PAC1 receptor internalization and endosomal MEK/ERK activation is essential for PACAP-mediated neuronal excitability

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Abstract

Pituitary adenylate cyclase activating polypeptide (PACAP, *Adcyap1*) activation of PAC1 receptors (*Adcyap1r1*) can significantly increase the excitability of diverse neurons through differential mechanisms. For guinea pig cardiac neurons, the modulation of excitability can be mediated in part by PAC1 receptor plasma membrane G protein-dependent activation of adenylyl cyclase and downstream signaling cascades. By contrast, PAC1 receptor-mediated excitability of hippocampal dentate gyrus granule cells appears independent of membrane delimited AC/cAMP/PKA and PLC/PKC signaling. For both neuronal types, there is mechanistic convergence demonstrating that endosomal PAC1 receptor signaling has prominent roles. In these models, neuronal exposure to Pitstop2 to inhibit β-arrestin/clathrin-mediated PAC1 receptor internalization eliminates PACAP modulation of excitability. β-arrestin is a scaffold for a number of effectors especially MEK/ERK and notably, paradigms that inhibit PAC1 receptor endosome formation and ERK signaling also blunt the PACAP-induced increase in excitability. Detailed PAC1 receptor internalization and endosomal ERK signaling mechanisms have been confirmed in HEK PAC1R-EGFP cells and shown to be long lasting which appear to recapitulate the sustained electrophysiological responses. Thus, PAC1 receptor internalization/endosomal recruitment efficiently and efficaciously activates MEK/ERK signaling and appears to represent a singular and critical common denominator in regulating neuronal excitability by PACAP.

Keywords

INTRODUCTION

Pituitary adenylate cyclase activating polypeptides (PACAP, *Adcyap1*) are evolutionarily well-preserved trophic and intercellular signaling molecules that are widely distributed within neural and endocrine tissues (Arimura, 1998; Hannibal, 2002; Jaworski and Proctor, 2000; Vaudrey et al., 2009). PACAP peptides are members of the vasoactive intestinal peptide (VIP)-secretin-glucagon family of bioactive peptides and were first isolated from ovine hypothalami based on their abilities to stimulate adenylyl cyclase (AC) activity in anterior pituitary cells (Kimura et al., 1990; Miyata et al., 1989). Alternative posttranslational processing of the precursor molecule gives rise to two α-amidated forms of the peptide, PACAP38 and PACAP27. PACAP38 has 38 amino acid residues [rat pro-PACAP(131-168)], while PACAP27 corresponds to the amino terminal segment of PACAP38 [proPACAP(131-157)]. The levels of PACAP38 predominate in most tissues, although PACAP38 : PACAP27 ratio appears tissue-specific (Arimura et al., 1991). PACAP27 exhibits 68% amino acid identity with the 28-amino acid peptide VIP (Kimura et al., 1990; Miyata et al., 1989).

The actions of PACAP are mediated through several heptahelical G-protein-coupled receptor (GPCR) subtypes including the PACAP-selective PAC1 receptor (*Adcyap1r1*) and PACAP/VIP VPAC receptors (*Vipr1* and *Vipr2*; also VPAC1 and VPAC2, respectively) (Harmar and Lutz 1994; Arimura, 1998; Braas and May, 1999; Vaudry et al., 2009). There are multiple PAC1 receptor isoforms from alternative splicing depending on the absence or presence of Hip and/or Hop cassette inserts into the third cytoplasmic loop that may serve to fine-tune the second messenger responses. The PAC1Null (neither Hip nor Hop insert) and PAC1Hop receptor variants predominate in the CNS and PNS (Spengler et al., 1993; Braas and May, 1999). There is duality in canonical membrane delimited PAC1 receptor signaling through the recruitment of Gαs and Gαq/11 leading to adenylyl cyclase (AC) and phospholipase C (PLC) activation, respectively (Deutsch and Sun, 1992; Spengler et al., 1993; Pisegna and Wank, 1996; Braas and May, 1999). From these pathways, other intracellular signaling cascades, such as mitogen-activated protein kinase (MAPK) and Akt (also protein kinase B) (Pisegna and Wank, 1996; Barrie et al., 1997; Bouschet et al., 2003; May et al., 2010), may be engaged to diversify cellular responses. In addition to these well studied plasma membrane initiated signaling mechanisms, the PAC1 receptors, as in other GPCR systems, can internalize and initiate long-term ERK activation via endosomal signaling (May et al., 2010; May et al., 2014). These endosomal mechanisms are still not well understood but can be functionally critical in potentially delivering second messengers to intracellular sites with high spatial and temporal resolution. The ability for neurons to deliver the right signals to the right place and at the right time provides the mechanistic means to initiate and modulate cellular responses with high precision (Calebiro et al., 2010; Scita and Di Fiore, 2010; McMahon and Boucrot, 2011; Irannejad et al., 2013).

Among its many physiological effects, PACAP can play key roles in sensory and autonomic functioning, learning and memory, and responses to injury and stress through actions on peripheral and central nervous systems neurons (Braas et al., 1998; Cho et al., 2012; Hammack et al., 2009; Hammack and May, 2014; Hill et al., 2011; Legradi et al., 2007; Stroth et al., 2012; Tompkins et al., 2007; Vaudry et al., 2009). As peripheral and central neuronal models, we have investigated and compared, using guinea pig intracardiac parasympathetic postganglionic neurons and rat hippocampal dentate gyrus granule cells, the importance of PACAP-induced PAC1 receptor internalization and endosomal MEK/ERK signaling in modulating neuronal excitability. Both neuron types express PAC1 receptors and are innervated by PACAP containing fibers.

PACAP increases cardiac neuron excitability through multiple signaling pathways

PACAP is present in parasympathetic cholinergic preganglionic nerve terminals innervating guinea pig cardiac ganglia neurons (Braas et al.,1998; Calupca et al., 2000). The effects of PACAP on cardiac neurons were studied using intracellular recordings from neurons in whole mount preparations of the guinea pig intracardiac ganglia (Braas et al., 1998; Merriam et al., 2013); both neurally-released and exogenous PACAP application depolarized and increased excitability of the cardiac neurons via selective PAC1 receptor activation (Braas et al.. 1998; Hoover et al., 2009; Tompkins et al., 2007). The primary focus of our past studies involved elucidating mechanisms contributing to the peptide-induced increase in excitability, which was evident from the shift in excitability curves, as determined by plotting the number of action potentials generated by 1 second depolarizing current steps of increasing magnitude before and in the presence of PACAP (Braas et al., 1998; Merriam et al., 2013; May and Parsons, 2016; Parsons and May, 2019). In intracellular recordings of control unstimulated whole mount preparations, the majority of the cardiac neurons exhibited a phasic or accommodating firing pattern in response to long suprathesthold depolarizing current steps; approximately 10% of cardiac neurons exhibited tonic firing patterns. The application of PACAP to these preparations markedly enhanced excitability in all cardiac neurons regardless of control response phenotype.

Modulation of multiple ionic conductances contributed to the PACAP-induced increase in cardiac neurons excitability (Parsons et al., 2016; May and Parsons, 2016; Parsons and May, 2019; Tompkins et al., 2006; Tompkins et al., 2016), which appeared to reflect concerted plasma membrane delimited and endosomal signaling mechanisms. While activated PAC1 receptor recruitment of $G\alpha q/11$ for PLC-mediated signaling did not appear to play any role in PACAP-induced increase in cardiac neuron excitability (Parsons et al., 2008; Parsons et al., 2016), plasma membrane PAC1 receptor $G\alpha s$/adenylyl cyclase activation and cAMP-induced enhancement of the hyperpolarization-induced nonselective cationic current I_h contributed to the PACAP-stimulated increase in excitability in some cardiac neurons. The PACAP responses were suppressed following treatments with cesium to block I_h (Merriam et al., 2004; Tompkins et al., 2009); further, the PACAP effect was reduced by nickel treatment, indicating that PACAP activation of the low voltage-activated calcium current It also participated in the PACAP-induced increase in excitability (Tompkins et al., 2015). In coherence, protein kinase A (PKA) phosphorylation of T-type channel α subunits has been shown to enhance I_T (Talavera and Nilius, 2006; Chemin et al., 2007; Iftinca and Zamponi,

2008; Simms and Zamboni, 2014). Hence in sum, cardiac neuron membrane PACAP/PAC1 receptor activation of the canonical downstream AC/cAMP/PKA pathway has abilities to enhance dual I_h and I_T, contributing synergistically to PACAP regulation of cardiac neuron excitability.

Importantly however, in more detailed surveys to probe underlying mechanisms using pathway inhibitors, cardiac neuron pretreatments with the MEK inhibitor PD98059 also significantly suppressed PACAP enhancement of excitability. Cardiac neuron PAC1 receptor activation of MEK/ERK signaling can be generated through both cAMP/PKA and endosomal signaling (Clason et al., 2016; see schematic) and the ERK-induced enhancement of an inward current appeared to be generated through the voltage-dependent sodium channel Nav1.7 (Tompkins et al., 2016). To delineate which mechanistic route to ERK activation was more prominent, the conditions that blunt clathrin-mediated receptor endocytosis, including treatment with the clathrin inhibitor Pitstop2 or the dynamin inhibitor dynasore, as well as decreasing ambient temperature, strikingly eliminated the PACAP effect on cardiac neuron excitability (Merriam et al., 2013; May et al., 2014;Tompkins et al., 2018; Parsons and May, 2019). From these observations, PAC1 receptor internalization and recruitment of endosomal signaling appeared to be a critical mechanism underlying the PACAP effect on cardiac neuron excitability. Although plasma membrane AC/cAMP production and endosomal ERK signaling may act synergistically to modulate the currents necessary to enhance excitability, recent studies have shown that a significant fraction of total cellular AC activity and cAMP levels can be produced by neuronal endosomes following receptor endocytosis (Calebiro et al., 2009; Ferrandon et al., 2009; Calebiro et al., 2010, Irannejad et al., 2013; Vilardaga et al., 2014; Di Fiore and von Zastrow, 2016). While the concept of a unifying mechanism remains to be tested, these results may implicate endosomal signaling platforms as the primary means of sustained cAMP and activated ERK generation underlying PAC1 receptor-mediated neuronal excitability.

PACAP increases granule cell excitability through endosomal ERK activation.

High levels of PAC1 receptor transcripts in the granule cell layer of the dentate gyrus in the hippocampus were initially determined by *in situ* hybridization studies (Jaworski and Proctor, 2000; Hannibal, 2002) and hippocampal PACAP hilar mossy neurons were shown to send fiber projections to the DG inner molecular layer (IML) (Condro et al., 2016; Johnson et al., 2020b); hence, there are short PACAPergic synaptic circuits between PACAP-expressing hilar mossy cells and the proximal dendrites on PAC1 receptor-expressing DG granule cells. Accordingly, PACAP application to hippocampal dentate gyrus slice preparations increased the excitability of granule cells (Johnson et al., 2020a; Johnson et al., 2020b). In whole-cell recordings in current clamp mode, the granule cells fired tonically in response to suprathreshold 1 second depolarizing current injections; the action potential frequency increased with increasing current intensity (Johnson et al., 2020a; Johnson et al., 2020b). PACAP enhanced the excitability of DG granule cells assessed by determining the shift in excitability curves determined by plotting action potential frequency before and in PACAP with increasing current steps. The increase in excitability was associated with a negative shift in the threshold for action potential generation and appeared to be independent of changes in input resistance and changes in resting membrane potential

(Johnson et al., 2020a). Some hippocampal interneurons express VIP, but as VIP did not mimic the actions of PACAP, PACAP increased DG granule cells excitability through PAC1 receptor signaling.

In contrast to cardiac neurons, neither AC/cAMP/PKA nor PLC/DAG/IP$_3$ signaling appeared to be required for the PACAP modulation of DG cell excitability. But to a greater extent than cardiac neurons, treatments with the MEK inhibitor PD98059, virtually eliminated PACAP-enhanced induced excitability of DG granule cells (Johnson et al., 2020a). The effect of PACAP on DG granule cells was also blunted markedly by treatment with the cell-permeable clathrin-mediated endocytosis inhibitor Pitstop2; hence the effects of PD98059 or Pitstop2 to block the PACAP-mediated responses appeared comparable. Thus for hippocampal dentate granule cells, PAC1 receptor internalization and endosomal recruitment of MEK/ERK signaling appeared to represent the primary if not the sole second messenger mechanism contributing to the PACAP modulation of neuronal excitability in DG granule cells (Johnson et al., 2020a; see schematic). The PACAP effect remained in the presence of a number of well-established blockers of voltage-dependent potassium and calcium currents, suggesting modulations by these conductances were not essential (Johnson et al., 2020a). However, PACAP failed to increase excitability in DG cells pretreated with the persistent sodium channel blocker riluzole, indicating that the PACAP effect required this component of the inward sodium current (Johnson et al., 2020a).

PACAP induces PAC1 receptor internalization in HEK cell cultures

Whole mount and ex vivo tissue preparations are not always amenable to detailed mechanistic and visualization studies, and accordingly, the electrophysiological studies were coupled with PACAP/PAC1 receptor internalization and activated MEK/ERK assessments using HEK cells stably expressing a human PAC1 receptor C-terminally tethered to EGFP (HEK PAC1R-EGFP receptor cells) (Merriam et al., 2013; May et al., 2014). From Western blots, PACAP-mediated ERK activation was BimI-sensitive, which unlike the neurons above, implicated roles for PLC/PKC signaling in HEK cells. But notably, Pistop2 and dynasore blocked PACAP/PAC1 receptor internalization/endosomal signaling that contributed to pERK generation (May et al., 2014). These biochemical measures were correlated with PACAP induced PAC1 internalization determined using confocal microscopy (Merriam et al., 2013; May et al., 2014). Under control conditions at 37°C, the receptor remained primarily in the cell membrane, whereas within a few minutes of exposure to nanomolar PACAP concentrations, there was extensive PAC1R-EGFP endocytosis into intracellular vesicles with a concomitant reduction in cell surface fluorescence. In contrast, PACAP exposures of similar duration at 22 - 24°C resulted in scant receptor translocation into intracellular vesicles and no loss of membrane fluorescence, indicating that receptor internalization was suppressed at room temperature. Similar to the reduction in activated PAC1 receptor trafficking at ambient temperature, pretreatment of the cells at 37 C with Pitstop2, a clathrin inhibitor (von Kleist et al., 2011), or dynasore, a dynamin I/II inhibitor (Maca et al., 2006) also blunted markedly PACAP-stimulated PAC1R-EGFP internalization resulting in receptor retention on the cell surface.

From these observations, the HEK PAC1R-EGFP internalization studies aligned with the neuronal results and allowed more detailed analyses of the endosome signaling process. In treating the HEK PAC1R-EGFP with PACAP and signaling inhibitors, the characteristics of PAC1 receptor internalization and endosomal ERK signaling were determined. A brief 5 minute pulse exposure to 25 nM PACAP alone produced a sustained elevation of pERK that peaked at 10 –15 minutes and returned to baseline levels in about 60 minutes (Figure 1), demonstrating a long lasting generation of pERK by PACAP. Additional experiments tested whether either membrane delimited PLC/PKC or internalization/endosomal signaling was most prominent. Across treatment times and PACAP concentrations, Pitstop 2 blocked PACAP-stimulated ERK activation more than 70% whereas BimI attenuated activation approximately 40 – 60% (Figure 2). By these measures, the endosomal signaling pathway appeared more prevalent. Even with low PACAP concentrations (1 nM), a 15 minute exposure significantly increased pERK levels that was sensitive to Pitstop 2 and not by BimI (Figure 2, right panels), further implicating receptor internalization and endosomal signaling as predominant mechanisms for PAC1 receptor long-term MEK/ERK signaling.

Conclusion

In addition to membrane delimited G protein-dependent signaling, GPCRs undergo a sequence of steps leading to receptor internalization and endosomal signaling (Calebiro et al., 2009; Ferrandon et al., 2009; Calebiro et al., 2010, Irannejad et al., 2013; Vilardaga et al., 2014). GPCR endocytosis had initially been associated with receptor desensitization and recycling pathways, but is now recognized to represent a mechanism supporting sustained second messenger generation via signaling endosomes (Calebiro et al., 2009; Ferrandon et al., 2009; Calebiro et al., 2010, Irannejad et al., 2013; Vilardaga et al., 2014). A significant component of pERK generation by PACAP is dependent on PAC1 receptor internalization and endosomal signaling (Figure 3, schematic). Further, the MEK/ERK signaling cascade has critical roles in synaptic plasticity and regulation of neuronal excitability (Sweatt, 2004). We propose that PAC1 receptor internalization/endosomal recruitment of MEK/ERK signaling is a critical mechanism supporting the PACAP modulation of neuronal excitability.

Acknowledgements:

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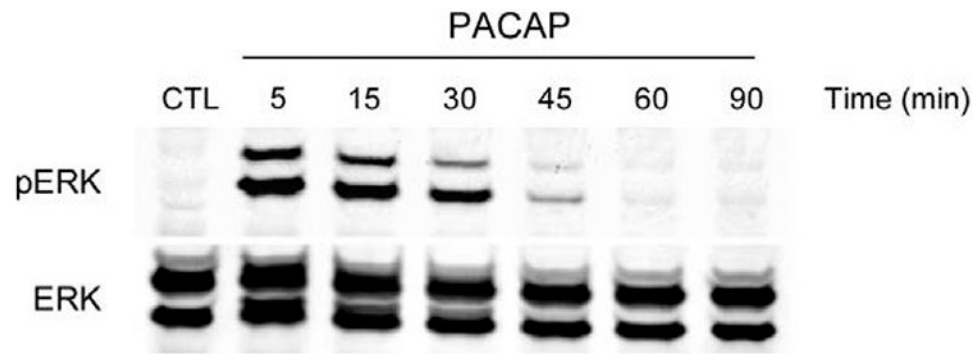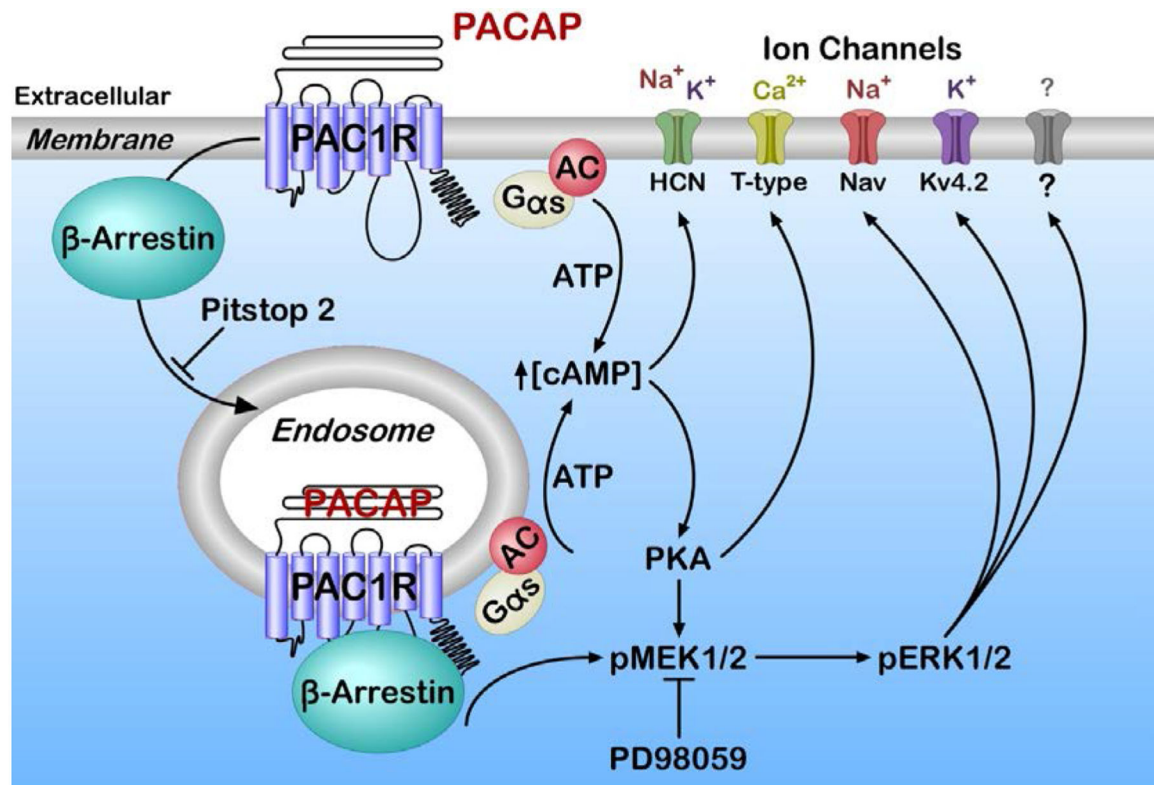
Figure 1.
Brief PAC1 receptor activation results in long term ERK activation duration. Stable HEK PAC1 receptor-EGFP cell lines were acutely pulse treated with 25 nM PACAP27 for 5 min followed by washout, incubation in control medium without peptide and harvest at the times shown (5 – 90 min). Even after brief PACAP exposure, the duration of ERK activation (pERK) was extended markedly over 30 min and attenuated to baseline levels at 60 min. The same Western blot was reprobed with a pan ERK antibody to illustrate equal sample loading.
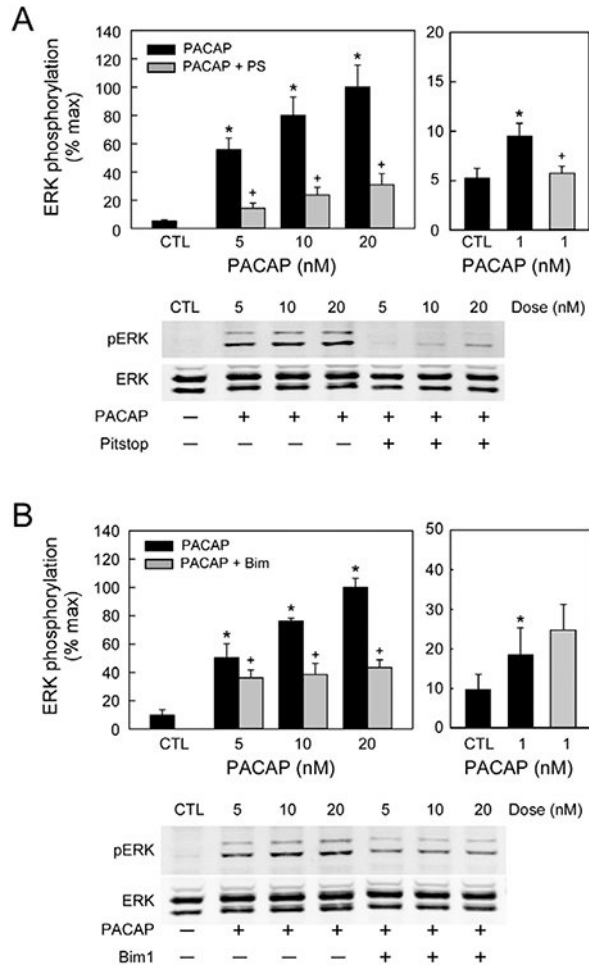
Figure 2.
Pitstop2 and BimI blocked PACAP-mediated ERK activation at varying PACAP concentrations. HEK PAC1 receptor-EGFP cells were pretreated with 20 μM Pitstop 2 (A) or 15 μM BimI (B) for 15 min before the addition of PACAP (5 - 20 nM) for 15 min. Pitstop and Bim I inhibited ERK activation at all PACAP peptide concentrations tested, suggesting that receptor internalization/endosomal signaling occurred even at the low peptide concentrations. Even at 1 nM PACAP, ERK activation was more sensitive to receptor internalization/endosome-mediated mechanisms than PKC signaling. Representative Western blots for each treatment shown in replicate. *, significantly different from untreated control (CTL); +, different from sample at same PACAP concentration. Data represent mean ± SEM, n = 3.

Figure 3.
Schematic of PAC1 receptor plasma membrane and endosomal signaling. PACAP/PAC1 receptor activation can generate plasma membrane delimited and endosomal signals following β-arrestin-mediated receptor internalization. PACAP/PAC1 receptor-stimulated AC/cAMP production is generated largely from the plasma membrane although a smaller fraction can also be derived from endosomes; both can contribute to the gating of hyperpolarization-activated cyclic nucleotide (HCN) channel and I_h. Cyclic AMP pathways can lead to ERK signaling, although sustained ERK activation may result from effectors such as MEK, docked to endosomal receptor/β-arrestin signaling platforms to regulate T-type, sodium, potassium and/or yet uncharacterized channels. Pitstop 2 blocks receptor/β-arrestin internalization and endosomal signaling. MEK inhibitor PD98059 blocks ERK signaling and channel activation.