GATTTTGGAAAATTTCTTGGGATGT-3′, reverse primer 5′-TTTGTCTTCATGATTTGCTATCA-3′; TRPC3 forward primer 5′-GACATATTCAAGTTCATGGTCCTC-3′, reverse primer 5′-ACATCACTGTCATCCTCAATTTC-3′; TRPC4 forward primer 5′-TCTGCAAATATCTCTGGGAAGAATGCT-3′, reverse primer 5′-AAGCTTTGTTCGTGCAAATTTCCATTC-3′; TRPC5 forward primer 5′-ATCTACTGCCTGGTACTACTAGC-3′, reverse primer 5′-TCAGCATGATCGGCAATAAGCTG-3′; TRPC6 forward primer 5′-AAAGACATCTTCAAGTTCATGGTC-3′, reverse primer 5′-CACATCAGCGTCATCCTCAATTTC-3′. The expected size of the PCR products for the TRPC was between 323 to 415 bp. The PCR DNA products were subcloned into a pCR II vector (Invitrogen, Carlsbad, California), and their sequences were verified by double-stranded DNA sequencing (Biomedical Resource Center, University of California, San Francisco).

TRPC anti-sense cDNA vector construction and transfection of keratinocytes To construct a vector expressing antisense RNA for human TRPC, a 374 bp TRPC1 cDNA fragment, a 315 bp TRPC3 cDNA fragment, and a 415 bp TRPC4 cDNA fragment were subcloned in an antisense orientation into a mammalian expression vector pcDNA3.1 (Invitrogen) that contained a hygromycin resistance gene. Keratinocytes cultured on glass coverslips were transfected with these human TRPC antisense cDNA constructs in KGM containing 0.03 mM calcium using TransIT keratinocyte transfection reagent (Mirus, Madison, Wisconsin) according to the manufacturer's protocol. This results in an initial transfection efficiency of 30%–40%. Two days after transfection, transfected cells were selected with 100 μg per mL of hygromycin (Roche Molecular Biochemicals) for 24 h to enrich the transfected cells. In the case of knockdown of PLCγ1, an expression vector containing the full-length PLCγ1 gene in an antisense orientation (Xie and Bikle, 1999) was used for transfection. Transfected cells were selected with 200 μg per mL of neomycin analog G418 for 48 h. These selection protocols kill essentially all non-transfected cells. Afterwards, transfected cells were loaded with Fura-2/AM and measured for intracellular Ca^{2+} as described above. For the immunoblotting analyses in the antisense knockdown studies, keratinocytes were grown in 100 mm tissue culture dishes until 35%–50% confluent. Cells were then transfected with the desired antisense cDNA construct and selected with G418 or hygromycin B as described above. Cells were then lysed in RIPA buffer and protein content in total lysates was determined by bicinchoninic acid (BCA) assay. Equivalent amounts of protein (75 μg) were loaded per lane in 5% polyacrylamide gels for immunoblotting analyses as described in the following section.

Immunoblotting analysis of membrane proteins Pre-confluent keratinocytes were cultured in KGM containing 0.03 mM $CaCl_2$. Crude plasma membranes were isolated from these cultures as described. Briefly, the cells were rinsed twice and scraped into PBS. Cells were then collected by centrifugation and the cell pellet was sonicated in homogenization buffer (containing 20 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 4 mM $MgCl_2$, 5 mM EGTA, and protease inhibitor cocktail (Complete protease inhibitors, Roche Molecular Biochemicals)). The homogenate was centrifuged at 100,000 $\times$ *g* for 30 min, and the resultant pellet was extracted with RIPA buffer (50 mM Hepes, pH 7.4, 1% deoxycholate acid, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, and Complete protease inhibitor cocktail) for 30 min at 4°C. The soluble membrane protein fraction was separated from the insoluble cytoskeleton fraction by centrifugation at 100,000 $\times$ *g* for 30 min. All steps were carried out at 4°C. Protein concentration in these membrane

preparations was determined by the BCA Protein Assay Kit (Pierce, Rockford, Illinois) and equivalent amounts (75 μg) per sample were analyzed as follows. Membrane protein samples were electrophoresed through 5% polyacrylamide gels and electroblotted onto polyvinylidene fluoride membranes (Immobilon-P, 0.45 μm; Millipore, Bedford, Massachusetts). After blocking with 5% milk in TBS (20 mM Tris- HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA), the blots were incubated with 1 μg per mL of primary polyclonal antibodies at 4°C overnight. Subsequently, the blots were incubated with 0.1 μg per mL horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham Pharmacia Biotech, Piscataway, New Jersey) for 1 h at room temperature. The bound antibody was visualized using the SuperSignal West Dura Chemiluminescent Kit (Pierce) and subsequent exposure to X-ray film. The data presented are representative of three independent experiments. Anti-TRPC antibodies were purchased from Chemicon International Inc. (Temecula, California). The corresponding sequences of the peptides used to raise antibodies for TRPC proteins are as follows: human TRPC1 (Accession P48995) residues 557–571, mouse TRPC3 (Accession Q9QXC1) residues 822–835, mouse TRPC4 (Accession AAC05179) residues 943–958, human TRPC5 (Accession Q9UL62) residues 959–973, and mouse TRPC6 (Accession Q61143) residues 24–38. All epitopes are specific for their designated TRPC proteins and not present in any other known proteins. Antibodies against PLCγ1 and PLCβ1 were obtained from Santa Cruz Biotechnology (Santa Cruz, California).

Co-immunoprecipitation Pre-confluent keratinocytes were scraped and lysed in ice-cold lysis buffer (1% NP-40, 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA supplemented with Complete protease inhibitor cocktail) with gentle tumbling for 2 h. The lysates were then centrifuged at 10,000 × *g* for 10 min, and the supernatants were collected and used in immunoprecipitation experiments. All steps were carried out at 4°C. Protein concentration in lysates was determined by the BCA assay. 500 μg of total proteins was immunoprecipitated by 5 μg of monoclonal antibody against PLCγ1 (Santa Cruz Biotechnology) or IP_3R (Calbiochem-Novabiochem), followed by Sepharose-conjugated protein G (Immuno-Link Immobilized Protein G, Pierce) in 0.5 mL of lysis buffer with gentle tumbling at 4°C overnight. Immunoprecipitants were collected, washed, eluted, and separated by SDS-PAGE. In subsequent immunoblotting analyses, polyclonal antibodies were used to detect the presence of TRPC1, TRPC3, and TRPC4 in the immunoprecipitants.

Fluorescence Immunolocalization of TRPC in Keratinocytes Keratinocytes were cultured on coverslips and were fixed with 4% paraformaldehyde for 20 min at 37°C. Cells were permeablized with 1% NP-40 in PBS. After blocking with 5% goat serum in PBS with 0.01% Tween-20, cells were incubated with 10 μg per mL of polyclonal antibodies against TRPC proteins (Chemicon) at 4°C for overnight. Subsequently, cells were incubated with fluorescein-conjugated anti-rabbit IgG antibody (Molecular Probes) at room temperature for 1 h. Finally, coverslips were washed in PBS, mounted on glass slides using Gel-Mount (Biomeda, Foster City, California) and examined with a Leica TCS NT/SP confocal microscope (Leica Microsystems, Heidelberg, Germany).

This study was conducted according to Declaration of Helsinki Principles and the medical ethical committee of the University of California approved all described methods.

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Address correspondence to: Dr Chia-Ling Tu, Endocrine Unit (111N), 4150 Clement Street, San Francisco, CA 94121, USA.
Email: ctu@itsa.ucsf.edu

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