Stress peptide PACAP engages multiple signaling pathways within the carotid body to initiate excitatory responses in respiratory and sympathetic chemosensory afferents.

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ABSTRACT

Consistent with a critical role in respiratory and autonomic stress responses, the carotid bodies are strongly excited by pituitary adenylate cyclase-activating polypeptide (PACAP), a neuropeptide implicated in stress responses throughout the sympathetic nervous system. PACAP excites isolated carotid body glomus cells via activation of PAC1 receptors, with one study suggesting PAC1-induced excitation is due entirely to protein kinase A (PKA)-mediated inhibition of TASK channels. However, in other systems, PAC1 is known to be coupled to multiple intracellular signaling pathways including PKA, phospholipase C (PLC), phospholipase D (PLD) and protein kinase C (PKC), and that trigger multiple downstream effectors including increased Ca\(^{2+}\) mobilization, inhibition of various K\(^+\) channels and activation of non-selective cation channels. This study tests if non-PKA/TASK channel signaling helps mediate PACAP’s stimulatory effects on the carotid body. Using an \textit{ex-vivo} arterially-perfused rat carotid body preparation, we show that PACAP-38 stimulates carotid sinus nerve (CSN) activity in a biphasic manner (peak response, falling to plateau). PKA blocker H-89 only reduced the plateau response (~41%) whereas the TASK-1 like K\(^+\) channel blocker/ TRPV1 channel agonist anandamide only inhibited the peak response (~48%), suggesting involvement of additional pathways. PLD blocker, CAY10594 significantly inhibited both peak and plateau responses. PLC blocker U73122, decimated both peak and plateau responses. Brefeldin A, a blocker of Epac (cAMP-activated guanine exchange factor, reported to link Gs-coupled receptors with PLC/PLD), also reduced both phases of the response, as did blocking signaling downstream of PLC/PLD with PKC inhibitors chelerythrine chloride and GF 109203X. Suggesting the involvement of non-TASK ion channels in PACAP’s effects, A-type K\(^+\) channel blocker 4-aminopyridine (4-AP) and putative TRPC/T-type calcium channel blocker SKF 96365 each significantly inhibited the peak and steady-state responses. These data suggest the stimulatory effect of PACAP-38 on carotid body sensory activity is mediated through multiple signaling pathways: the PLC-PKC pathways predominates, with TRPC and/or T-type channel activation and Kv channel inactivation; only partial involvement is attributable to PKA and PLD activation.
INTRODUCTION

Pituitary adenylate cyclase-polypeptide (PACAP) is a 27 or 38 amino acid neuropeptide, widely expressed within the central and peripheral nervous systems with diverse and important physiological functions [50, 60]. In adults, systemic infusion of PACAP into anesthetized animals reveals potent acute actions on a wide range of critical physiological systems, including effects on cardiac output, vasodilation, glucose homeostasis and bronchodilation [50]. The importance of PACAP signaling to survival is particularly evident in neonatal life [62]. Mice deficient in PACAP, or the PACAP-specific receptor PAC1, are more prone to sudden neonatal death than their wild type littermates, a phenotype exacerbated dramatically by mild reductions (3°C) in ambient temperature [17]. In neonates, PACAP-deficiency reduces baseline breathing, blunts responses to both hypoxia and hypercapnia and, under conditions of anesthetic-induced hypothermia, leads to long-duration life-threatening apneas [12,13]. These data support an important role for PACAP as an “emergency transmitter” in modulating stress responses including those initiated by respiratory distress.

Consistent with PACAP’s involvement in stress responses in general, PACAP is present throughout the sympathetic nervous system including brainstem catecholamine neurons that innervate both the hypothalamus and preganglionic sympathetic neurons in the spinal cord [2,16]. Spinal preganglionic sympathetic neurons innervating the adrenal gland also contain PACAP [22], as do at least half the sympathetic neurons of the superior cervical ganglia [36]. Consistent with PACAP’s involvement in initiating responses to respiratory stress, several studies have demonstrated an acute effect of PACAP on breathing [20]. When administered intravenously, PACAP increases respiratory frequency in anesthetized dogs [49]. This effect is largely dependent on intact carotid sinus nerves (CSN) demonstrating that PACAP activates the carotid bodies, the major peripheral respiratory and sympathetic chemoreceptor organs [46].

The carotid bodies, which are themselves innervated by the sympathetic neurons of the superior cervical ganglia, sense changes in arterial PO$_2$, PCO$_2$, pH and temperature. Hypoxia, hypercapnia, low pH and elevated temperature increase sensory discharge of the carotid bodies, triggering reflex pathways which involve brainstem cardiorespiratory centers and sympathetic
pathways crucial for maintaining homeostasis [24, 46]. Using isolated chemosensitive glomus cells, Xu et al [63] demonstrated that PACAP stimulates the carotid body via the PACAP-specific G-protein coupled receptor PAC1 and suggested the mechanism was independent of oxygen sensing. They proposed that PACAP’s excitatory effects is mediated entirely through an adenylate cyclase-PKA pathway, resulting in reduction in TASK-1 like K⁺ currents which, in turn, leads to glomus cell depolarization and [Ca^{2+}]ᵢ rise [63]. Although elegant in its simplicity, this explanation is incongruent with previous studies of carotid body function that consistently emphasise the PLC-PKC pathway while downplaying the adenylate cyclase-PKA pathway in acute responses [7, 10, 40, 41, 42, 53, 54, 61]. In addition, the importance of TASK channels in carotid body function has not yet been solidified. For example, while some studies suggest that TASK-like channels are an important component of the oxygen sensing machinery [4], in a recent study we showed that the potent stimulatory effects of the TASK-1 channel blocker and TRPV1 agonist anandamide (ANA) on intact carotid body chemosensory discharge is mediated entirely via direct activation of TRPV1 receptors on petrosal afferents [47]. On the one hand, if PACAP modulates TASK-like channels in the intact carotid body and TASK-like channels are involved in oxygen sensing, then responses to hypoxia and PACAP are unlikely to be independent. On the other hand, if the effects of TASK blocker (ANA) on the intact carotid body are not mediated by TASK, then PACAP’s stimulatory effect is likely by a different mechanism.

As summarized in Fig 1, PAC1 signaling in other systems can invoke multiple G-proteins (Gs and Gq), activating cAMP-PKA [34, 43], cAMP-Epac [23, 52], PLC-IP₃ and/or PLC-PKC pathways [23, 25, 35]. The PAC1 receptor can also modulate the activity of phospholipase D (PLD) in recombinant cell lines through a small G-protein independent of the PLC-PKC pathway [30]. Once activated, these pathways do PACAP’s bidding by changing gene expression, releasing Ca²⁺ from internal stores and/or activating / phosphorylating a range of ion channels including T-type Ca²⁺ channels [8, 19, 38, 55, 57, 58]. The cellular mechanisms by which PACAP leads to secretion have been particularly well studied in the rat PC12 cell line which share the oxygen sensing and catecholaminergic phenotype of carotid body glomus cells [68]. The PACAP-induced catecholamine secretion from PC-12 is time dependent, consisting of an initial peak secretory event followed by a sustained secretion of lower magnitude. The initial even triggered by PACAP requires influx of Ca²⁺, likely through L-type VGCCs, whereas the sustained secretion is suppressed by the PLC inhibitor U73122 and non-selective cation channels.
Whether a similar mechanism contributes to carotid body chemosensory excitation in response to PACAP remains to be investigated.

The aim of the present study was to explore the mechanisms by which PACAP-38 modulates rat carotid body chemosensory afferents. To achieve this, we assessed the effects of PACAP-38 on CSN activity of an ex vivo arterially-perfused carotid body preparation. We then tested if the response to PACAP was mitigated by a PLC inhibitor (U73122), a PKA inhibitor (H-89), two PKC inhibitors (chelerythrine chloride and GF109203X), an Epac inhibitor (Brefeldin A) or two PLD inhibitors (0.5% butanol and CAY10594). We also examined whether the PACAP response was exacerbated by forskolin, a cAMP activator. Further, to assess the possible ionic mechanisms underlying PACAP-38 induced effects, we used the TASK-1 like K+ channel blocker ANA, the Kv channel blocker 4-aminopyridine (4-AP) and the TRPC / T-type Ca2+ channel blocker SKF 96365. Our data demonstrate that the stimulatory effect of PACAP-38 on CSN activity is mediated through multiple cellular-signaling pathways (though dominated by PKC) and likely involves at least two types of ion channel.
MATERIALS AND METHODS

Animals: Experiments were conducted using forty-nine 150-250g male adult Sprague-Dawley albino rats (Charles River, Quebec, Canada). Experimental procedures were approved by the University of Calgary Animal Care Committee and were in accordance with national guidelines.

Chemicals and solutions: PACAP-38 was purchased from AnaSpec, Inc. (San Jose, CA). H-89, cherelythrine chloride, GF10923X, U73122, SKF96365 and brefeldin A were purchased from Tocris Bioscience (Minneapolis, MN). Anandamide was purchased from EMD Biosciences, USA. 4-aminopyridine (4-AP) and forskolin were obtained from Sigma, USA. CAY10594 was purchased from Cayman Chemical (Michigan, USA).

Physiological saline for dissection and perfusion consisted of the following (in mM): 115 NaCl, 4 KCl, 24 NaHCO3, 1.25 NaH2PO4, 2 CaCl2, 10 D-glucose and 12 sucrose.

Ex-vivo CB-CSN preparation: The carotid bifurcation, including the CB-CSN-superior cervical ganglion, was isolated en-bloc from halothane-anesthetized rats for in vitro perfusion as described previously [47]. Briefly, the isolated tissue was transferred to saline equilibrated with 95% O2, 5% CO2. After 20 min, the isolated tissue was transferred to a recording chamber with a built-in water-fed heating circuit, the common carotid artery was cannulated for luminal perfusion and the tissue overlying the carotid body was carefully removed to free the carotid sinus nerve.

A peristaltic pump was used to set the perfusion rate through the common carotid body at ~ 15 ml/min, which was sufficient to maintain a constant pressure of ~100 mmHg at the tip of the cannula. The perfusate was equilibrated with computer-controlled gas mixtures monitored using CO2 and O2 gas analyzers (models CA-2A and PA1B, respectively, Sable Systems, Las Vegas, NV). A gas mixture of 100 Torr PO2 and 36 Torr PCO2 balanced with N2 was used throughout the experiments (yielding pH~7.4) unless otherwise stated. The temperature of the perfusate was maintained at 37 ± 0.5°C. The effluent from the chamber was re-circulated.

Electrophysiology: Chemosensory discharge was recorded extracellularly from the whole desheathed CSN, which was placed on a platinum electrode and lifted into a thin film of paraffin oil. A reference electrode was placed close to the bifurcation. CSN activity was monitored using a differential AC amplifier (model 1700, AM Systems) and a secondary amplifier (model
AM502, Tektronix, Beaverton, OR). The neural activity was amplified, filtered (300-Hz low cutoff, 5-kHz high cutoff), displayed on an oscilloscope, rectified, integrated (200-ms time constant), and stored on a computer using an analog-to-digital board (Digidata 1322A, Axon Instruments) and data acquisition software (Axoscope 9.0). Preparations were exposed to a brief hypoxic challenge (4 min, PO2 = 60 Torr) to determine viability. Preparations that failed to show a clear-cut increase in activity during this challenge were discarded. After this challenge, preparations were left undisturbed for 60 min to stabilize before initiation of the experimental protocol.

**Data Analysis**: Data were analyzed offline using custom software (written by R. J. A. Wilson). CSN activity was divided into 60 s time bins, and activity in each bin was rectified and summed (expressed as integrated neural discharge). The peak CSN response was taken as the value of the bin with the highest level of activity; the plateau response was the last bin during PACAP exposure, just prior to washout. The neural responses for different conditions in the protocol were normalized to the baseline (normoxic) condition. All data were expressed as mean ± S.E.M. One-way ANOVA with Student-Newman-Keuls *post hoc* tests were used to analyze the data (SigmaStat 2.03). *P* < 0.05 was considered significant.
RESULTS

Effect of PACAP-38 on CSN activity.

To confirm the excitatory effect of PACAP on CSN activity, the isolated carotid body was perfused with normoxic saline (PO$_2$ = 100 Torr; PCO$_2$ = 36 Torr) containing 100 nM PACAP-38 for 30 minutes. PACAP-38 stimulated CSN activity in a biphasic manner with an initial sharp transient rise, peaking within 3 minutes of PACAP-38 application, followed by a slow decay that transitions into a plateau phase (Fig 2A & B). The plateau phase persisted after washout of PACAP-38. In some experiments, there was a rebound effect immediately after washout. This was not explored further. The effects of PACAP concentration on the peak and plateau phase are summarized in Fig 2C. The transient increase in CSN activity was much less prominent at 50 nM PACAP and completely lost at 25 nM (with 25 nM, the average peak and steady state CSN responses were not significantly different from baseline). At a higher concentration of PACAP-38 (200 nM) the onset of the transient phase was slightly faster; however the overall peak and steady state responses were not significantly different from 100 nM of PACAP-38 ($P = 0.25 & 0.10$, respectively; Fig 2A & C). For all remaining experiments, we used 100nM PACAP-38 in order to investigate both transient and plateau phases. This was the same concentration as used to study PACAP-induced second messenger pathways in isolated glomus cells [63] and PC-12 cells [35]. According to Xu et al. [63], the glomus cell [Ca$^{2+}$]$_i$ responses to PACAP and hypoxia are additive, indicating independence. To confirm this finding in the intact carotid body, we tested the ability of carotid body afferents to respond to a moderate hypoxic challenge (PO$_2$ = 60 Torr) during the plateau phase of the PACAP-38 response (100 nM). As predicted by Xu et al.’s [63] data, the responses to hypoxia and PACAP-38 were approximately additive (Fig 2D).

Effect of PKA blocker H89 on PACAP-38 induced CSN activity.

To test the involvement of PKA in the intact carotid body, we blocked PKA activity using H-89. As illustrated in Fig 3A & 3B, H-89 pre-treatment (10 μM) for 10 minutes had no effect on CB hypoxic chemosensitivity compared to control hypoxic response, suggesting that hypoxic CSN response is not PKA sensitive. In the presence of H-89, PACAP-38 continued to evoke the characteristic biphasic CSN response. The peak and steady-state CSN responses of
PACAP-38 in the presence of H-89 were significantly different from each other ($P=0.04; n=5$) (Table 1). Although PKA inhibition had no significant inhibitory effect on the PACAP-38 peak response, it marginally reduced the amplitude of the plateau ($1.95\pm 0.20$ vs $1.56 \pm 0.14; P=0.034$) (Fig 10). Since PKA activation can be induced by cAMP production, we tested the possibility that the effects of PACAP-38 are mimicked and/or amplified by the cAMP activator forskolin. Forskolin (10 μM) alone had no effect on baseline CSN activity or the peak response to PACAP-38. However, the plateau phase of the PACAP-38 response was significantly higher than the control ($1.95 \pm 0.20$ vs $2.41 \pm 0.10; P = 0.01, n=5$) (Fig 3C & 3D). These data demonstrate that PACAP-38 induced excitation involves PKA to some degree, partially confirming Xu et al.'s study in isolated glomus cells [63]. However, the fact that forskolin does not induce a large PACAP-like transient and most of the response to PACAP remains in the presence of a PKA inhibitor suggests other pathways are more important.

**Effect of TASK channel blocker anandamide (ANA) on PACAP-38 induced CSN activity.**

In their model, Xu et al. [63] proposed that PACAP-38 induced increases in glomus cell $[Ca^{2+}]$, via depolarization caused solely by inhibition of TASK1-like $K^+$ channels. To test this aspect of Xu et al.'s model in an integrative way, we pre-treated the carotid body for 10 minutes with the TASK-1 channel blocker ANA (5 μM). In accordance with our previous study [47], ANA elevated baseline normoxic CSN activity but did not increase the hypoxic response (Fig 4A & 4B). Subsequent challenge with PACAP-38 in the presence of ANA further increased CSN activity, which remained elevated throughout the exposure time. The typical biphasic PACAP-38 CSN response pattern that normally includes a well-defined peak was not evident in the presence of ANA; the average peak and plateau responses were not significantly different ($P = 0.051; n=3$) (Table 1). Compared to PACAP-38 response alone, ANA significantly suppressed the peak response (PACAP-38: $3.30 \pm 0.51$vs PACAP-38 + ANA: $2.20 \pm 0.16$; $P<0.001$), however, ANA had no effect on the plateau (PACAP-38: $1.95 \pm 0.20$vs PACAP-38 + ANA: $1.93 \pm 0.06; P=0.83$) (Fig 10). Considering that ANA increases CSN activity through TRPV1 activation [47] and did not block the response to either hypoxic or PACAP-38, it is unlikely that the TASK-1 background $K^+$ current plays a major role in PACAP-38 mediated signaling in the intact carotid body. The above observation further indicates that other pathways mediate the stimulatory effect of PACAP-38 on CSN activity.
Effect of PLC blocker U73122 on PACAP-38 induced CSN activity.

Previous studies have shown that PACAP-evoked intracellular Ca^{2+} increase in PC-12 cells [35] and catecholamine secretion in adrenal chromaffin cells [23] involve the upstream regulatory enzyme PLC. We considered the prospect of a PLC-dependent pathway being responsible for PACAP-38 induced CSN excitation. Pretreatment with the PLC blocker U73122 (10 μM) alone appeared to reduce hypoxic CSN activity, however this apparent reduction was not significant compared to control (Hx = 1.55 ± 0.14 vs Hx+U73122 = 1.39 ± 0.12; n=5; \( P=0.20 \)) (Fig 5A & 5B). With regards to the response to PACAP-38, U73122 diminished overall response magnitude but the biphasic nature remained: the average peak and plateau responses were significantly different from one another (\( P=0.032; n=5 \)) (Table 1). Compared to PACAP-38 alone, U73122 significantly reduced the peak response to PACAP-38 (PACAP-38 peak = 3.30 ± 0.51 vs U73122 + PACAP-38 peak = 1.43 ± 0.14, \( P<0.001 \)) and the plateau was almost abolished (PACAP-38 plateau = 1.95± 0.20 vs U73122 + PACAP-38 plateau = 1.07 ± 0.07, \( P=0.006 \)) (Fig 10). Thus, acute stimulation with PACAP-38 elicits a CSN response mediated, in large part, through a PLC-dependent pathway.

Effect of PKC blockers, chelerythrine chloride (CC) and GF 109203X on PACAP-38 induced CSN activity.

PLC activation results in generation of two second messenger molecules, IP3 to release Ca^{2+} from intracellular ER stores and DAG to activate PKC (Fig 1). Because inhibition of PLC attenuated markedly the PACAP-38 induced CSN response, we proposed that PLC dependent PKC activation might be important for PACAP-induced CSN excitation. We tested the potential involvement of PKC using two inhibitors chelerythrine chloride (CC) and GF 109203X. In Fig 6A the hypoxic CSN response appears partially blunted in the presence of CC (10 μM), but this apparent effect was not significant compared to control (Hx= 1.82 ± 0.2 vs Hx+CC = 1.53 ± 0.13; n=5, \( P = 0.27 \)) (Fig 6B). CC significantly reduced both peak and plateau responses caused by PACAP-38 (PACAP-38 peak: 3.30± 0.5 vs CC+PACAP-38 peak : 1.40 ± 0.11; \( P < 0.001 \) and PACAP-38 steady-state: 1.95 ± 0.2 vs CC+PACAP-38 steady state: 1.09 ± 0.05; \( P=0.007 \))(Fig 10) with the plateau response barely above baseline activity. Consequently, despite being heavily attenuated, the peak response remained significantly higher than the plateau (\( P =
The above observation was corroborated using another PKC blocker, GF109203X (10 μM). The result was similar to CC (Table 1; Fig 6C & 6D): GF109203X significantly blunted the peak ($P<0.001$; n=6) and plateau ($P=0.001$; n=6) responses to PACAP-38 (Fig 10). Thus, these data suggest the excitatory effect of PACAP-38 is primarily mediated through PKC activation.

**Effect of Epac inhibitor brefeldin A on PACAP-38 induced CSN activity**

Studies indicate that PACAP-38 signaling can be mediated through activation of exchange protein activated by cAMP (Epac) pathway through the elevation in cAMP [52], triggering PLC and PKC for catecholamine release from adrenal chromaffin cells [23]. To test whether PACAP-38 effects are Epac mediated, we used Epac inhibitor brefeldin A (100 μM). Brefeldin A had no significant effect on the mean hypoxic CSN response (1.65 ± 0.10 vs 1.52 ± 0.12; $P = 0.26$, n=5). However, the peak and plateau responses to PACAP-38 were significantly reduced (PACAP-38 peak = 3.30 ± 0.51 vs Brefeldin A + PACAP-38 peak = 2.20 ± 0.17, $P=0.001$; PACAP-38 plateau = 1.95 ± 0.20 vs Brefeldin A + PACAP-38 plateau = 1.32 ± 0.10, $P=0.01$) (Fig 7A & 7B). These findings suggest PACAP-38 signaling in the carotid body is mediated in part by the Epac pathway.

**Effect of PLD blocker butanol on PACAP-38 induced CSN activity.**

PLD has also been implicated in PACAP-signaling, through activation of PKC and/or via direct upstream activation of the small G-protein ARF [30]. In the present study, butanol (0.5%) and CAY10594 were used to block PLD. Fig 8A & 8B shows that butanol had a mild, brisk and short-lived stimulatory effect, but CAY10594 (10 μM) did not (Fig 8D). Neither drug affected the hypoxic response. With regards to the PACAP response, both butanol and CAY10594 appear to slow the decay from the peak response to the plateau. Statistically, the average peak and plateau responses were not significantly different (Butanol: $P=0.01$; n=6, CAY10594: $P=0.12$; n=4) (Table 1). Compared to PACAP-38 alone, butanol significantly suppressed the peak response only (PACAP-38 peak: 3.30± 0.5 vs butanol + PACAP-38 peak : 2.12 ± 0.20; $P =0.008$)(Fig 10). CAY10594, being a more selective PLD blocker, significantly suppressed both peak and steady state PACAP-38 responses (PACAP-38 peak: 3.30 ± 0.51 vs CAY10594+PACAP-38 peak: 1.47 ± 0.15, $P <0.001$; PACAP-38 plateau: 1.95 ± 0.20 vs
CAY10594 + PACAP-38 plateau: 1.33 ± 0.10, \( P =0.012 \) (Fig 10). Thus, these data suggest PACAP-38 induced CSN excitation is partially mediated through PLD activation.

**Effect of Kv channel blocker 4-AP on PACAP-38 induced CSN activity.**

PACAP-induced excitability of rat sacral pre-ganglionic neurons is attributed to inhibition of the 4-AP-sensitive fast inactivating potassium conductance \( (I_A) \) [33]. Since, carotid body glomus cells express a 4-AP sensitive A-type K\(^+\) current \([11,59]\), we used 4-AP to appraise the role of this channel in PACAP-38-induced carotid body activity. Pretreatment with 4-AP (5 mM) produced oscillations in CSN activity: a rapid transient increase, followed by a rapid decay, a second rapid transient increase, followed by a slower decay with activity remaining above the basal CSN activity (Fig. 9A & 9B). During the second decay phase, the carotid body retained its sensitivity to hypoxia but the response to PACAP-38 was severely blunted (PACAP-38 peak: 3.30 ± 0.5 vs 4-AP+PACAP-38 peak: 1.31 ± 0.11, \( P<0.001 \); and PACAP-38 plateau: 1.95 ± 0.20 vs 4-AP + PACAP-38 plateau: 1.21 ± 0.08, \( P=0.017 \)) (Fig 10). 4-AP abolished the difference between peak and steady-state responses to PACAP-38 \( (P = 0.52; n=5 \) (Table 1). Thus, 4-AP effectively and significantly attenuated the response to PACAP-38, suggesting that inhibition of a fast inactivating K\(^+\) conductance could be one of the potential mechanisms by which PACAP-38 increases CSN activity.

**Effect of TRPC/Ca\(^{2+}\) channel blocker SKF96365 on PACAP-38 induced CSN activity.**

Previous reports in PC-12 cells suggest PACAP-induced catecholamine secretion involves a store-operated Ca\(^{2+}\) channel (SOCC) / transient receptor potential C (TRPC) channel that facilitates capacitive Ca\(^{2+}\) entry [35]. Since rat carotid body is endowed with various TRPC channels [5] we probed a possible contribution of SOCC/TRPC to the PACAP-induced increase in CSN activity. We pretreated the carotid body with imidazole-derivative SKF 96365, a SOCC/TRPC blocker that also blocks T-type calcium channels [32,51], before PACAP-38 treatment. SKF96365 transiently stimulated CSN activity (Fig 9C & 9D) and had no effect on hypoxic response (Fig 9C). However, in the presence of SKF96365, after the transient activity subsided, PACAP-38 caused only mild stimulation (Fig 9D). Thus, SKF96365 significantly suppressed the stimulatory effect of PACAP-38 (PACAP-38 peak: 3.3 ± 0.5 vs SKF96365 + PACAP-38 peak: 1.35 ± 0.14, \( P <0.001 \), n=5; PACAP-38 steady-state: 1.95 ± 0.20 vs
SKF96365 + PACAP-38 steady-state: 1.12 ± 0.04, \( P < 0.001 \) (Fig 10) and there was no significant difference between peak and steady-state responses \( (P = 0.26; n=5) \) (Table 1). This suggests that PACAP-mediated PLC activation may trigger capacitative Ca\(^{2+}\) influx through TRPC channels, or calcium entry through T-type calcium channels, leading to enhanced neurotransmitter release and a robust CSN response.
DISCUSSION

This study identifies cellular mechanisms through which stress peptide PACAP-38 increases rat carotid sinus nerve activity in an arterially-perfused ex vivo rat carotid body preparation. Our data indicate that PLC-PKC dependent pathways dominate both peak and plateau phases of the response to PACAP, though we also found evidence for a PKA-mediated mechanism contributing to the plateau phase. Once triggered, these pathways appear to lead to activation of TRPC and/or T-type Ca\(^{2+}\) channels and inhibition of the fast inactivating K\(^{+}\) current (I\(_{A}\)). These results contrast to a previous study on the effects of PACAP on isolated glomus cells, which proposed the major mechanism by which PACAP stimulates the carotid body involves PKA-mediated inhibition of TASK-like K\(^{+}\) channels.

Second messengers mediating the effects of PACAP

PACAP has three principle receptors, PAC1, VPAC1 and VPAC2. PAC1 is specific to PACAP, whereas VPAC1 and VPAC2 bind PACAP and VIP with equal affinity [60]. In isolated glomus cells [63] and in our preparation, only PACAP causes stimulation (VIP data not shown). Therefore, we assume that PACAP’s excitatory effects on carotid body afferents are mediated entirely by PAC1 activation. PAC1 is commonly coupled with Gs, and thereby capable of activating the canonical adenylate cyclase-cAMP-PKA pathway [14]. However, like most other G-protein coupled receptor, PAC1 signaling is likely promiscuous. In the hippocampal, superior cervical ganglia and adrenal medulla for example, PAC1 activation also triggers PLC suggesting the receptor is coupled to Gq [23,28,60,64] and/or Gs activation triggers the non-canonical Epac (exchange protein activated by cAMP) pathway [52]. PAC1 receptors are also reported to couple to small G-protein ARF (ADP- ribosylation factor) leading to PLD activation [30].

Congruent with PACAP’s ability to activate PLC in other systems, our data suggest PLC is the major (but not only) pathway by which PACAP stimulates the carotid body. Specifically, we show that blocking PLC activity with U73122 suppressed the peak and plateau response to PACAP-38 by ~ 81% and ~ 93%, respectively. U73122 is a widely used PLC inhibitor, but data from experiments using reconstituted PAC1hop expressing-PC12 cells suggest U73122 may have additional non-specific effects [35]. To mitigate this concern, and to better understand how
PLC activation leads to increased neuronal activity, we investigated the two prominent signaling pathways downstream of PLC: (a) upregulation of DAG leading to activation of PKC, and (b) IP₃ production causing IP₃-R activation and TRPC channel activation [1, 3]. Blocking TRPC channels with SKF96365 significantly inhibited both phases of the PACAP response. Blocking PKC activity with CC and GF, suppressed PACAP-38 peak response by ~83% and ~66% respectively, and blocked the plateau response by ~90% and ~95% respectively. In addition, we note that blocking PLC with U73122 reduced the hypoxic response, an effect mimicked by PKC blockers CC and GF. These results are consistent with previous studies by Summers et al. [54] who used different PKC blockers to show the importance of the PLC-PKC pathway in mediating hypoxia-induced calcium influx in isolated glomus cells. Together, these data support an effect of U73122 on PLC activity and thus the importance of a PLC-dependent pathway in mediating the carotid sinus nerve response to PACAP-38.

We have yet to resolve exactly how PACAP binding to PAC1 leads to PLC production. We know that PACAP activates Gs because the PKA inhibitor H-89 causes a reduction in the plateau phase. Thus, it seems highly likely that PACAP activates soluble adenylate cyclase within the carotid body leading to cAMP production. While effects through the canonical PKA pathway were moderate and limited to the plateau phase, an increase in cAMP production has the ability to activate the Epac pathway that, in turn, triggers PLC. Consistent with this line of reasoning, we found that Epac blocker Brefelin A significantly reduced both peak and plateau phases. These data map well to recent studies in other organs that have homed in on Epac as an important, PKA independent, pathways for PACAP-38 signaling [23, 52] they also re-enforce findings in the carotid body that suggest that most cAMP effects are Epac mediated [45].

PAC1 receptors are also reported to couple to small G-protein ARF (ADP-ribosylation factor) leading to PLD activation [30]. PLD is also reported to be activated by the PLC-PKC pathway. Our data support a role for PLD in the peak carotid sinus nerve response to PACAP because PLD inhibitors CAY10594 and butanol significantly reduced the magnitude of peak phases. The involvement of PLD in the plateau phase is less clear: CAY10594 reduced the magnitude of the plateau phase by 38%, whereas butanol had no effect on the plateau. To the best of our knowledge, these are the first data suggesting carotid body activity is modulated by
PLD, likely warranting detailed future investigation. In addition to PLC, PKC and PLD pathways, our data also suggest a role for PKA in sustaining the plateau response (discussed below).

**Comparison to previous study of PACAP on the carotid body**

PACAP was purported to stimulate the carotid body entirely via a PKA-TASK1 dependent mechanisms. Thus, Xu et al. [63] used the small molecule PKA inhibitor H89 and lipid soluble ANA to argue that PACAP’s effects were mediated by a PKA - TASK dependent pathway. However, in our preparation, we found that high concentrations of H89 in the range that might inhibit other kinases [26] caused significant reduction in the plateau response (~41%) but had no statistical effect on the peak response.

We also tested the effects of ANA, used by Xu et al [63] to block TASK-like K+ channels. In contrast, to the effect of H89, ANA had a moderate effect on the peak response but no statistical effect on plateau activity. The qualitative difference in effect of H89 and ANA on the PACAP response in our preparation suggests that in the intact carotid body these two reagents affect the PACAP response through different mechanisms. Thus, if PKA is involved in sustaining plateau activity, then TASK channels are not, and visa-versa: if TASK channels are involved in the peak response, PKA is not. Hence, we found no evidence to suggest PKA-mediated modulation of TASK channels was involved in either phase of the response.

As ANA is a TASK channel blocker and abolishes the peak response to PACAP, it is conceivable that the peak response is the product of PACAP inhibiting TASK channels -- and thereby exciting the carotid body -- by a mechanism that does not involve PKA. However, we note that ANA is a TASK1 blocker, a TRPV1 agonist and a CB1 receptor agonist. In a previous study we showed that the excitatory effect of ANA on carotid body afferent activity (e.g., see increase in baseline activity in **Fig 4A**) was mediated entirely by TRPV1 receptors on petrosal neurons; we found no evidence for involvement of TASK-like channels in ANA-mediated excitation [47]. This observation makes it difficult to reconcile a role for TASK-like channels in the response to PACAP based solely on the effects of ANA. In our previous study [47], we also found no effect of the CB1 receptor agonist oleamide on baseline or moderate hypoxia-induced
carotid sinus nerve activity. However, the excitatory effect of ANA was greatly enhanced in CB1 knockout mice. Therefore, we speculate that CB1 receptors may limit maximum excitation of the carotid body and thus, CB1 receptors when activated by ANA, may blunt the peak PACAP response. Additional studies are required to validate this explanation and to confirm or eliminate a role for TASK.

While Xu et al. [63] used isolated glomus cells to study the effects of PACAP on the carotid body, we employed an *ex vivo* arterially perfused rat carotid body preparation in which we could monitor the effects of PACAP on carotid sinus nerve activity. Our preparation has many key physiological advantages over more reduced preparations. These include preserving (a) the spatial and functional relationship between glomus and type II cells; (b) bi-directional synapse between glomus cells and sensory afferents; (c) vascular perfusion, allowing tissue to be maintained under normoxic, normocapnic and normothermic conditions; and (d) use of afferent activity, not changes in cellular calcium, as the output measure. However a possible disadvantage of our preparation in comparison to using isolated glomus cells, is reduced accessibility of glomus cells to pharmacological agents. This is an important caveat in interpreting our results because, if H89 did not gain access to glomus cells in our preparation, we might be underestimating the importance of the PKA pathway. In considering this caveat, we note that while H89 had no effect on the peak response in our preparation, it did have a statistical effect on the plateau. Also, we used a relatively high concentration of this small molecule inhibitor that would be expected to have effects deep within tissue (10 µM; IC50: 135 nM) [26]. Finally, blockade of the PLC pathway with U73122 greatly diminished both peak and plateau responses: even if our results underestimate the involvement of the PKA pathway, clearly other pathways are also important.

Our findings, suggesting involvement of multiple intracellular signaling pathways in mediating PACAP’s excitatory effects on CSN activity, may reflect the fact that our preparation involves three cell types (glomus cells, glial-like sustenticular cells and nerve endings). While PACAP is known to stimulate glomus cells directly [63], PACAP may also modulate the post-synaptic terminals of petrosal neurons that transmit chemoreceptor information to the brainstem. Thus, our data suggesting involvement of multiple second messenger pathways may simply
reflect cell-type specific differences in PACAP signaling. Alternatively, PACAP may activate multiple signaling cascade within glomus cells, akin to the effects of PACAP on neuroepithelial cells were regulation of cytosolic Ca^{2+} during early neuronal development proceeds through both a cAMP/PKA and PLC/PKC-dependent pathways [67].

**Possible ion channels involved in PACAP-mediated increase in carotid body afferent activity**

If a PKA-TASK channel pathway is not the main mechanisms by which PACAP excites chemosensory activity, what is? Our data demonstrates that the excitation caused by PACAP is severely diminished by the classic A-type voltage-gated K^{+} channel blocker 4-AP [21]. This observation is reminiscent of PACAP’s effects on lumbosacral preganglionic neurons, where PACAP quells a fast-inactivating K^{+} conductance (I_A) that is also suppressed by 4-AP [33]. A role for I_A in the carotid body’s response to PACAP is also supported by several studies demonstrating the presence of a 4-AP sensitive voltage-gated K^{+} current in glomus cells [11,15,39,59].

The role I_A plays in regulating glomus cell membrane potential, catecholamine release and/or afferent activity resulting from hypoxic stimulation is not entirely clear. A few studies have suggested 4-AP directly suppresses a PO_2-sensitive K^{+} current, implicating I_A in oxygen sensing [11, 27, 59]. However, using a superfused rat carotid body, Donnelly [15] showed no increase baseline CSN activity with 1 mM 4-AP (although 4-AP did suppress an outward K^{+} current in glomus cell). Using a perfused cat carotid body preparation, Roy et al. also demonstrated that 1 mM 4-AP failed to stimulate CSN activity or catecholamine release, but reduced the CSN activity and catecholamine release with hypoxia [48]. Using the arterially-perfused rat carotid body preparation, here we found that 5mM 4-AP increases normoxic carotid sinus nerve activity for tens of seconds. Interestingly, the increase in activity included an initial rapid transient (of similar magnitude to that produced by a moderate hypoxic challenge) followed immediately by a slower-rising transient, which decayed gradually towards normoxic levels. A second hypoxic challenge during this decay phase netted a robust response but the delta was slightly smaller than the initial challenge. Thus, while the 4-AP blocked current is not essential for the hypoxic response, some aspect of the mechanism mediating the hypoxia
response is compromised. These divergent results with 4-AP likely warrant further studies to elucidate the precise role of the 4-AP sensitive current in oxygen sensing.

We did not investigate how PACAP-38 modulates A-type voltage-gated K⁺ channels in the ex vivo carotid body preparation, but in other systems, K⁺ channels are often demonstrated to be phosphorylated by PKC [9, 66]. In olfactory neurons for example, PACAP reduces a rapidly inactivating K⁺-current (A type) through a mechanism that can be blocked by inhibiting the PLC pathway, but not the PKA pathway [18]. Interestingly, 4-AP reduced the PACAP-38 peak response substantially (~87%) with a sizable, but lesser effect on the plateau phase (~78%), such that the two phases could not be statistically distinguished. These data suggest that voltage-gated potassium currents may be particularly important for initiating the PACAP response.

TRPC and/or T-type Ca²⁺ channels may mediate PACAP-38 induced CSN activity.

We showed that PLC, and therefore IP₃ production, was likely involved in mediating the response to PACAP. There is compelling evidence that activation of IP₃ receptors regulates calcium-permeable non-selective cation channels, i.e., through direct interaction with TRPC channels, causing Ca²⁺ influx [3, 29]. One member of the TRPC family, TRPC1, has also been identified as the channel involved in ‘capacitive’ Ca²⁺ entry whereby IP₃ triggers calcium release from internal stores which, in turn, causes store depletion and triggers TRPC1 activation resulting in calcium entry (i.e., store operated calcium entry, SOCE) [3]. We demonstrated that the best-of-class TRPC channel antagonist SKF96365, suppressed the peak response of PACAP-38 by ~87% and reduced the plateau response by ~79%, suggesting the CSN response to PACAP-38 may involve TRPC.

In evaluating these results one must consider how specific SKF96365 is to TRPC channels. In a cell culture system, SKF96365 blocks capacitive Ca²⁺ entry, which is likely mediated by TRPC. However, some data suggest that this compound also blocks non-capacitive calcium entry by acting directly on low voltage activated T-type calcium channel in the membrane [51]. In addition, a recent study suggests SKF96365 may function as a non-competitive GABAₐ antagonist [44]. While the carotid body expresses GABAₐ receptors, the role in petrosal neurons is one of shunting inhibition [65]: assuming endogenous release of GABA, antagonism should stimulate carotid sinus nerve activity. The initial transient increase in
carotid sinus nerve activity caused by SKF9365 may be the result of this disinhibition. However, the suppressive effect of SKF96365 on the PACAP response cannot be explained by disinhibition and is therefore likely caused by SKF96365 antagonism of TRPC and/or T-type calcium channels. Given the infancy of TRPC and T-type calcium channel pharmacology and our reliance on SKF96365, caution is required in interpreting our results as strict evidence for activation of anyone species of ion channel.

Both TRP and T-type Ca$^{2+}$ channels are implicated in PACAP signaling elsewhere. In sympathetic post-ganglionic neurons of the superior cervical ganglia, PACAP causes cytosolic calcium elevation. As suggested by the data herein for carotid sinus nerve activity, the signaling cascade in the superior cervical ganglia appears to involve PLC activation and IP$_3$ production that in turn opens TRP channels in the plasma membrane [3]. Contrasting with the effects on carotid sinus nerve activity, PACAP appears to act in the superior cervical ganglia through a signaling cascade independent of PKA and PKC. In PC-12 cells, PACAP signaling also includes IP$_3$ production and TRP channel activation, but in this case the species implicated (i.e., TRPC1) is activated by depletion of calcium from the endoplasmic reticulum [35]. Recently, TRPC channels have been suggested to underlie the excitatory effects of PACAP on cardiac neurons; excitation that was suppressed by SKF96365 [31]. Further, corroborating an important role for TRPC channels in regulating carotid sinus nerve responses to PACAP, multiple TRPC channel proteins (TRPC1/3/4/5/6/7) are strongly expressed on glomus cells and/or the petrosal afferent nerve terminals that innervate them [5]. T-type Ca$^{2+}$ channels are also expressed in the carotid body [6] (though see Overholt and Prabhakar, 1997 [37]) and these too are implicated in PACAP signaling. In neurosecretory chromaffin cells of the adrenal medulla for example, PACAP causes catecholamine release by triggering calcium entry through T-type channels via a PKC dependent mechanism [19]. Only with the advent of new pharmacological tools will the relative importance of non-specific cation (TRPC) and T-type Ca$^{2+}$ channels in the response of the carotid sinus nerve to PACAP be determined.

Conclusion
In conclusion, we show that the response of the carotid sinus nerve to PACAP is concentration-dependent and at higher concentrations is biphasic. Multiple second messenger systems contribute to this response. PLC and PKC-dependent pathways dominate the response, with additional PKA and PLD components. PACAP’s effects require 4-AP and SKF96365-sensitive mechanisms, suggesting second messenger pathways triggered by PACAP converge on Kv and non-specific cation (TRPC)/T-type Ca$^{2+}$ channels. As the effects of PACAP were only partially suppressed by the TASK channel blocker ANA, activation of TASK channels appear to play only a minor role. These results contrast to previous findings suggesting PACAP stimulates the glomus cells of the carotid body mainly through a PKA-TASK mediated mechanism.

**Perspectives and Significance**

PACAP is associated with every relay in the stress pathway, including the carotid bodies which send afferents baring information about metabolic state to respiratory and sympathetic centers in the CNS. As PACAP is present in the superior cervical ganglia which projects to the carotid body and increases activity of carotid body afferents, it likely helps adjust the autonomic and respiratory system to stress. Previously the effects of PACAP on the carotid body were thought to be mediated by PKA and TASK channels. We show here that PACAP acts on additional pathways, most noticeably those that are PLC and PKC-dependent and involve 4-AP and SKF96365 sensitive mechanisms. Thus, the modulation of the carotid body by PACAP is much richer than we previously thought, demonstrating the carotid body has a plethora of targets for pharmaceuticals aimed at modulating autonomic and respiratory stress responses.
ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1: Possible PACAP-38 induced intracellular signaling pathways in the carotid body and drugs used herein for their pharmacological interrogation. A. PACAP-38 can induce activation of PLC resulting in IP$_3$-R and PKC activation. Activation of IP$_3$ may directly/indirectly gate TRPC channels. PKC activation can inhibit the A-type Kv channels. B. PACAP-38 can activate PKA, via cAMP. PKA may then inhibit a TASK-1 like K$^+$ channel. PACAP-38 can also activate PKC, via Epac. C. PACAP-38 can stimulated PLD activity via ARF and PKC sensitive pathways. Abbreviations: PAC1-R, PACAP selective PAC1 receptor; PLC, phospholipase C; IP$_3$R, IP$_3$ receptor; ER, endoplasmic reticulum; DAG, diacylglycerol; PKC, protein kinase C; Kv, voltage-gated K$^+$ channel; VGCC, voltage gated calcium channel; AC, adenylatecyclase; PKA, protein kinase A; TASK, TASK-1 like K$^+$ channel; Epac, exchange protein activated by cAMP; ARF, ADP- ribosylation factor; PC, Phosphatidylcholine; PA, Phosphatidic acid; CC, cherelytrine chloride; 4-AP, 4-aminopyridine; ANA, anandamide; Gs and Gq, G-proteins. Modified from Beaudet MM et al., 2000 [2]; Dickson L & Finlayson 2009 [14] and based on signalling pathways downstream of PAC1 deduced in other systems [2, 13, 23, 31, 49, 58].

Figure 2: PACAP-38 leads to an acute enhancement of CSN activity in the ex vivo artificially perfused rat carotid body preparation. A. Examples of integrated CSN responses to four different concentrations of PACAP-38 perfused through the common carotid artery (25, 50, 100 & 200 nM). Each example is from a separate preparation. In each experiment, after a 5-minute baseline recording at normoxia (PO$_2$ = 100 Torr; PCO$_2$ = 40 Torr), the CB was challenged with hypoxia (Hx) (PO$_2$ = 60 Torr; PCO$_2$ = 40 Torr) for 4 minute and then returned to normoxic conditions for 5-minutes to allow activity to return to baseline levels before exposure to PACAP-38. Perfusing with 100 nM PACAP-38 for 30 minutes caused a vigorous increase in CSN activity, reaching a peak within 3 min, then decaying to a plateau that persisted after washout. A higher concentration of PACAP-38 (200 nM) caused rapid onset with no additional increase in CSN activity compared to 100 nM. Preparations perfused with lower concentrations of PACAP-38 had attenuated responses. B. Summary graph of mean data from multiple preparations (see key for details). C. Dose response for peak and steady state phases. Note peak response was taken as the average activity of the 1 minute time bin with the highest average
activity; steady state response was measured over the last minute of PACAP exposure, just prior to washout. D. In presence of PACAP-38, hypoxia could induce additional increase in integrated CSN activity. * & ** indicate significantly higher CSN responses than 25 nM and 50 nM of PACAP-38 respectively.

**Figure 3: PACAP-38 induced stimulation of CSN was only partially reduced by PKA inhibitor and activated by forskolin.** A. Example of integrated CSN activity showing response to PACAP-38 in presence of the PKA blocker, H-89 (10 μM). H-89 had no effect on the hypoxic response (Hx) compared to control and failed to abolish the typical PACAP-38 (100 nM) response. The only effect of H-89 on the PACAP-38 response was a small reduction in the magnitude of the steady state phase. B. Summary graph showing mean data from 5 preparations treated with H-89. C. Integrated CSN activity showing mild increase in CSN activity with cAMP activator forskolin with no effect on the hypoxic response. The steady state PACAP-38 response was amplified by forskolin. D. Summary data of forskolin from 5 preparations.

**Figure 4: PACAP-38 induced stimulation of CSN was partially reduced by TASK-1 like K+ channel blocker.**

A. Putative TASK-1 blocker, ANA (5 μM) augmented CSN activity during normoxia, but had no effect on the hypoxic response. ANA practically abolished the peak phase of the CSN response to PACAP-38 but the steady state phase of the response persisted. B. Summary graph showing mean data from 3 preparations. Note, ANA is also a TRPV1 and CB1 agonist [47].

**Figure 5: PACAP-38 induced CSN stimulation involves activation of PLC.** A. Example of integrated CSN responses to PACAP in the presence of PLC blocker, U73122 (10 μM). U73122 partially decreased hypoxic responses (Hx) prior to PACAP exposure. However, in the presence of U73122, the peak response to PACAP-38 was greatly reduced and the plateau phase was practically abolished. B. Summary graph showing mean data from 5 preparations.

**Figure 6: PACAP-38 induced stimulation of CSN is highly dependent on PKC.** A. Example of integrated CSN responses in presence of PKC blocker, CC (10 μM). CC partially blunted the hypoxic response (Hx) and massively attenuated the response to PACAP-38: both the peak and steady state phases of the response to PACAP-38 were greatly reduced. B. Summary graph
showing mean data from 5 preparations treated with CC. C. Another PKC blocker GF 109203X (10μM) had similar effects to CC. D. Summary graph showing mean data from 6 preparations treated with GF 109203X.

**Figure 7: PACAP-38 induced stimulation of CSN involves the Epac pathway.**
A. Example of integrated CSN response in presence of Epac inhibitor, brefeldin A (100 μM), which marginally reduced the hypoxic response and also suppressed the peak as well as the steady state PACAP-38 responses. B. Summary graph showing the mean data from 5 preparations treated with brefeldin A.

**Figure 8: PACAP-38 induced stimulation of CSN also involves PLD.**
A. Example of integrated CSN response to PACAP in the presence of PLD blocker, butanol. Butanol (0.5%) caused a mild transient increase in normoxic CSN activity with no effect on hypoxic CSN response. B. In another separate experiment, in the presence of butanol, the response to PACAP was reduced with the peak phase most affected. C. Summary graph showing mean data from 6 preparations treated with butanol. D. A specific PLD blocker, CAY10594 (10 μM), caused no change in normoxic and hypoxic CSN responses, but reduced both peak and steady state PACAP-38 responses. E. Summary graph showing mean data from 4 preparations treated with CAY10594.

**Figure 9: PACAP-38 induced stimulation of CSN requires A-type K⁺ channels and TRPC / T-type calcium channel.** A. Example of integrated CSN response to PACAP-38 in the presence of the A-type K⁺ channel blocker, 4-AP (5 mM). Perfusion with 4-AP caused a rapid transient increase in CSN activity followed by a second transient that had a slower decay phase. The magnitude of the hypoxic response (delta) was slightly reduced during the decay phase of the second transient. In the presence of 4-AP, the response to PACAP was tepid: the peak phase was completely abolished and only a small plateau response remained. B. Summary graph showing mean data from 5 preparations treated with 4-AP. C. Example of integrated CSN responses in presence of TRPC channel / T-type Ca²⁺ channel blocker, SKF 96365 (50 μM). Perfusion with SKF 96365 produced a transient increase in CSN activity that rapidly decayed back to baseline, followed by a hypoxic response that remained unaltered. D. In the presence of SKF96365, PACAP-38 had only a small effect: peak and steady state phases of the response were evident.
but both were greatly attenuated. **D.** Summary graph showing mean data from 5 preparations treated with SKF 96365.

**Figure 10: Comparative summary of CSN responses to PACAP-38 in the presence of different drugs.** Peak responses (*filled circles*) were taken as the average activity of 60 s time bins having maximum average activity; steady state responses (*open circles*) were taken as the average activity of the 60s time bin at the end of PACAP exposure, just prior to washout. Peak responses in the presence of a drug that differs significantly from the peak response with PACAP-38 (100 nM) alone (left column) are indicated by ‘*’. Similarly, ‘Ψ’ indicate significant differences in steady state responses.
Table 1: Effects of PACAP-38 and different drugs on PACAP-38 induced CSN responses (Mean ± SEM).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Peak response</th>
<th>Steady state response</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PACAP-38 (200 nM)</td>
<td>3.36 ± 0.28*</td>
<td>2.02 ± 0.17</td>
<td>0.002 (n=3)</td>
</tr>
<tr>
<td>PACAP-38 (100 nM)</td>
<td>3.30 ± 0.5*</td>
<td>1.95 ± 0.2</td>
<td>&lt; 0.001 (n=8)</td>
</tr>
<tr>
<td>PACAP-38 (50 nM)</td>
<td>1.79 ± 0.2*</td>
<td>1.28 ± 0.1</td>
<td>0.043 (n=3)</td>
</tr>
<tr>
<td>PACAP-38 (25 nM)</td>
<td>1.20 ± 0.06</td>
<td>1.12 ± 0.05</td>
<td>0.31 (n=3)</td>
</tr>
<tr>
<td>H-89 (10 µM) + PACAP-38 (100 nM)</td>
<td>3.03 ± 0.4*</td>
<td>1.56 ± 0.14</td>
<td>0.04 (n=5)</td>
</tr>
<tr>
<td>forskolin (10 µM) + PACAP-38 (100 nM)</td>
<td>3.17 ± 0.2*</td>
<td>2.41 ± 0.14</td>
<td>&lt;0.001 (n=5)</td>
</tr>
<tr>
<td>ANA (5 µM) + PACAP-38 (100 nM)</td>
<td>2.20 ± 0.16</td>
<td>1.93 ± 0.06</td>
<td>0.051 (n=3)</td>
</tr>
<tr>
<td>U73122 (10 µM) + PACAP-38 (100 nM)</td>
<td>1.43 ± 0.14*</td>
<td>1.07 ± 0.07</td>
<td>0.032 (n=5)</td>
</tr>
<tr>
<td>CC (10 µM) + PACAP-38 (100 nM)</td>
<td>1.40 ± 0.11*</td>
<td>1.09 ± 0.05</td>
<td>0.041 (n=5)</td>
</tr>
<tr>
<td>GF109203X (10 µM) + PACAP-38 (100 nM)</td>
<td>1.79 ± 0.26*</td>
<td>1.05 ± 0.05</td>
<td>0.008 (n=6)</td>
</tr>
<tr>
<td>Brefeldin A (100 µM) + PACAP-38 (100 nM)</td>
<td>2.20 ± 0.17*</td>
<td>1.32 ± 0.11</td>
<td>&lt;0.001 (n=5)</td>
</tr>
<tr>
<td>Butanol (0.5%) + PACAP-38 (100 nM)</td>
<td>2.12 ± 0.20</td>
<td>1.72 ± 0.20</td>
<td>0.01 (n=6)</td>
</tr>
<tr>
<td>CAY10594 (10 µM) + PACAP-38 (100 nM)</td>
<td>1.47 ± 0.15</td>
<td>1.33 ± 0.10</td>
<td>0.12 (n=4)</td>
</tr>
<tr>
<td>4-AP (5mM) + PACAP-38 (100 nM)</td>
<td>1.31 ± 0.11</td>
<td>1.21 ± 0.08</td>
<td>0.52 (n=5)</td>
</tr>
<tr>
<td>SKF96365 (50 µM) + PACAP-38 (100 nM)</td>
<td>1.35 ± 0.14</td>
<td>1.12 ± 0.04</td>
<td>0.26 (n=5)</td>
</tr>
</tbody>
</table>

* indicates significant difference (P<0.05) between peak and steady state CSN responses. Values normalized to basal CSN response.
Fig 2

A

CSN activity (normalized)

Time (min)

200 nM PACAP-38
100 nM PACAP-38
50 nM PACAP-38
25 nM PACAP-38

R-89-12
R-82-09
R-83-10

B

CSN activity (normalized)

Time (min)

200 nM-PACAP (n=3)
100nM PACAP-38 (n=8)
50nM PACAP-38 (n=3)
25nM PACAP-38 (n=3)

C

CSN activity (normalized)

PACAP-38 (nM)

Peak response
Plateau response

D

CSN activity (normalized)

Time (s)

PACAP-38 (100 nM)

Hx

R-68-11
Fig 3

A. H-89 (10 μM) and PACAP-38 (100 nM) treatment.

B. H-89 (10 μM) and PACAP-38 (100 nM) treatment.

C. Forskolin (10 μM) and PACAP-38 (100 nM) treatment.

D. Forskolin (10 μM) and PACAP-38 (100 nM) treatment.
A) ANA (5 μM) normalized CSN activity over time. Hx PACAP-38 (100 nM) treatment.

B) ANA (5 μM) normalized CSN activity over time. Hx PACAP-38 (100 nM) treatment.
Fig 5

A

Hx U73122 (10 μM)
PACAP 1-38 (100 nM)

CSN activity (normalized)

Time (min)

B

Hx U73122 (10 μM)
PACAP-38 (100 nM)

CSN activity (normalized)

Time (min)
Fig 7

A

Brefeldin A (100 μM)
PACAP-38 (100 nM)

CSN activity (normalized)

Time (min)

B

Brefeldin A (100 μM)
PACAP-38 (100 nM)

CSN activity (normalized)

Time (min)
Fig 8

A

B

C

Contd.
**Fig 8**

### D

![Graph showing CSN activity (normalized) over time](image)

- **CAY 10594 (10 μM)**
- **PACAP-38 (100 nM)**

### E

![Graph showing CSN activity (normalized) over time](image)

- **CAY 10594 (10 μM)**
- **PACAP-38 (100 nM)**
Fig 9

A

B

CSN activity (normalized)

Time (min)

0 10 20 30 40 50 60 70 80 90

0.8 1.0 1.2 1.4 1.6 1.8 2.0 2.2

4-AP (5 mM)

Hx

PACAP-38 (100 nM)

CSN activity (normalized)

Time (min)

0 10 20 30 40 50 60 70 80 90

0.8 1.0 1.2 1.4 1.6 1.8 2.0 2.2

4-AP (5 mM)

Hx

PACAP-38 (100 nM)

Contd.
Fig 9

C

D

E

CSN activity (normalized)

Time (min)

CSN activity (normalized)

Time (min)

CSN activity (normalized)

Time (min)

SKF 96365 (50 μM)
PACAP-38 (100 nM)

SKF 96365 (50 μM)
PACAP-38 (100 nM)

SKF 96365 (50 μM)
PACAP-38 (100 nM)
Fig 10

- Peak response
- Steady-state response

CSN activity (normalized)

Groups

PACAP-38
H-89 + PACAP-38
Forskolin + PACAP-38
ANA + PACAP-38
UT3122 + PACAP-38
GF + PACAP-38
Brefeldin A + PACAP-38
Butanol + PACAP-38
CA Y10594 + PACAP-38
4AP + PACAP-38
SKF 96365 + PACAP