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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PITUITARY ADENYLATE CYCLASE-activating 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 the involvement of PACAP 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 the involvement of PACAP 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 Po2, Paco2, pH, and temperature. Hypoxia, hypercapnia, low pH, and elevated temperature increase sensory discharge of the carotid bodies, triggering reflex pathways that 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 the excitatory effects of PACAP are 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 intracellular Ca2+ concentration ([Ca2+]i) rise (63). Although elegant in its simplicity, this explanation is incongruent with previous studies of

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carotid body function that consistently emphasize the PLC-PKC pathway while downplaying the adenylyte 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 receptors on petrosal afferents (47). On the one hand, if TASK 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 the stimulatory effect of PACAP is likely by a different mechanism.

As summarized in Fig. 1, PAC1 signaling in other systems can invoke multiple G proteins (G₁ and G₃), activating cAMP-PKA (34, 43), cAMP-exchange protein activated by cAMP (Epac; Refs. 23, 52), PLC-inositol 1,4,5-trisphosphate (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 pathway in acute responses (7, 10, 40, 41, 42, 53, 54, 61). In addition, the importance of TASK channels in carotid body chemosensory discharge are mediated entirely via direct activation of TRPV1 receptors on petrosal afferents (47).

![Figure 1. Possible pituitary adenylyte cyclase-activating polypeptide (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 phospholipase C (PLC) resulting in inositol 1,4,5-trisphosphate receptor (IP₃-R) and protein kinase C (PKC) activation. Activation of IP₃-R may directly/indirectly gate transient receptor potential channel (TRPC) channels. PKC activation can inhibit the A-type voltage-gated K⁺ channel (Kᵥ) channels. B: PACAP-38 can activate protein kinase A (PKA), via cAMP. PKA may then inhibit a TASK-1-like K⁺ channel. PACAP-38 can also activate PKC, via exchange protein activated by cAMP (Epac). C: PACAP-38 can stimulate PLD activity via ADP-ribosylation factor (ARF)- and PKC-sensitive pathways. PAC1-R, PACAP-selective PAC1 receptor; ER, endoplasmic reticulum; DAG, diacylglycerol; VGCC, voltage-gated calcium channel; AC, adenylylate cyclase; TASK, TASK-1-like K⁺ channel; PC, phosphatidylycholine; PA, phosphatidic acid; CC, cherythrine chloride; 4-AP, 4-aminopyridine; ANA, anandamide; G₁, and G₃, G proteins. [Modified from Beaudet et al. (2) and Dickson and Finlayson (14) and based on signaling pathways downstream of PAC1 deduced in other systems (2, 13, 23, 31, 49, 58).]
SKF96365. Our data demonstrate that the stimulatory effect of PACAP-38 on CSN activity is mediated through multiple cellular-signaling pathways (although dominated by PKC) and likely involves at least two types of ion channel.

MATERIALS AND METHODS

Animals. Experiments were conducted using forty-nine 150- to 250-g 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 (San Jose, CA). H-89, chelerythrine chloride, GFT0923X, U73122, SKF96365, and brefeldin A were purchased from Tocris Bioscience (Minneapolis, MN). ANA was purchased from EMD Biosciences. 4-AP and forskolin were obtained from Sigma. CAY10594 was purchased from Cayman Chemical.

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

Ex vivo carotid body–CSN preparation. The carotid bifurcation, including the carotid body-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-fac heating circuit, the common carotid artery was cannulated for luminal perfusion, and the tissue overlying the carotid body was carefully removed to free the CSN.

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 37 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 recirculated.

Electrophysiology. Chemosensory discharge was recorded extra-cellularly 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 before washout. The neural responses for different conditions in the protocol were normalized to the baseline (normoxic) condition. All data are expressed as means ± SE. 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 (PO2 = 100 Torr; PCO2 = 36 Torr) containing 100 nM PACAP-38 for 30 min. PACAP-38 stimulated CSN activity in a biphasic manner with an initial sharp transient rise, peaking within 3 min of PACAP-38 application, followed by a slow decay that transitions into a plateau phase (Fig. 2, A and 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 and 0.10, respectively; Fig. 2, A and C). For all remaining experiments, we used 100 nM PACAP-38 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 [Ca2+] 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 (PO2 = 60 Torr) during the plateau phase of the PACAP-38 response (100 nM). As predicted by the data of Xu et al. (63), 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. 3, A and B, H-89 pretreatment (10 μM) for 10 min had no effect on carotid body hypoxic chemosensitivity compared with control hypoxic 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 (PO2 = 60 Torr) during the plateau phase of the PACAP-38 response (100 nM). As predicted by the data of Xu et al. (63), the responses to hypoxia and PACAP-38 were approximately additive (Fig. 2D).

Effect of TASK channel blocker ANA on PACAP-38-induced CSN activity. In their model, Xu et al. (63) proposed that PACAP-38-induced increases in glomus cell [Ca2+] via depolarization...
caused solely by inhibition of TASK-1-like K⁺ channels. To test this aspect of the model of Xu et al. in an integrative way, we pretreated the carotid body for 10 min 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. 4, A and B). 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 with PACAP-38 response alone, ANA significantly suppressed the peak response (PACAP-38: 3.30 ± 0.51 vs. PACAP-38 + ANA: 2.20 ± 0.16; P < 0.001), however, ANA had no effect on the plateau (PACAP-38: 1.95 ± 0.20 vs. PACAP-38 + ANA: 1.93 ± 0.06; P = 0.83; see 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 Ca²⁺ 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 with control (Hx = 1.55 ± 0.14 vs. Hx + U73122 = 1.39 ± 0.12; n = 5; P = 0.20; Fig. 5, A and B). 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 with 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; see Fig. 10). Thus acute stimulation with PACAP-38 elicits a CSN response mediated, in large part, through a PLC-dependent pathway.

Effect of the PKC blockers CC and GF109203X on PACAP-38-induced CSN activity. PLC activation results in generation of two second messenger molecules, IP3 to release Ca2+ 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 CC and GF109203X. 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 with 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; see Fig. 10).
Table 1. Effects of PACAP-38 and different drugs on PACAP-38 induced CSN responses

<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</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</td>
</tr>
<tr>
<td>PACAP-38 (50 nM)</td>
<td>1.79 ± 0.2*</td>
<td>1.28 ± 0.1</td>
<td>0.043</td>
</tr>
<tr>
<td>PACAP-38 (25 nM)</td>
<td>1.20 ± 0.06</td>
<td>1.12 ± 0.05</td>
<td>0.31</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</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</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</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</td>
</tr>
<tr>
<td>CC (10 µM) + PACAP-38 (100 nM)</td>
<td>1.40 ± 0.1*</td>
<td>1.09 ± 0.05</td>
<td>0.041</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</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</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</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</td>
</tr>
<tr>
<td>4-AP (5 µM) + PACAP-38 (100 nM)</td>
<td>1.31 ± 0.11</td>
<td>1.21 ± 0.08</td>
<td>0.52</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</td>
</tr>
</tbody>
</table>

Values are means ± SE and normalized to basal carotid sinus nerve (CSN) response. PACAP, pituitary adenylate cyclase-activating polypeptide; ANA, anandamide; CC, chelythrine chloride; 4-AP, 4-aminopyridine. *P < 0.05, significant difference between peak and steady-state CSN responses.

with the plateau response barely above baseline activity. Consequently, despite being heavily attenuated, the peak response remained significantly higher than the plateau (P = 0.041; n = 5; Table 1). The above observation was corroborated using another PKC blocker, GF109203X (10 µM). The result was similar to CC (Table 1; Fig. 6, C and D); GF109203X significantly blunted the peak (P < 0.001; n = 6) and plateau (P = 0.001; n = 6) responses to PACAP-38 (see 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 Epac pathway activation 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. 7, A and B). 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 ADP-ribosylation factor (ARF; Ref. 30). In the present study, butanol (0.5%) and CAY10594 were used to block PLD. Figure 8, A and B, 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

Fig. 4. PACAP-38-induced stimulation of CSN was partially reduced by TASK-1-like K⁺ channel blocker. A: the 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 transient receptor potential vanilloid 1 (TRPV1) and cannabinoid receptor type 1 (CB1) agonist (47).
were not significantly different (butanol: $P = 0.01$; $n = 6\); CAY10594: $P = 0.12$; $n = 4\); Table 1). Compared with 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; $P = 0.008$; see 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; $P = 0.012$; see Fig. 10). Thus these data suggest PACAP-38-induced CSN excitation is partially mediated through PLD activation.

**Effect of $K_v$ channel blocker 4-AP on PACAP-38-induced CSN activity.** PACAP-induced excitability of rat sacral preganglionic neurons is attributed to inhibition of the 4-AP-sensitive fast inactivating potassium conductance ($I_{Ks}$; Ref. 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. 9, A and B). 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/Ca2+ channel blocker SKF96365 on PACAP-38-induced CSN activity.** Previous reports in PC-12 cells suggest PACAP-induced catecholamine secretion involves a store-operated Ca2+ channel (SOCC)/TRPC channel that facilitates capacitative Ca2+ 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 SKF96365, a SOCC/TRPC blocker that also blocks T-type calcium channels (32, 51), before PACAP-38 treatment. SKF96365 transiently stimulated CSN activity (Fig. 9, C and D) 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 Ca2+ 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 CSN 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, although 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 Ca2+ channels and inhibition of the fast inactivating K+ current ($I_{Ks}$). 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 the excitatory effects of PACAP 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 receptors, 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 noncanonical Epac pathway (52). PAC1 receptors are also reported to couple to small G protein ARF leading to PLD activation (30).

Congruent with the ability of PACAP 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 responses 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 nonspecific 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: 1) upregulation of DAG leading to activation of PKC, and 2) 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 GF109203X, 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

Fig. 6. PACAP-38-induced stimulation of CSN is highly dependent on PKC. A: example of integrated CSN responses in the presence of the PKC blocker CC (10 µM). CC partially blunted the hypoxic response 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 GF109203X (10 µM) had similar effects to CC. D: summary graph showing mean data from 6 preparations treated with GF109203X.
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 CSN 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 adenylyl 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 the Epac blocker brefeldin 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 pathway for PACAP-38 signaling (23, 52); they also reenforce 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 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 CSN response to PACAP because the 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-TASK-1-dependent mechanisms. Thus Xu et al. (63) used the small molecule PKA inhibitor H89 and lipid-soluble ANA to argue that the effects of PACAP 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 TASK-1 blocker, a TRPV1 agonist, and cannabinoid receptor type 1 (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 CSN 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 re-
Fig. 8. PACAP-38-induced stimulation of CSN also involves PLD. A: example of integrated CSN response to PACAP in the presence of the 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.
Fig. 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 Kv 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 the presence of the TRPC channel/T-type Ca²⁺ channel blocker SKF96365 (50 µM). Perfusion with SKF96365 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. E: summary graph showing mean data from 5 preparations treated with SKF96365.
that would be expected to have effects deep within tissue (10). A relatively high concentration of this small molecule inhibitor, 4-AP (21), was used in our preparation, we might be underestimating the importance of our preparation compared with using isolated glomus cells. 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; Ref. 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 the excitatory effects of PACAP on CSN activity, may reflect the fact that our preparation involves three cell types (glomus cells, glial-like sustentacular cells, and nerve endings). While PACAP is known to stimulate glomus cells directly (63), PACAP may also modulate the postsynaptic 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 neuroepethelial cells were regulation of cytosolic Ca2+ 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 mechanism by which PACAP excites chemosensory activity, what is? Our data demonstrate 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 the effects of PACAP on lumbosacral preganglionic neurons, where PACAP quells a fast-inactivating K+ conductance (Iv) that is also suppressed by 4-AP (33). A role for Iv in the carotid body 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 Iv 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 P02-sensitive K+ current, implicating Iv in oxygen sensing (11, 27, 59). However, using a superfused rat carotid body, Donnelly (15) showed no increase in 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. (48) 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. Using the arterially perfused rat carotid body preparation, here we found that 5 mM 4-AP increases normoxic CSN 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 Ca$^{2+}$ channels may mediate PACAP-38-induced CSN activity. We showed that PLC, and therefore IP$_3$ production, were likely involved in mediating the response to PACAP. There is compelling evidence that activation of IP$_3$ receptors regulates calcium-permeable nonselective cation channels, i.e., through direct interaction with TRPC channels, causing Ca$^{2+}$ influx (3, 29). One member of the TRPC family, TRPC1, has also been identified as the channel involved in “capacitive” Ca$^{2+}$ entry whereby IP$_3$ 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; Ref. 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 Ca$^{2+}$ entry, which is likely mediated by TRPC. However, some data suggest that this compound also blocks noncapacitive 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 noncompetitive GABA$_A$ antagonist (44). While the carotid body expresses GABA$_A$ receptors, the role in petrosal neurons is one of shunting inhibition (65): assuming endogenous release of GABA, antagonism should stimulate CSN activity. The initial transient increase in CSN activity caused by SKF96365 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 any species of ion channel.

Both TRP and T-type Ca$^{2+}$ channels are implicated in PACAP signaling elsewhere. In sympathetic postganglionic neurons of the superior cervical ganglia, PACAP cause cytosolic calcium elevation. As suggested by the data herein for CSN 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 CSN 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 CSN 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) [although see Overholt and Prabhakar (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 nonspecific cation (TRPC) and T-type Ca$^{2+}$ channels in the response of the CSN to PACAP be determined.

Conclusion. In conclusion, we show that the response of the CSN 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. The effects of PACAP require 4-AP- and SKF96365-sensitive mechanisms, suggesting second messenger pathways triggered by PACAP converge on K$_v$ and nonspecific 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 appears 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 that send afferents bar ing information about metabolic state to respiratory and sympathetic centers in the CNS. As PACAP is present in the superior cervical ganglia that project to the carotid bodies 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.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.R. conception and design of research; A.R. performed experiments; A.R., F.D., and R.J.W. analyzed data; A.R. and R.J.W. interpreted results of experiments; A.R. and F.D. prepared figures; A.R. drafted manuscript; R.J.W. edited and revised manuscript; R.J.W. approved final version of manuscript.

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