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Termination and activation of store-operated cyclic AMP production

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Abstract

Diverse pathophysiological processes (e.g. obesity, lifespan determination, addiction, and male fertility) have been linked to the expression of specific isoforms of the adenylyl cyclases (AC1-AC10), the enzymes that generate cyclic AMP (cAMP). Our lab recently discovered a new mode of cAMP production, prominent in certain cell types, that is stimulated by any maneuver causing reduction of free [Ca^{2+}] within the lumen of the endoplasmic reticulum (ER) calcium store. Activation of this "store-operated" pathway requires the ER Ca^{2+} sensor, STIM1, but the identity of the enzymes responsible for cAMP production and how this process is regulated is unknown. Here we used sensitive FRET-based sensors for cAMP in single cells combined with silencing and overexpression approaches to show that store-operated cAMP production occurred preferentially via the isoform AC3 in NCM460 colonic epithelial cells. Ca^{2+} entry via the plasma membrane Ca^{2+} channel, Orai1, suppressed cAMP production, independent of store refilling. These findings are an important first step towards defining the functional significance and to identify the protein composition of this novel Ca^{2+}/cAMP crosstalk system.

INTRODUCTION

Many intracellular organelles, including the endoplasmic reticulum (ER), actively sequester calcium ions [1]. Transient loss of Ca^{2+} from the ER is an inherent consequence of intracellular Ca^{2+} signaling. However, dysregulation of ER Ca^{2+} homeostasis also occurs pathologically following exposure to toxic insults, ischemia, and trauma [2]. Alteration (be it of physiological or pathological origin) in free [Ca^{2+}] within these internal Ca^{2+} storage compartments is often sensed and translated into downstream signaling activities, such as ER stress responses or engagement of store-dependent Ca^{2+} entry channels [3].

In a previous report from our lab, we showed that cAMP could become elevated in the cytosol of certain cell types following depletion of Ca^{2+} from intracellular stores (principally the ER), but this increase in cAMP did not depend whatsoever on the corresponding changes

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Conflict of Interest: M.P.M. holds part ownership of InCell Corporation LLC, which sells M3:10 medium. The other authors declare no conflicts.

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in cytosolic [Ca^{2+}]. The increase in cAMP, rather, directly mirrored the decrease of free [Ca^{2+}] inside the ER lumen and required a transmembrane ER Ca^{2+} sensor, STIM1 [4].

STIM1 has been shown to translocate from the bulk ER to specialized ER/plasma membrane junctions following store depletion [5-7]. STIM1 has also been firmly established to interact with Orai1, the pore-forming unit of a plasma membrane channel that permits Ca^{2+} entry from the extracellular environment [8-11]. Activation of Orai1 (and possibly other channels) *via* STIM1 allows a sustained Ca^{2+} signal, and refilling of the store. This ubiquitous, widely studied phenomenon known as "store-operated Ca^{2+} entry" is activated by any maneuver that lowers the free [Ca^{2+}] in the ER lumen [12].

In our studies on ER-dependent cAMP signaling we found that silencing STIM1 or preventing its translocation reduced cAMP production caused by treatments that lower the levels of free Ca^{2+} within the ER. Because of the many parallels with store-operated Ca^{2+} entry, we named this process "Store-Operated cAMP Signaling" (SOcAMPS). So far SOcAMPS has been described in several cell types, notably in NCM460 cells [4], a model of normal colonic crypt epithelial cells [13] and CaLu-3 cells (normal human airway epithelia cells) [14]. While the physiological meaning of SOcAMPS in NCM460 cells is not known, in CaLu-3 this process has been shown to participate in cAMP-dependent chloride and fluid secretion induced by Ca^{2+}-mobilizing products secreted from the bacterium *Pseudomonas aeruginosa*. This mechanism may contribute to airway clearance during bacterial infection. Using a model of mouse cholangiocytes, Spirli and colleagues also recently implicated SOcAMPS in the pathogenesis of polycystic liver disease [15]. Interestingly, certain other cells types, such as HeLa cells, appear to lack SOcAMPS [4]. This suggests that a specific panel of proteins must be expressed in order for SOcAMPS to be operational.

A pathway that links ER Ca^{2+} to cAMP signaling could have extremely important physiological implications, but apart from STIM1, little is known about the actual protein participants in SOcAMPS. For example, how does the principal target of STIM1, Orai1, fit into this process? Are proteins related to STIM1, such as STIM2, involved? Another key question concerns how exactly cAMP is generated following store depletion.

Our previous studies using pharmacological agents excluded the involvement of the phosphodiesterases, the enzymes that degrade cyclic nucleotides, in SOcAMP signaling, but did implicate the enzymes that produce cAMP, the adenylyl cyclases (ACs). To date nine closely related transmembrane isoforms of AC (tmAC1-9) and one soluble adenylyl cyclase (sAC or AC10) have been cloned and characterized [16]. Classically, tmACs are activated by heterotrimeric G–proteins in response to stimulation of G-protein coupled receptor (GPCRs). In contrast, the sAC is directly activated by cellular metabolites (CO_2, bicarbonate and ATP), consistent with a role as an intracellular metabolic sensor [17]. A panel of different AC isoforms are generally co-expressed in a given cell type.

In the present study we used genetically encoded cAMP reporters in single NCM460 cells in order to define whether a singular isoform of adenylyl cyclase is responsible for store-dependent cAMP production in this cell type. We overcame the limitation imposed by the lack of pharmacological inhibitors for specific AC isoforms, and lack of suitable antibodies for protein chemistry by using overexpression and knockdown approaches to show a preferential link between the AC isoform AC3 in this store-operated process. In addition, while the presence of the plasma membrane Ca^{2+} entry channel Orai1 (or the STIM1 releative, STIM2) did not affect SOcAMPS, Ca^{2+} entering through this store-operated pathway did have a strong effect in terminating cAMP production. The physiological

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circumstances under which SOcAMPS is expected to become the predominant second messenger pathway following loss of ER Ca^{2+} stores are discussed.

RESULTS

We observed previously that diverse strategies culminating in the lowering of free [Ca^{2+}] within the ER resulted in cAMP production in NCM460 cells, measured using both a panel of FRET-based cAMP sensors and conventional cAMP immunoassays. These maneuvers included: (i) inhibition of Ca^{2+} uptake by SERCA inhibitors (thapsigargin and tert-butyl hydroquinone), (ii) $InsP_3$-dependent release of stores using native Ca^{2+} mobilizing agonists working through $G\alpha_q$-coupled receptors (ATP and carbachol), (iii) buffering ER Ca^{2+} with high concentrations of membrane-permeant Ca^{2+} buffers (TPEN or BAPTA-AM), (iv) passive depletion of stores using high concentrations of EGTA, (v) treatment with Ca^{2+}-mobilizing compounds such as bile acid (deoxycholic acid) or eicosapentaenoic acid, and (vi) release of stores using Ca^{2+} ionophores such as ionomcyin.

In order to screen potential mediators or regulators of SOcAMPS, we developed a simplified protocol in which we released intracellular Ca^{2+} stores under Ca^{2+}-free conditions using ionomycin in NCM460 cells stably expressing a FRET-based cAMP sensor, EpacH30 [18] (**Figure 1A**). This resulted in a reproducible increase in cAMP production (as measured by FRET ratio change of EpacH30) that was typically ~35-40% of the maximal ratio change obtained following saturation of the cAMP sensor using forskolin (50μM) and IBMX (1mM). We also observed previously that this response to store depletion could be sustained for prolonged periods (measured longer than 60 minutes), provided internal Ca^{2+} stores were kept in an empty state [4]. It should be emphasized that this increase in cAMP was absolutely independent of the initial transient spike in Ca^{2+} elicited by ionomycin-induced store release [4]. When cells were loaded with the Ca^{2+} buffer BAPTA-AM (20μM for 30 minutes; conditions shown to eliminate the initial spike of cytosolic Ca^{2+} in NCM460 cells following ionomycin treatment, as measured by fura-2 in Ca^{2+}-free solutions), the increase in the FRET ratio following store release was not altered (**Figure 1B**), consistent with our previous findings that SOcAMPS is independent of cytosolic Ca^{2+}. In fact, maneuvers that caused Ca^{2+} to become elevated within the cytoplasm, such as re-addition of bath Ca^{2+}, strongly inhibited SOcAMPS (**Figure 1A**), and this was fully reversible upon superfusion of cells with Ca^{2+}-free solutions (not depicted). This effect of Ca^{2+} re-addition was slowed dramatically but not eliminated in the BAPTA-AM pretreated cells, consistent with the fact that Ca^{2+} entering from the extracellular space will eventually overwhelm the Ca^{2+} buffering capacity of BAPTA, resulting in elevation of free Ca^{2+} within the cytoplasm and inhibition of SOcAMPS. Note too that these experiments indicated that alterations in cationic metals other than Ca^{2+} (e.g. Zn^{2+})[19] were likely not responsible for this effect since BAPTA-AM chelates many trace heavy metals. Moreover, the effect of ionomycin was not altered by pretreatment with TPEN (N,N,N',N'-Tetrakis-(2-pyridylmethyl)ethylenediamine; 10μM) a membrane permeant compound that is extremely effective at scavenging heavy metals (not depicted; n=4 expts/26 cells).

Orai channels and STIM2 are not required for SOcAMPS

We first examined how the presence of the major Ca^{2+} entry channel, Orai1, affected this process. Knockdown by RNAi of the channel did not alter the FRET response to store depletion compared to scramble controls (**Figures 1C-1E** and **Supp. Figure 1A**), but the inhibitory action of Ca^{2+} re-addition was dramatically attenuated in the absence of Orai1 (**Figure 1F**), showing that Ca^{2+} entry *via* store-operated pathways was important for this effect. These findings are consistent with the fact that ionomycin-elicited Ca^{2+} transport across the plasma membrane derives mostly from activation of store-operated Ca^{2+} entry,

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with a smaller component arising from the ability of the ionophore to act as a Ca^{2+} carrier [20].

Overexpression of Orai1 alone or STIM1 alone is known to cause only slight alterations in store-operated Ca^{2+} entry. However, it has been shown that the co-expression of STIM1 and Orai1 in a specific ratio can enhance the Ca^{2+} entry current known as "I_{CRAC}" as much as 103-fold, creating the so called "monster" I_{CRAC} [21-23]. Overexpression of STIM1 alone [4], Orai1 alone (**Figures 1G** and **1H**), or of STIM1 + Orai1 (**Figure 1I**) did not significantly enhance the amplitude of the FRET response to ionomycin, although we did note that co-expression of both Orai1 and STIM1 caused the rate of cAMP increase following store depletion to become slightly faster. Furthermore, no effect was observed when the related channel, Orai3, which is also expressed endogenously in NCM460 cells, or Orai3 + STIM1 were overexpressed (**Supplemental Figures 1B and 1C**). The overexpresssion or knockdown of the STIM1 relative, STIM2, also had no major impact on store-dependent cAMP signaling (**Supplemental Figure 2**).

The chief conclusion of the preceding is that Orai1 is not necessary for store-operated cAMP production, but Ca^{2+} entry through Orai1 does have a significant inhibitory action on SOcAMPS. We considered the Orai1-dependent sensitivity of SOcAMPS to Ca^{2+} entry as an important clue towards identifying the potential players in SOcAMPS. For example, all AC isoforms are actually inhibited by high (10-25 μM, *i.e.* non-physiological) [Ca^{2+}] [24]. However lower concentrations (0.2-0.6 μM), such as those attained during Ca^{2+} signaling events, can either activate or inhibit certain ACs. Ca^{2+} entry through store-operated Ca^{2+} channels is known in particular to have a strong impact on AC1 and AC8 (isoforms activated by Ca^{2+} *via* calmodulin) and AC5 and AC6 (directly inhibited by Ca^{2+}) [25-29]. More controversial has been the role of Ca^{2+} and Ca^{2+}-calmodulin on the modulation of AC3 activity, which however has been shown in some cell systems to be inhibited by CaM kinase II (CaMKII)[30,31].

In our previous paper, RT-PCR identified seven different ACs isoforms expressed in NCM460 cells (AC3, AC4, AC5, AC6, AC7, AC9, and sAC) [4]. Isoform-specific inhibitors for the different ACs are not generally available [32,33], nor are specific antibodies that permit immunoprecipitation of many of the ACs with their potential interacting proteins. Our prior data showed that SOcAMPS was prevented by P-site inhibitors specific for tmACs, such as SQ22536, and was enhanced by forskolin, an activator of most tmAC isoforms, except AC9. We therefore focused our efforts on overexpression and silencing of AC3-AC7, but also tested sAC.

Overexpression of AC isoforms

Plasmids coding each of the isoforms of interest were cotransfected one at a time with the marker mCherry in NCM460 cells stably expressing EpacH30. To confirm that the tmAC plasmids were expressed and working properly, control experiments in HeLa cells (which don't have SOcAMPS) showed that only in mCherry positive cells was it possible to visualize enhanced cAMP production when challenging the cells with low doses of forskolin (10 nM). Using the protocol illustrated in **Figure 2A**, the FRET response to ionomycin was compared between mCherry-expressing and control cells in the same microscope field. Only single cells not touching other cells were analyzed, to avoid artifacts due to gap-junction-mediated cAMP transfer between cells [34].

Only overexpression of AC3 was able to consistently increase both the amplitude ($p<0.01$) and slope (a measure of cAMP production velocity; $p<0.05$) of the ionomycin response. Data from the other ACs are summarized in **Figure 2B**, showing that the amplitude (but not the speed) was also somewhat enhanced by transfection with plasmids for AC5 and AC6.

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Interestingly, AC3 (but not AC5 or AC6) co-expressed with STIM1-mCherry produced an ionomycin effect that was markedly greater than AC3 alone (**Figure 2C;** summary data in **Figure 2D**), reminiscent of the "monster" I_{CRAC} reported for Orai1 and STIM1 co-expression.

Knockdown of AC3, but not AC5 or AC6, attenuates SOcAMPS

Based on our findings above, we next tested the effect of knocking down AC3, AC5, and AC6. We first used shRNA plasmids that also encode the red fluorescent marker, dsRed, allowing us to distinguish knockdown cells from control cells in the same microscope field. As illustrated in **Figure 3A** cells expressing dsRed and shRNA against AC3 gave significantly reduced responses to ionomycin compared to both neighboring control cells and cells expressing a dsRed + scramble construct. As summarized in **Figure 3B**, this action was not observed for AC5 shRNA. It should be pointed out that the presence of dsRed produced a highly variable fluorescence artifact leading to a small downward shift in the FRET ratio of EpacH30. This might be due green fluorescent maturation intermediates of dsRed or aggregate formation, but the comparison of ratio changes between shRNA for AC3 and the scramble control nevertheless showed a significant effect of knocking down AC3.

These findings were further confirmed by examining FRET responses of populations of cells treated with siRNA for AC3, AC5, and AC6. Although all siRNA constructs were effective in knocking down their respective targets, only siRNA against AC3 was able to significantly reduce the amplitude of the ionomycin-induced ratio change (**Figures 3C-3E**).

Rescue of SOcAMPS phenotype in HeLa cells

We next tested whether the SOcAMPS phenotype could be rescued in HeLa cells by simply overexpressing AC3, AC5, or AC6. While there was generally no effect of introducing these ACs into these cells (**Supplemental Figure 3**), even when inhibiting PDE activity with IBMX, it should be noted that there were occasional HeLa cells that gave a credible response to ionomycin after transfection with AC3 (2/11 transfected cells in three experiments) or AC3 + STIM1 (10/45 transfected cells in n=8 experiments), but not STIM1 alone. The reasons for this inconsistency are unknown, but this could suggest that there is an unknown factor required to reconstitute the SOcAMPS phenotype that is intermittently expressed in HeLa cells.

Effects of PTX, CaMKII and PKC inhibitors on SOcAMPs

NCM460 cells pre-incubated for 18 hrs with pertussis toxin (PTX; 200ng/ml) yielded typical responses to ionomycin treatment (78/79 cells in n=9 expts). This indicates that PTX-sensitive $G\alpha_i$-dependent signaling is not required for the initiation of SOcAMPS.

AC3 has been reported to be a target of CaMKII and PKC [24]. Thus we questioned whether SOcAMPS might also be affected by pharmacological modulation of these two kinases. As shown in **Figure 4A**, acute treatment with the CaMKII inhibitor KN-62 (10 μM) after SOcAMPS had been initiated by ionomycin caused a slight enhancement of the response. However, treatment of NCM460 cells with KN-62 in the absence of ionomycin also caused a comparable elevation of the baseline FRET ratio. While KN-62 did not prevent the subsequent cAMP response when ER stores were depleted with ionomycin (**Figure 4B**), the amplitude of the response was partially inhibited.

Similarly, acute addition of the PKC activator PMA (phorbol 12-myristate 13-acetate; 100nM) caused minor increases in the FRET ratio by itself, but did not prevent the subsequent response to ionomycin (**Figure 4C**). PMA, like KN-62, also slightly augmented the FRET ratio when added on top of ionomycin (**Figure 4D**). Long term pretreatment with

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100nM PMA (15 min, 3 and 12 hours), conditions expected to downregulate PKC activity, also did not abolish SOcAMPS (see **Supplemental Figure 4** for summary of data at 15 min and 3hr time points). From these data we can conclude that neither CaMKII nor PKC serve as the mediators linking store depletion to cAMP production, although both of these effectors can slightly modulate cAMP signaling in NCM460 cells, as might be expected from a process mediated by AC3.

DISCUSSION

Most cells express not one, but rather a panel of multiple AC isoforms [35], the coexistence of which was formerly considered redundant. However, the striking phenotypes of isoform-specific knockout animals and of humans with polymorphisms and mutations in AC genes have revealed highly specialized physiological functions for the various ACs [16]. For example, AC5 is largely expressed in heart and brain, and when silenced in mice, prolongs lifespan and protects against stress [36]. AC6 is also expressed in heart, yet the deletion of AC6 compromises left ventricular function in a manner that is not replicable by eliminating AC5 [37]. Meanwhile, AC8 has been demonstrated to be a modulator of anxiety [38], whereas sAC is involved in male fertility, since mice lacking this gene have defects in sperm motility [39]. AC1, AC5, AC6, and AC8 have been reported to be uniquely susceptible to “superactivation”, a form of sensitization seen in model systems of drug withdrawal and recovery [32,40].

Of note are the dramatic phenotypes associated with knocking down AC3, which in mouse induces an obese phenotype [41]. Ablation of AC3 also leads to alterations in murine maternal behavior [42] that are likely related to defects in olfaction [43]. Genome-wide association studies have linked AC3 to the genetic etiology of major depressive disorder [44]. Interestingly, in mouse hippocampal and cortical neurons, AC3 is localized exclusively to the primary cilium, where it generates a cAMP signal that is essential for certain types of learning and memory such as novel object recognition and contextual fear extinction [45].

The collective evidence presented here indicates that store-operated cAMP production occurs preferentially *via* AC3 in NCM460 colonic epithelial cells. It is noteworthy that recent data from Spirli and colleagues implicated AC6 as the likely source of cAMP in mouse cholangiocytes during SOcAMPS [15]. However, although some of our overexpression experiments suggested that AC5 and AC6 might also participate in this process, AC3 was the only isoform to consistently yield positive results in both overexpression and knockdown studies. Concurrent expression of AC3 plus STIM1 produced an even larger change in cAMP in response to store depletion. AC3 is the dominant AC in NCM460 cells (as measured by mRNA), but is widely expressed in other cell types and tissues, including at least one cell type that does not exhibit SOcAMPS, the HeLa cell. Moreover, overexpression of AC3 (or AC3 + STIM1) in HeLa cells did not consistently rescue the SOcAMPS phenotype. Therefore the presence of AC3 is likely not a unique factor that defines SOcAMPS, and other, as yet unidentified, components of the pathway must exist.

Lowering of free ER [Ca^{2+}] leads to dissociation of Ca^{2+} from the luminal EF hand domain of STIM1, which drives the clustering of STIM1 and Orai1 into aggregates at closely apposed junctions between ER and plasma membrane. The recruitment of other plasma membrane proteins (e.g. STIM2, L-type channels, TRP family members etc.) to this complex has also been described. Because of the dependence of SOcAMPS on the expression of STIM1, we similarly envisioned a scenario in which Orai1, by virtue of its physical interaction with STIM1, might serve to stabilize a complex of AC3-STIM1-Orai1 at the plasma membrane after ER [Ca^{2+}] was reduced. However the expression level of

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Orai1 (or Orai3 or STIM2, which are also abundant in NCM cells) did not affect cAMP production following store depletion. Unfortunately we were unable to determine whether there might be a physical linkage between AC3 and STIM1 during SOcAMPS, due to the lack of suitable antibodies for immunoprecipitation of AC3. We also could not find any evidence for clustering of a GFP-tagged AC3 [46] into punctae following store depletion (Hofer and Maiellaro, *unpublished*). Thus the nature of the interaction between STIM1 and AC3 during SOcAMPS remains to be determined.

Cooper and colleagues recently suggested a functional and perhaps physical connection between exogenously expressed AC8, a Ca^{2+}-activated AC, and Orai1 [47]. This intimate arrangement allows Ca^{2+} entering through Orai1 to potently activate AC8 in a localized microenvironment beneath the plasma membrane. While Orai1 does not appear to be necessary for SOcAMPS, Ca^{2+} entering *via* this store-operated Ca^{2+} entry channel did reproducibly attenuate store-dependent cAMP production, although did not completely eliminate it. The mechanism whereby Ca^{2+} exerts its inhibitory action is not known, and may be indirect. For example, using total internal reflection fluorescence (TIRF) microscopy of STIM1-YFP, we found that the translocation of STIM1 is diminished by high cytosolic [Ca^{2+}] in NCM460 cells (A.M. Hofer, *unpublished*), as it is in other cell types [48,49].

The existence of a cAMP-generating pathway that is simultaneously activated by store depletion but inhibited by the concomitant store-dependent Ca^{2+} influx is unusual, but may provide some clues as to the function of SOcAMPS. In many cell types, Ca^{2+} signaling events elicited by physiological doses of agonists are oscillatory, with nominal release and refilling of the ER during each oscillatory cycle. While the repetitive Ca^{2+} spiking in the cytosol may be optimally tuned to activate Ca^{2+} effectors, this type of ER depletion will not be of sufficient duration to elicit SOcAMPS. On the other hand, strong agonist stimulation typically results in profound ER store depletion sufficient to activate SOcAMPS, but also elicits robust Ca^{2+} entry through Orai channels, and this would attenuate cAMP production. While such signals are normally terminated quite effectively by removal of the initiating stimulus, during persistent Ca^{2+} signaling Ca^{2+} entry can eventually become downregulated, for example *via* feedback phosphorylation of Orai1 by Ca^{2+}-dependent PKC [50]. Under such conditions SOcAMPS would then become the dominant second messenger pathway. ER Ca^{2+} stores are also vulnerable to wide variety of environmental stressors, toxic insults, drugs, and viral and bacterial pathogens that cause unregulated, even permanent, loss of ER Ca^{2+}. We propose that the unorthodox mode of AC3-dependent cAMP production described here may signal the initiation of adaptive responses (such as cAMP-dependent fluid secretion [51] or compensatory programs of gene expression) in the face of persistent non-physiological loss of ER Ca^{2+} stores. Certain cell types (e.g. colon and airway epithelia) are at the frontline in the barrier against these noxious agents, and perhaps not coincidentally, are the same cell types that manifest the SOcAMPS phenotype.

MATERIALS and METHODS

Reagents

BAPTA-AM (1,2-bis(o-Aminophenoxy)ethane-N,N,N′,N′-tetraacetic Acid Tetra(acetoxymethyl) Ester) was purchased from Molecular Probes/Invitrogen (Carlsbad, CA, USA), and ionomycin from Calbiochem (USA). Unless otherwise noted, all other reagents were obtained from Sigma-Aldrich (USA).

Cell culture

NCM460 cells, a model of normal human colonic crypt epithelial cells [13] were obtained by licensing agreement from INCELL, San Antonio TX, and were grown in M3:10 medium

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(INCELL, San Antonio TX). For most experiments NCM460 cells stably transfected with the FRET-based cAMP probe, CFP(nd)-EPAC(dDEP/CD)-YFP(nd) x pCDNA3 (H30) Amp or "EpacH30" [18] were used. HeLa cells were grown in DMEM + 10% FCS; both lines were maintained in a humidified CO_2 (5%) incubator at 37°C. Cells were split every two days after reaching 80% confluence.

Transfection with shRNA plasmids and siRNAs

All siRNA tranfections were performed using Lipofectamine (Invitrogen) according to manufacturer's instructions, while plasmids were transfected using Effectene (Qiagen, Valencia, CA USA). Briefly, 300ng of the plasmid of interest plus 300 ng of mCherry (used as marker of transfected cells), were mixed in 180 μl of buffer reagent, to which 8 μl of Enhancer and 10 μl of Effectene reagent were added. After 20 minutes the entire mixture was added to cells grown to 60-70% confluency into 6-well plates.

For efficient knockdown of the different ACs we used a mix of 4 different HuSH 29mer shRNAs (500 ng of each plasmid per 6-well plate) specific against AC3 or AC5. The pRFP-C-RS vector (Origene) expressing the shRNAS also encodes the red fluorescent protein dsRED, used as a marker of transfected cells. As an alternative we used siRNAs against AC3, AC5, AC6 and their effects were compared to that of non-effective siRNA "scramble". The day after transfection cells were trypsinized and plated on glass coverslips, a step that resulted in a high proportion of isolated single cells in each microscope field, thereby minimizing gap-junction-mediated cAMP diffusion between cells [34].

Two days after transfection, imaging experiments were performed. Additionally total RNA was collected using RNeasy kit (Qiagen). One microgram of RNA was converted into cDNA using random hexamers (Invitrogen SuperScript First-Strand Synthesis System). Total AC3, AC5 and AC6 mRNA levels were calculated by Real-time RT-PCR using a TaqMan-based assays (Applied Biosystems) and normalized to the endogenous 18S rRNA or GAPDH levels (amplified using a standard primer sets, Applied Biosystems) in 3 independent experiments.

Ratio Imaging of FRET-based cAMP sensors

Glass coverslips containing cells were mounted in a home-built flow-through perfusion chamber on top of the fluorescence imaging microscope stage as described previously [34,52]. Two different FRET ratio imaging set ups were used for this study: (1) a Nikon TE200 inverted fluorescence microscope equipped with a Photometrics QuantEM EMCCD camera, and (2) a Nikon Eclipse TE2000-U microscope with attached Hamamatsu ORCA-ER CCD camera. Metafluor software (Molecular Devices) was used to control filter wheels (Sutter Instruments) in the excitation and emission paths and to control image acquisition (one image pair every 5 sec.) for both systems.

Cyclic AMP measurements were performed on single cells using the 480nm/535nm FRET emission ratio (440nm excitation) of EpacH30 [18] and either a 40X Plan Fluor 1.30 NA or a 60X Plan Apo TIRF 1.45 NA oil immersion objective. Cells were bathed in a HEPES-buffered Ringer's-like solution containing (in mM): 125 NaCl, 25 HEPES, 10 Glucose, 5 K_2HPO_4, 1 $MgSO_4$ and 1 $CaCl_2$, pH, 7.40. For Ca^{2+}-free solutions (0 Ca^{2+}), the $CaCl_2$ was omitted and EGTA (25 μM) added to minimize contaminating Ca^{2+}.

Statistics and Analysis

Data are presented as mean ± SEM. Paired or unpaired Student's t-test was performed as appropriate to compare the means of two different groups of experiments. A value of $p<0.05$ was considered statistically significant. Analysis of the change in initial slope during the two

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minutes following ionomycin addition was fitted by linear regression using Kaleidagraph software, and expressed as a percentage of the change in slope of the corresponding control response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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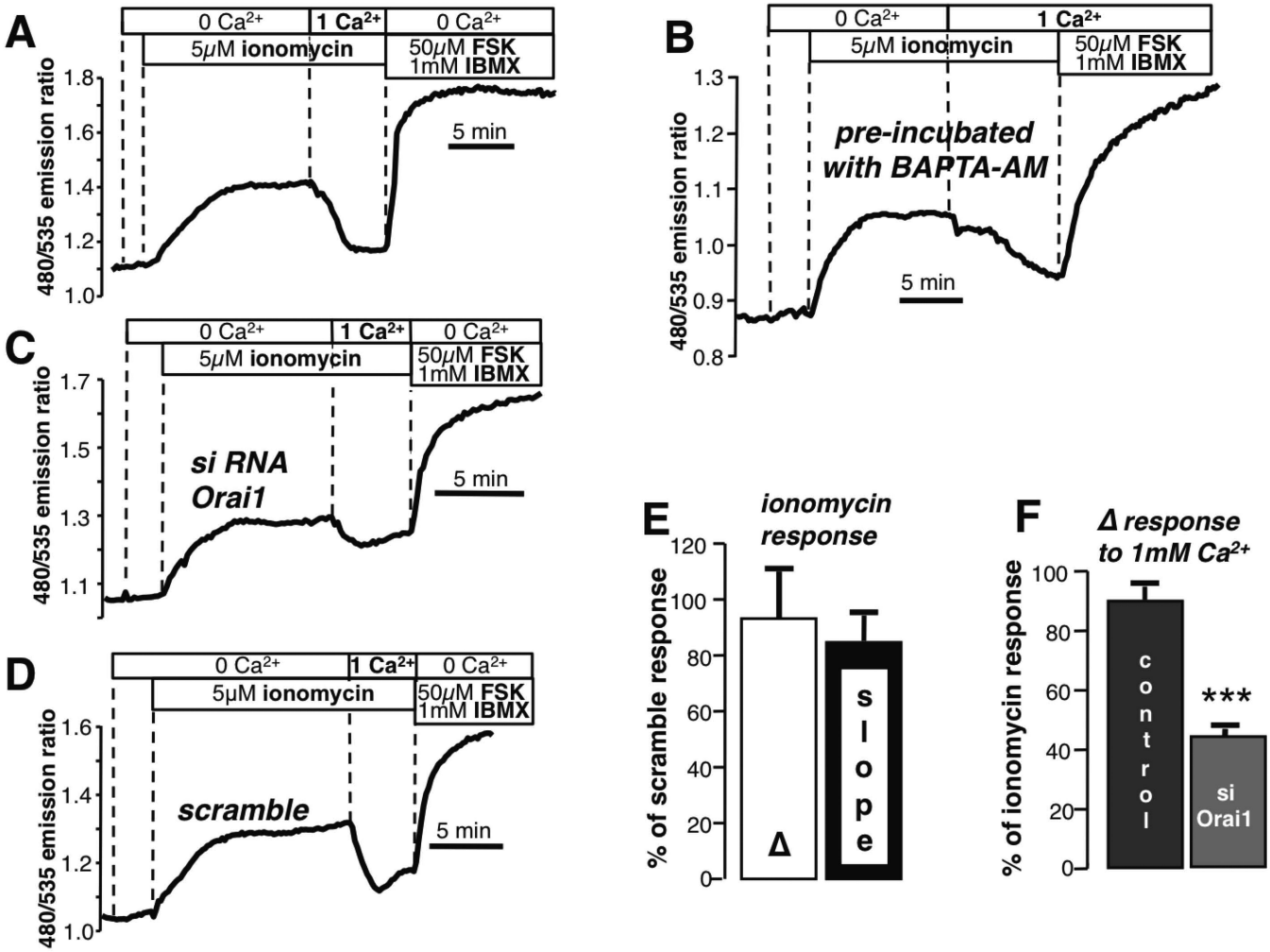

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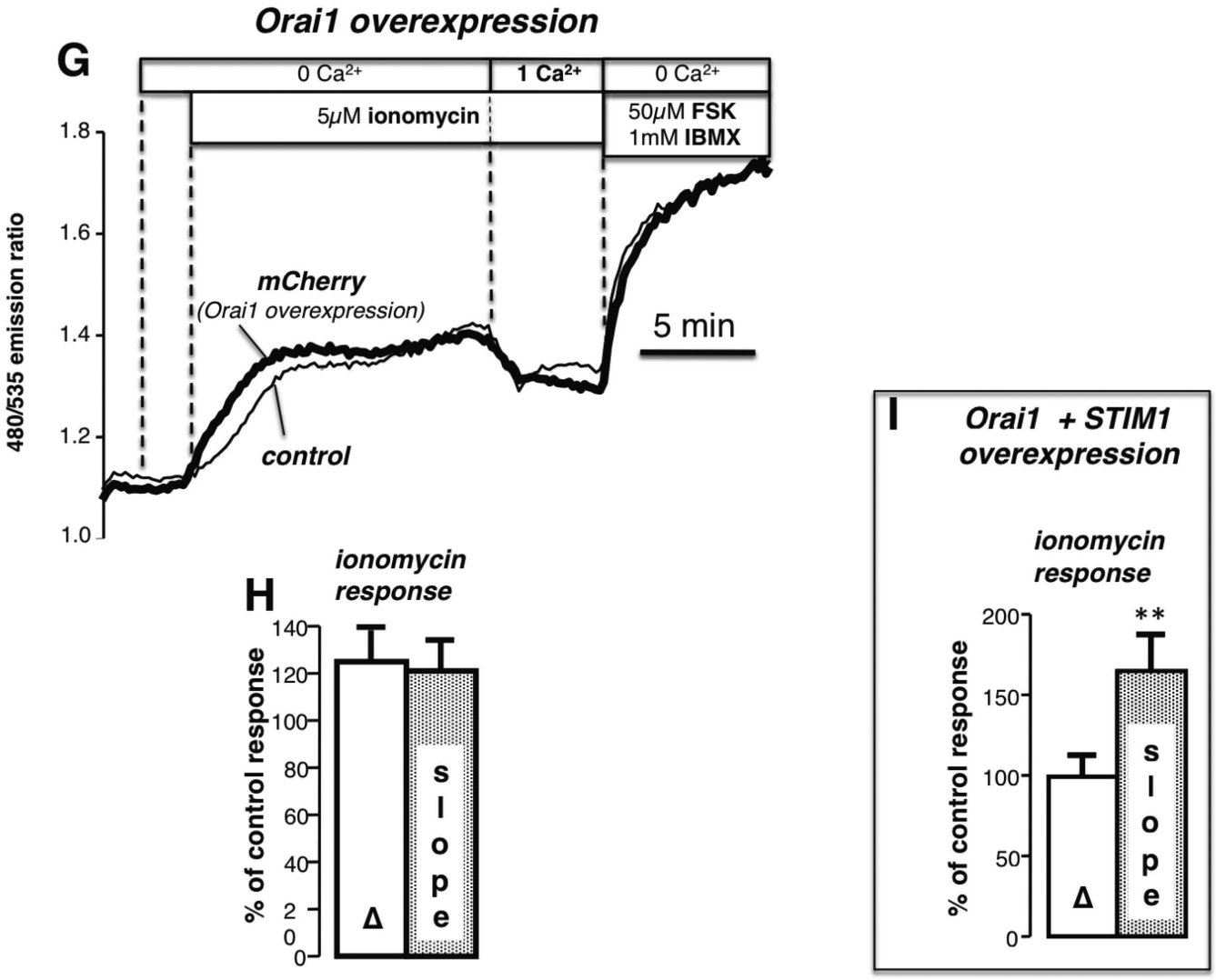

Figure 1. Evaluation of possible players involved in SOcAMPs in NCM460 cells stably expressing EpacH30

A- Effect of ionomycin (5μM) in the absence of external Ca^{2+}. Addition of 1mM Ca^{2+} resulted in a reduction in the FRET ratio (typical of 5 exp./29 cells). At the end of all experiments forskolin (FSK; 50μM) and IBMX (1mM) were added to obtain a supramaximal FRET response. **B**- Effect of ionomycin in cells pre-loaded with BAPTA-AM (20μM, 30 min; typical of 6 exp./49cells). **C**- Knockdown by RNAi of the Orai1 channel did not alter the response to ionomycin, however, the inhibitory action of 1mM Ca^{2+} was significantly reduced as compared to scramble controls (6 exp./33 cells). **D**- Response to ionomycin in scramble controls (6 exp./97 cells). **E-F** Summary of experiments in panel C and D: **E**- Amplitude (Δ) and slope of the ionomycin response expressed as percentage of the response in scramble controls. **F**- Response to 1 mM Ca^{2+} expressed as a % of the ionomycin response in scramble and siOrai1-treated cells. **G**- Effect of overexpression of Orai1 on the response to ionomycin (5 exp./25 mCherry cells/7 control cells). **H**- Summary of Orai1 overexpression experiments. Amplitude and slope of the ionomycin response expressed as a percentage of the response in control cells. **I**- Summary of Orai1 + STIM1 overexpression experiments. (5 exp./9 control cells/10 mCherry cells) **p<0.02, *** p<0.0001.

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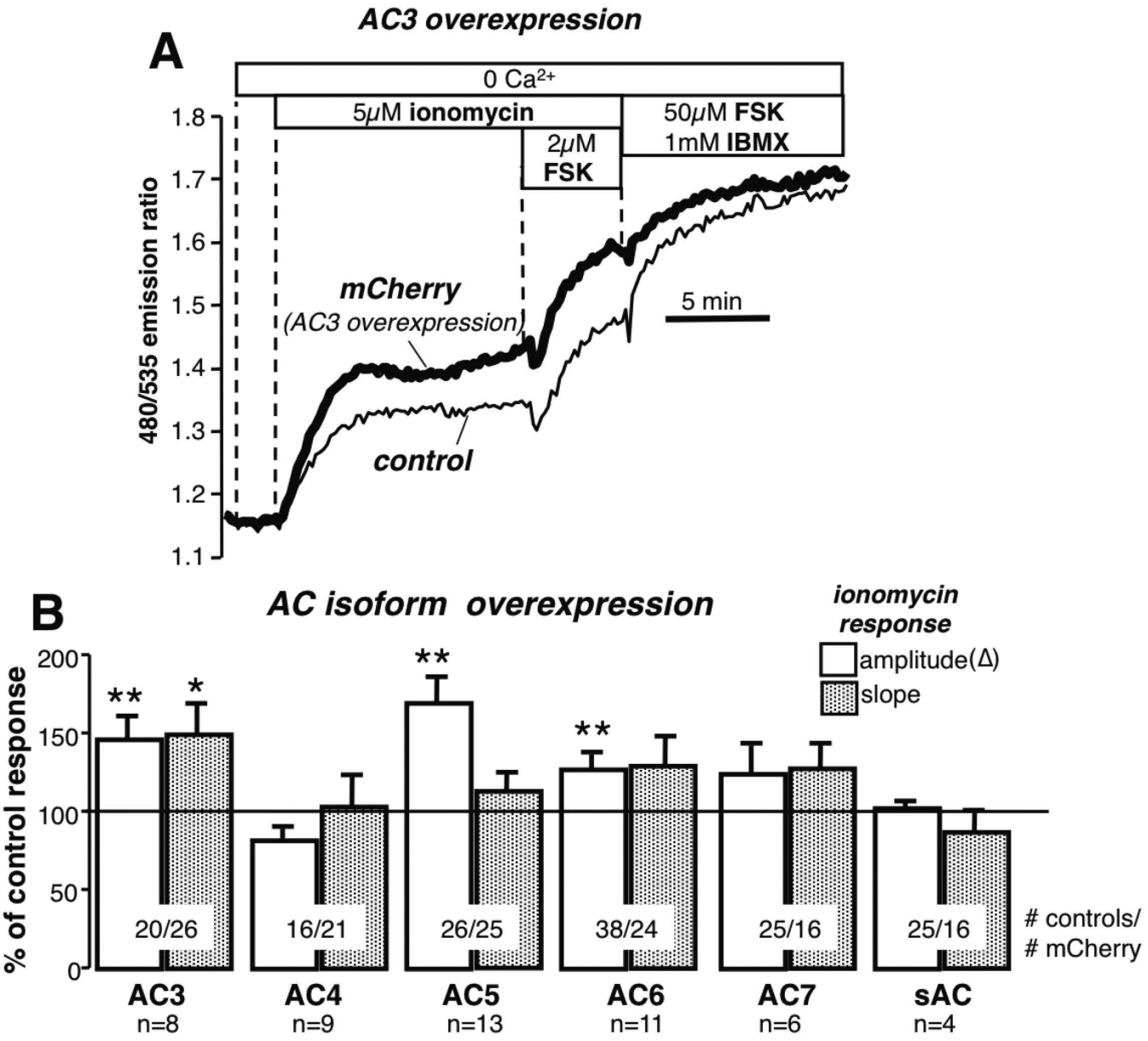

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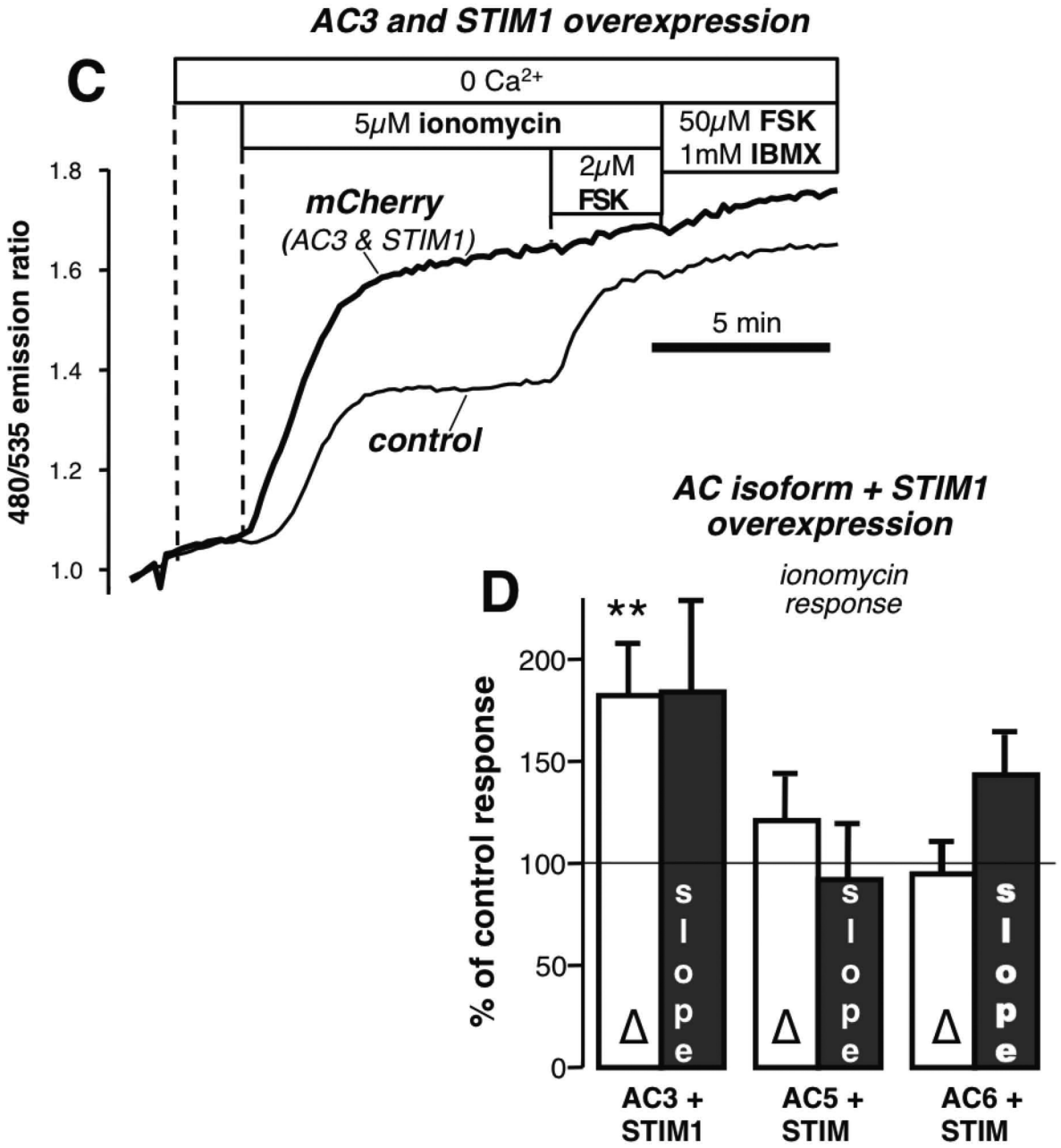

Figure 2. Overexpression of AC isoforms

A- Overexpression of AC3; response to ionomycin in cells cotransfected with AC3 and mCherry (used as a marker for transfection) and in control cells. **B**- Summary of experiments on AC isoforms. Amplitude and slope of the ionomycyn response expressed as % of control response. AC3: 8 exp./20 control/26 mCherry cells; AC4: 9 exp./16 control/ 21mCherry cells; AC5: 13 exp./26 control/25 mCherry cells; AC6: 11 exp./38 control/ 24 mCherry cells; AC7: 6 exp./ 25 control/ 16 mCherry cells; sAC: 4 exp./11 control/ 10 mCherry cells. **C**- Effect of co-expression of AC3 and STIM 1 on the response to ionomycin. **D**- Summary of results of overexpression of AC isoforms and STIM1. The response to ionomycin is expressed as % of the response in control cells. AC3+STIM1: 6 exp./22control /8 mCherry cells; AC5+ STIM1: 8 exp./8 control/8 mCherry cells; AC6+STIM1: 10 exp./18 control/14 mCherry cells. * $p<0.05$, ** $p<0.02$, ***$p<0.01$.

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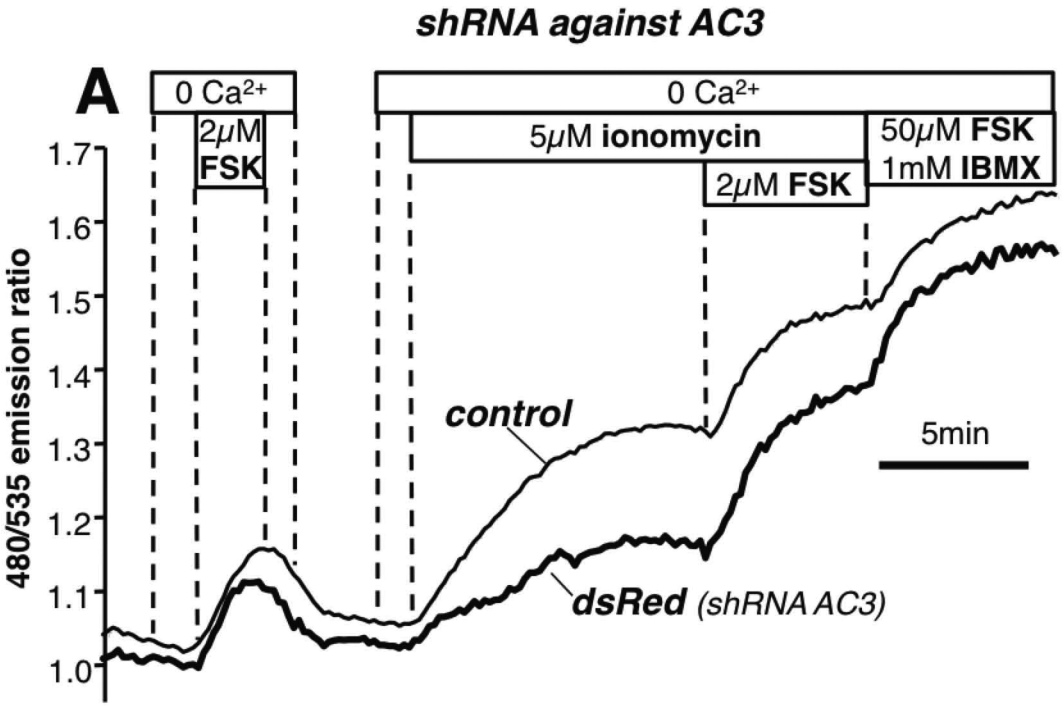

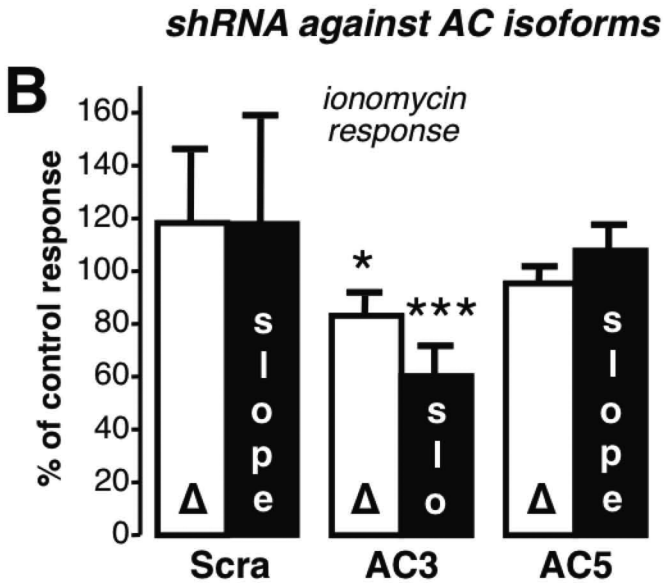

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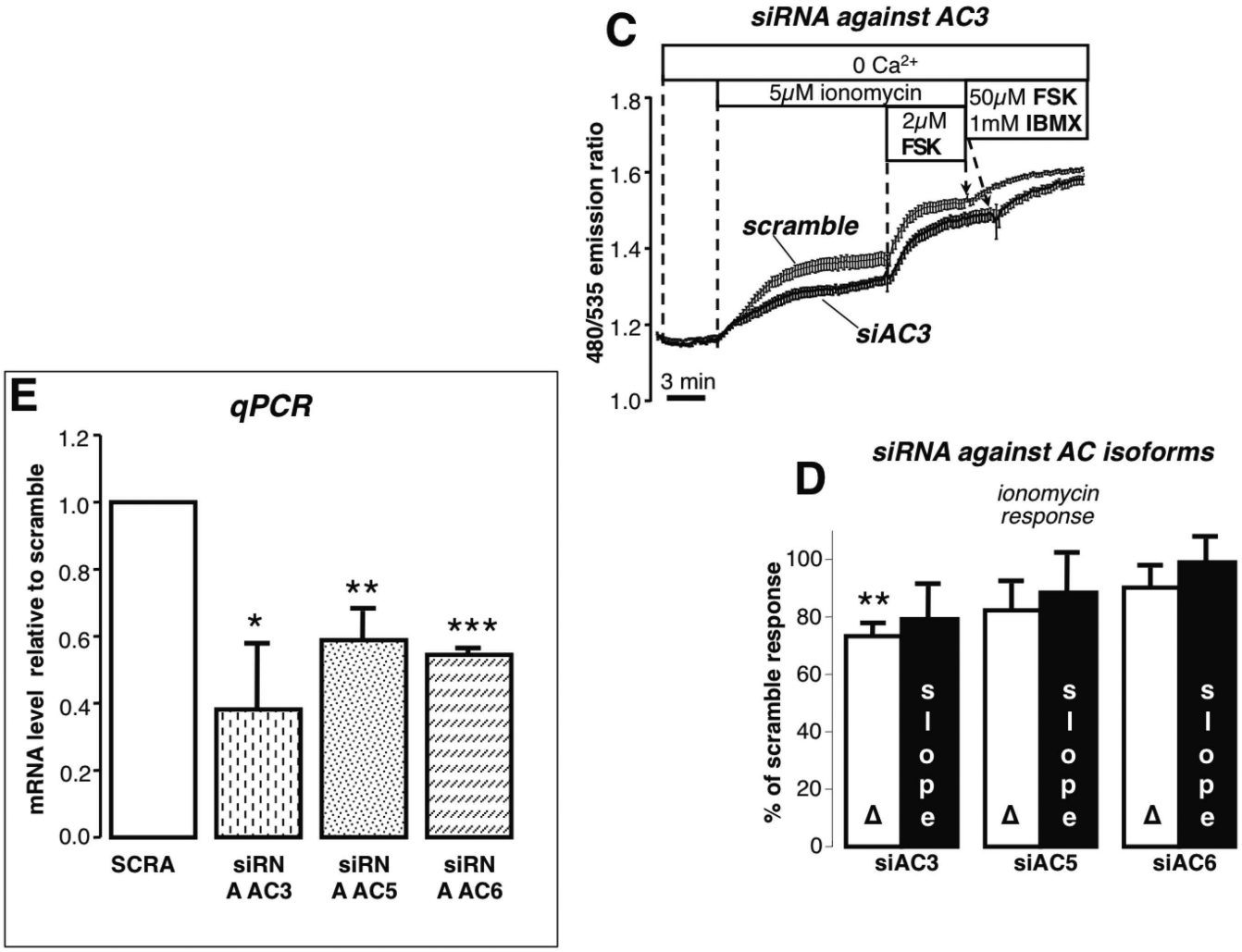

Figure 3. Effect of knockdown of AC isoforms

A- Response to ionomycin in cells expressing shRNA against AC3; dsRed was used as a marker for transfection with shRNA. **B**- Summary of experiments in cells expressing dsRed and shRNA against AC3 (13 exp./16 cells), AC5 (Δ: 7 exp., 12 cells; slope: 6 exp./11 cells), and treated with scramble (Δ: 10 exp./16 cells; slope: 9 exp./15 cells). Amplitude and slope of ionomycin response are expressed as % of control response. **C**- Response to ionomycin in cells treated with siRNA for AC3 compared to cells treated with scramble (average of 14 scramble and 12 dsRed cells) **D**- Summary of experiments with cells treated with siRNA for AC3 (6 exp./80 cells), AC5 (7 exp./68 cells) and AC6 (8 exp./91 cells) expressed as % of the response in scramble cells (scramble controls for siAC3 and siAC5: 13 exp./126 cells; scramble controls for siAC6 16 exp./202 cells). **E**- Summary of qPCR experiments. siRNA AC3: 3 independent experimental samples; siRNA AC5: 3 exp.; siRNA AC6: 2 exp. *p<0.05; **p<0.01; *** p<0.001.

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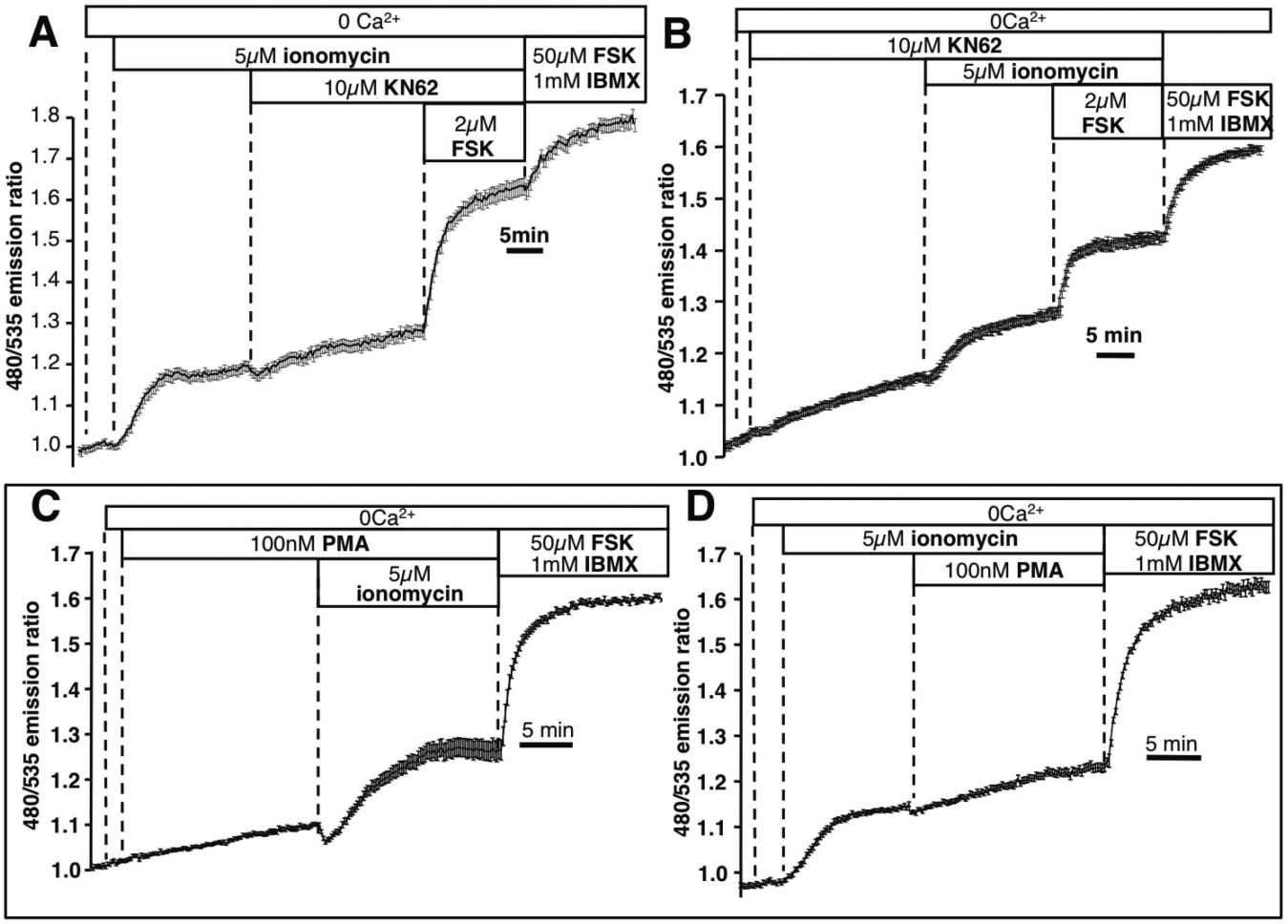

Figure 4. Effects of CaMKII and PKC inhibitors

A- Treatment with KN-62 (10μM) after ionomycin (average of 25 cells), typical of 8 exp./115 cells. **B**- KN-62 added before ionomycin (average of 16 cells); typical of 4 exp./44 cells. **C**- 100nM PMA added before ionomycin (average of 20 cells); typical of 5 exp./95 cells. **D**- Effect of 100nM PMA added during the response to ionomycin (average of 18 cells); typical of 6 exp./93 cells.