Pituitary adenylate cyclase-activating peptide enhances electrical coupling in the mouse adrenal medulla

Jacqueline Hill, Seong-Ki Lee, Prattana Samasilp, and Corey Smith

Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio

Submitted 9 April 2012; accepted in final form 15 May 2012

Hill J, Lee SK, Samasilp P, Smith C. Pituitary adenylate cyclase-activating peptide enhances electrical coupling in the mouse adrenal medulla. Am J Physiol Cell Physiol 303: C257–C266, 2012. First published May 16, 2012; doi:10.1152/ajpcell.00119.2012.—Neuroendocrine adrenal medullary chromaffin cells receive synaptic excitation through the sympathetic splanchnic nerve to elicit catecholamine release into the circulation. Under basal sympathetic tone, splanchnically-released acetylcholine evokes chromaffin cells to fire action potentials, leading to synchronous phasic catecholamine release. Under elevated splanchnic firing, experienced under the sympathoadrenal stress response, chromaffin cells undergo desensitization to cholinergic excitation. Yet, stress evokes a persistent and elevated adrenal catecholamine release. This sustained stress-evoked release has been shown to depend on splanchnic release of a peptide transmitter, pituitary adenylate cyclase-activating peptide (PACAP). PACAP stimulates catecholamine release through a PKC-dependent pathway that is mechanistically independent of cholinergic excitation. Moreover, it has also been reported that shorter term phospho-regulation of existing gap junction channels acts to increase junctional conductance. In this study, we test if PACAP-mediated excitation upregulates cell-cell electrical coupling to enhance chromaffin cell excitability. We utilize electrophysiological recordings conducted in adrenal tissue slices to measure the effects of PACAP stimulation on cell coupling. We report that PACAP excitation increases electrical coupling and the spread of electrical excitation between adrenal chromaffin cells. Thus PACAP acts not only as a secretagogue but also evokes an electrical remodeling of the medulla, presumably to adapt to the organism’s needs during acute sympathetic stress.

Catecholamine secretion after desensitization of cholinergic signaling in the sympathoadrenal stress response (22, 26, 47). PACAP excitation acts through a series of signaling steps that include an acute subthreshold membrane depolarization (24, 26) and parallel PKC phosphorylation-dependent recruitment of a low voltage-activated T-type calcium channel conductance to supply calcium necessary for secretion (24). In the longer term (>10 min), PACAP-mediated adrenal excitation further depolarizes the membrane and recruits an L-type Ca2+ influx (35). Ultimately, long-term PACAP excitation elicits a broader modulation of adrenal exocytic function through the activation of secretion-associated genes (38, 41, 49). More recent studies (9) have suggested a wider role for PACAP in the sympathoadrenal stress response that may include increased intercellular electrical coupling within the medulla.

Gap junctions are intercellular channels formed by head-to-head docking of connexons; hexameric assemblies of connexin (Cx) proteins (19). Connexin channels bridge the cytoplasm of two cells and allow the diffusion of ions or second messengers. The prevalence and connexin composition of gap junction coupling in the adrenal medulla are dependent on age (30, 32), species (10, 20), gender (9, 33), stress state (12), or splanchnic innervation (30, 32). The role of gap junctions in normal adrenal physiology has been studied and implicated in modulation of adrenal excitation. Martin et al. (32) showed that rat gap junctions are under tonic inhibitory control during basal sympathetic tone, while chronic cold-stress dramatically up-regulates Cx36/Cx43 expression and enhances gap junction communication (12). Gap junctions also facilitate cholinergic-evoked catecholamine release, with nicotine treatment of a single chromaffin cell in situ eliciting catecholamine release from neighboring chromaffin cells (31). Thus chronic stress and cholinergic-mediated stress signaling enhance gap junction coupling.

Gap junction coupling is regulated by signaling events common to PACAP-evoked secretion. For example, PACAP binding to its high-affinity G12-coupled PACR1 receptor elicits an elevation in cAMP, which has also been shown to regulate connexin-43 (Cx43) through PKA-mediated phosphorylation (40). Other studies (16) show that the exchange protein activated by cAMP (Epac), which signals via PLC-mediated activation of PKC and is independent of PKA action, increases junctional conductance through Cx43-composed gap junctions. Common with a role for PKC, phorbol ester treatment also elevates electrical coupling (albeit with a parallel decrease in dye coupling; Refs. 27, 43). We hypothesize that PACAP excitation elicits a signaling cascade that intersects with gap junction regulatory processes. We further hypothesize that this signaling cascade positively regulates the electrical coupling observed during acute sympathetic stress. In testing this hypothesis, we provide data demonstrating that chromaffin cell

Additional information for this article can be found online at http://ajpcell.physiology.org.
stimulation by PACAP significantly increases the degree of electrical coupling in the mouse adrenal medulla.

MATERIALS AND METHODS

Adrenal slice preparation. Adult male and female C57BL/6 mice (4–8 wk old) from Jackson Laboratories (Bar Harbor, ME) were used in this study. Male and female mice were age matched within each experimental condition, and no significant variation in electrical coupling parameters was measured across the 4 wk age range. Anesthesia and euthanasia protocols were approved by the Case Western Reserve University Institutional Animal Care and Use Committee, a federal oversight body (Federal Welfare Assurance No. A3145–01). Mice were deeply anesthetized by isoflurane (USP; Halocarbon Products, River Ridge, NJ) inhalation and euthanized by decapitation. Adrenal glands were excised and submerged in ice-cold low-calcium bicarbonate-buffered saline (BBS) containing the following (in mM): 140 NaCl, 2 KCl, 5 MgCl₂, 26 NaHCO₃, and 10 glucose and continuously bubbled with 95% O₂–5% CO₂. All BBS components were purchased from Fisher Scientific, (Fair Lawn, NJ) except MgCl₂ (Sigma-Aldrich, St. Louis, MO). Osmolarity of the BBS was 280 mosM. Adrenal glands were trimmed of excess fat and embedded in low melting temperature agarose (Lona, Rockland, ME). Agarose was prepared by melting in low calcium BBS at 110°C following 15 min of equilibration in a 35°C water bath. Immediately after embedding, adrenal glands and agarose were placed on ice to gel. Gelled agarose was trimmed into 3- to 5-mm blocks, each containing a single adrenal gland. Agarose blocks containing the glands were glued to a sectioning stage (WPI, Sarasota, FL) with cyanoacrylate. The stage was placed in a vibrotome slicing chamber filled with ice-cold low calcium BBS that was continuously bubbled with 95% O₂–5% CO₂. Adrenal glands were vibratome sectioned at 200 μm. Sections containing medulla were collected and placed in a holding chamber containing low calcium BBS bubbled with 95% O₂–5% CO₂ at 25°C. Experiments were carried out within 6 h of slice preparation.

Cell isolation. Animals were deeply anesthetized and killed as described above. Adrenal glands were removed immediately and were placed in an ice-cold dissociation solution that contained the following (in mM): 80 Na glutamate, 55 NaCl, 6 KCl, 1 MgCl₂, 10 HEPES, and 10 glucose. The pH was adjusted to 7.0, and the osmolarity was adjusted with mannitol to 320 mosM, and pH was adjusted to 7.2 with 130 mosM with mannitol. All electrophysiological records were filtered at 10 kHz and digitized at 20 kHz using an EPC-9 amplifier controlled by “Pulse” software (v. 8.8; HEKA Electronik, Bellmore, NY). Igor Pro software (WaveMetrics Lake Oswego, OR) was used to analyze electrophysiological recordings.

The protocol used to quantify gap junction coupling was as follows: cells were held at −80 mV and delivered one depolarizing step to +50 mV, with a duration of 150 ms. The “echo” integral was measured using Igor Pro software, and an example of the analysis paradigm is shown in RESULTS (see Fig. 2), to determine membrane resistance, cells were stepped to a −130-mV potential, also 150 ms in duration. Membrane resistance was calculated under passive membrane conditions according to Ohm’s law. To provide internal control conditions and to assess the effect of reagents on echo integral and input resistance, electrical protocols were delivered twice: once in normal extracellular HEPES solution and then again in a HEPES solution supplemented with 100 μm carbenoxolone (CBX), 1 μm PACAP-38, 100 nm PMA, or 100 nm G6983 (all from Sigma-Aldrich) indicated in the text. A microperfusion system (Warner Instruments, Hamden, CT) was used for local rapid delivery of some pharmacological agents (see text) during recordings. Data were separated by gender of the donor animal for analysis.

Lucifer yellow (LY) fluorescent dye (LY-dipotassium salt; Sigma-Aldrich) was introduced into pipettes, and cells were patched in the whole cell patch configuration to allow complete diffusion of LY dye into patched chromaffin cells. Dye spread was visualized with 435-nm wavelength excitation using an Olympus BW50WI fixed stage upright microscope with ×40 water dipping objective (NA = 0.8). Excitation light was shuttered between exposures to limit photobleach and phototoxicity. Images were collected with a cooled charged-coupled device camera (RetigaEX; QImaging, Surrey, BC, Canada) at >520-nm emission at a fixed exposure duration and camera gain to allow for comparison between cells. The extent of LY dye spreading was estimated by counting the number of chromaffin cells to which LY dye spread 20-min post break-in. Signals were determined to have spread if the secondary cell increased fluorescence by more than three times over background.

Western blot analysis. Adrenal glands were extracted from male or female 6-wk-old mice. The adrenal cortex was removed, and the adrenal medulla was isolated and used for Western blot analysis. Samples were kept on ice or at 4°C. Male and female tissue were kept separate and blotted separately. When preparing samples were sampled, tissues were placed in PBS (Thermo Scientific, Logan, UT) solution containing 1% Triton X-100 (Sigma) and protease inhibitors (Roche Applied Biosciences, Indianapolis, IN). Tissue was disrupted using a pestle, and homogenized sample was passed through 22-gauge needle to completely solubilize the tissue. Samples were centrifuged at 16,000 g for 20 min at 4°C. The concentration of tissue lysate was determined by a BCA assay kit (Thermo Scientific), and 75 μg of protein were loaded in each well. Protein was resolved by SDS-PAGE.
Table 1. RT-quantitative PCR detection of connexins

<table>
<thead>
<tr>
<th>Gender/Connexin</th>
<th>Mean ΔCt ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male Cx36</td>
<td>12.27 ± 0.28</td>
</tr>
<tr>
<td>Female Cx36</td>
<td>12.76 ± 0.73</td>
</tr>
<tr>
<td>Male Cx43</td>
<td>8.38 ± 0.17</td>
</tr>
<tr>
<td>Female Cx43</td>
<td>7.42 ± 0.51</td>
</tr>
</tbody>
</table>

RT-quantitative PCR detection of connexin-36 and -43 (Cx36 and Cx43) message. Average change in cycle threshold (ΔCt) values are reported. No. of experiments = 3 for male and 4 for female.

We investigated the possibility that acute PACAP excitation enhances electrical coupling in the adrenal medulla. Studies in other tissues report an increase in junctional conductance mediated by signaling events common to the PACAP signaling pathway, including PKC (43) and Epac (16). In this study, we report a gender dimorphism in the input resistance of chromaffin cells, in the intercellular spread of electrical excitation, and in the connexin composition of gap junctions in the adrenal medulla. Furthermore, we report that PACAP, via PKC phospho-regulation, enhances cell-cell communication in either gender through a functional increase in gap junction conductance. Experiments were performed in acute adrenal tissue slices, unless noted otherwise, and electrophysiologically recorded signals were obtained in the perforated patch configuration to preserve native cytosolic composition and cell signaling processes.

Gap junctions in the mouse adrenal medulla. LY dye diffusion assays are a commonly utilized method of studying gap junction connectivity between cells (17). Thus, to obtain an estimate of basal coupling levels in the mouse adrenal medulla, we first investigated the extent of LY diffusion between chromaffin cells in situ (Fig. 1A, top) by loading the primary patched cell with LY and measuring spread of LY signal to
neighboring cells. LY diffusion was defined as spread of the fluorescence from the patched primary chromaffin cell to one or more unpatched secondary chromaffin cells to three times background at 20-min post break-in. As show in Fig. 1A, middle, LY dye does not diffuse to the neighboring chromaffin cells after 20 min. LY dye diffusion to one or more neighboring chromaffin cells (Fig. 1A, bottom) was only observed in 17.2% of patched cells. However, previous reports indicate the extent of LY dye diffusion can depend on connexin composition of gap junctions (6, 42) and may not provide a conclusive determination of electrical coupling (33). Thus we considered more sensitive electrophysiological methodologies to accurately estimate the degree of electrical coupling between chromaffin cells in situ. Moser (33) identified “echoing” action potentials from neighboring cells in electrical recordings of chromaffin cells in situ as a diagnostic determinant of cell-cell coupling. Echoing action potentials are elicited by a charge spreading from the primary voltage-clamped cell to neighboring unclamped cells. The charge injection into the secondary cell evoke an action potential that then conducts back through the gap junction to the primary, patched cell. These echoing action potentials are blocked by tetrodotoxin, confirming their dependence on voltage-activated Na+ channels and are measurable as inward current spikes on top of the primary cell current record (33). We tested if electrical echoes could be observed, even in cells that do not display LY dye diffusion. We devised an electrical protocol to depolarize the primary cell to the approximate reversal potential for inward Na+ and Ca2+ channels, and we conducted recordings in solutions that contained Cs+ to block K+ currents in the primary cell. Together, these steps minimized active currents in the primary cell, thus maximizing the resolution of “echoes” from secondary cells. The representative record provided in Fig. 1B is from the same cell as in Fig. 1A in which LY dye spreading was not observed (middle). As seen in Fig. 1B, inset, echoing action potentials were observed on top of step-evoked membrane current. Thus, despite the lack of LY dye diffusion, the primary cell is indeed electrically coupled to at least one other cell within the medulla.

Most studies examining electrical coupling in the adrenal medulla utilized slices from female mice only (12, 31–33). Yet, males and females indeed respond differently to stress excitation (48). Thus we repeated both LY and electrical protocols in males and females indeed respond differently to stress excitation (48). Thus we repeated both LY and electrical protocols in males and females. Despite the lack of LY dye diffusion, the primary cell is indeed electrically coupled if left unperturbed (Fig. 2Aii). CBX abolishes echo currents in chromaffin cells in situ. Next, to test that the observed electrical coupling is indeed due to gap junctions, slices were treated with 100 μm CBX, a commonly utilized gap junction decoupling agent (14). In the representative recording shown in Fig. 2Bi, a cell was patched and held at −80 mV and stepped to +50 mV (Fig. 2Bi, “CBX t = 0 min”) in normal HEPES ringer solution. The cell was treated with 100 μm CBX for 20 min, consistent with the pharmacokinetics of CBX gap junction block (32), and stepped to +50 mV at the 10-min time point (sample data not shown) and at the completion of the 20-min (“CBX t = 20 min”) treatment period. The sample recording provided shows a loss of all electrical echoes after 20 min of CBX treatment. The integrals of echoing action potentials from t = 0 min, t = 10 min, and t = 20 min conditions were pooled and plotted as means ± SE, shown in Fig. 2Bii (cells exhibiting no echoes were assigned an integral value = 0 pC). These data demonstrate that the echo currents are CBX sensitive as expected for electrical signaling through gap junctions from secondary unclamped cells back into the primary voltage-clamped chromaffin cell. However, it is possible that some chromaffin cells are weakly electrically coupled and do not fire action potentials in response to current injection from the primary cell. Thus we turned to a complementary electrical approach based on measuring the passive cell input resistance.

**Quantification of electrical coupling in the adrenal medulla.** A previous study (33) mathematically and experimentally established the relationship between amplitude of echoing action potentials and junctional conductance of neighboring cells. Similarly, the integral of echoing action potentials provides a measure of the charge passing through open gap junctions in series with the patch-clamped cell. However, accurate echo integral quantification may be complicated by factors such as a drifting or relatively high series resistance associated with the extended time frame of the perforated-patch recordings utilized here. To avoid issues relating to unstable series resistance, we eliminated cells that deviated >7 MΩ between sweeps from further analysis. Cells are treated with pharmacological agents and echoes are obtained at the 0- and 10-min time points (see Figs. 2–5). Thus, in an initial description of our system, we determined the inherent stability of echoes from single cells as a function of time. A representative electrical echo from the +50 mV step is shown in Fig. 2Ai, left (“t = 0 min”). After 10 (“t = 10 min”) and 20 min (“t = 20 min”), the patched cell was restimulated and example electrical echoes are shown again (Fig. 2Ai, middle and right). The shaded grey region indicates the region used for integral analysis. The echo integral does not significantly change between t = 0, t = 10, or t = 20 time points, thus demonstrating that cells express a stable and measurable coupling if left unperturbed (Fig. 2Aiit).
potential echoes observed under mary cell input resistance indicate that the electrical action neighboring cells (echoes) as well as the CBX-sensitive pri-
sensitive propagation of active action potential currents from

, Ai male”) is shown in Fig. 3
tative electrical echo from the resulting current trace (“fe-
repeated on a slice taken from a female mouse and a represen-
50 mV, as in Fig. 1. A representative electrical echo (“male”) is shown in Fig. 3

females in general express a greater increase in plasma catecholamine in response to stress (28, 48). In Fig. 2. Carbenoxolone (CBX) abolishes spike-like cur-
rents and increases input resistance in chromaffin cells. Ai: electrical echoes were evoked from a voltage-
clamped cell as in Fig. 1 over a 20-min time course to determine stability of the response (t = 0, 10, and 20 min). Region shaded in grey represents the integral of the echo, which is quantified in Ai and in subsequent figures. Aii: quantified data from each time point were plotted as means ± SE (n = 10 echoes/time point; P = 0.996 by ANOVA). Bii: effect of CBX on echo charge was tested. Cells were categorized as coupled if AP echoes were present at t = 0 min. An adrenal slice containing a coupled cell pair was bath-treated with 100 μm CBX. Echo charge was determined by current integration as above. Bii: echo integral (Int) data from all CBX time points (0, 10, and 20 min) were pooled, quantified, and plotted as means ± SE (n = 16 echoes/time point; *statistical significance of 0- and 20-min time points with P = 0.007 by Student’s t-test). C: input resistance (R_{memb}) was calculated using the same CBX treatment time course as in Bi. Cells were hyperpolarized to −130 mV from the holding potential of −80 mV to evoke a passive current to calculate membrane resistance (R_{memb}) of the cell. Representative current traces for the CBX t = 0-min and CBX t = 20-min time points are provided. Bii: this procedure was repeated at the 0-, 10-, and 20-min time points, and the R_{memb} was calculated, pooled, and presented as means ± SE (n = 25, 9, and 9 cells for 0-, 10-, and 20-min time points, respectively; *statistical significance of 0- and 20-min time points at P = 0.002).

Gender dimorphism of adrenal medullary electrical coupling.

In rats, females in general express a greater increase in plasma catecholamine in response to stress (28, 48). In Fig. 1, we reported a gender dimorphism in the extent of electrical coupling in the adrenal medulla. We considered the possibility that female mouse gap junctions are able to carry more charge than male mouse chromaffin cells. To investigate this possibility, a male mouse chromaffin cell in situ was voltage-clamped at −130 mV: electrical echoes were evoked from a voltage-
clamped cell as in Fig. 1 over a 20-min time course to determine stability of the response (t = 0, 10, and 20 min). Region shaded in grey represents the integral of the echo, which is quantified in Ai and in subsequent figures. Aii: quantified data from each time point were plotted as means ± SE (n = 10 echoes/time point; P = 0.996 by ANOVA). Bii: effect of CBX on echo charge was tested. Cells were categorized as coupled if AP echoes were present at t = 0 min. An adrenal slice containing a coupled cell pair was bath-treated with 100 μm CBX. Echo charge was determined by current integration as above. Bii: echo integral (Int) data from all CBX time points (0, 10, and 20 min) were pooled, quantified, and plotted as means ± SE (n = 16 echoes/time point; *statistical significance of 0- and 20-min time points with P = 0.007 by Student’s t-test). C: input resistance (R_{memb}) was calculated using the same CBX treatment time course as in Bi. Cells were hyperpolarized to −130 mV from the holding potential of −80 mV to evoke a passive current to calculate membrane resistance (R_{memb}) of the cell. Representative current traces for the CBX t = 0-min and CBX t = 20-min time points are provided. Bii: this procedure was repeated at the 0-, 10-, and 20-min time points, and the R_{memb} was calculated, pooled, and presented as means ± SE (n = 25, 9, and 9 cells for 0-, 10-, and 20-min time points, respectively; *statistical significance of 0- and 20-min time points at P = 0.002).

Gender dimorphism of adrenal medullary electrical coupling.

In rats, females in general express a greater increase in plasma catecholamine in response to stress (28, 48). In Fig. 1, we reported a gender dimorphism in the extent of electrical coupling in the adrenal medulla. We considered the possibility that female mouse gap junctions are able to carry more charge than male mouse chromaffin cells. To investigate this possibility, a male mouse chromaffin cell in situ was voltage-clamped at −130 mV: electrical echoes were evoked from a voltage-
clamped cell as in Fig. 1 over a 20-min time course to determine stability of the response (t = 0, 10, and 20 min). Region shaded in grey represents the integral of the echo, which is quantified in Ai and in subsequent figures. Aii: quantified data from each time point were plotted as means ± SE (n = 10 echoes/time point; P = 0.996 by ANOVA). Bii: effect of CBX on echo charge was tested. Cells were categorized as coupled if AP echoes were present at t = 0 min. An adrenal slice containing a coupled cell pair was bath-treated with 100 μm CBX. Echo charge was determined by current integration as above. Bii: echo integral (Int) data from all CBX time points (0, 10, and 20 min) were pooled, quantified, and plotted as means ± SE (n = 16 echoes/time point; *statistical significance of 0- and 20-min time points with P = 0.007 by Student’s t-test). C: input resistance (R_{memb}) was calculated using the same CBX treatment time course as in Bi. Cells were hyperpolarized to −130 mV from the holding potential of −80 mV to evoke a passive current to calculate membrane resistance (R_{memb}) of the cell. Representative current traces for the CBX t = 0-min and CBX t = 20-min time points are provided. Bii: this procedure was repeated at the 0-, 10-, and 20-min time points, and the R_{memb} was calculated, pooled, and presented as means ± SE (n = 25, 9, and 9 cells for 0-, 10-, and 20-min time points, respectively; *statistical significance of 0- and 20-min time points at P = 0.002).

Greater Cx43 and Cx36 expression in the female mouse adrenal medulla. We considered that the gender dimorphism in electrical coupling may be due to a differential expression of gap junction-forming connexins. Although high-conductance Cx43 and low-conductance Cx36 have both been shown to be present in the mouse adrenal medulla (15, 34), their gender-

2Ci. The trace labeled 0 min was before superfusion with a HEPES Ringer solution supplemented with 100 μm CBX while the 20-min trace represents complete CBX block. Data were also collected from the CBX 10-min time point. As expected, an increase in input resistance due to the CBX block is readily apparent. We repeated this protocol as above and the pooled data from the 0-, 10-, and 20-min time points are plotted as means ± SE in Fig. 2Cii. Taken together, both the CBX-sensitive propagation of active action potential currents from neighboring cells (echoes) as well as the CBX-sensitive primary cell input resistance indicate that the electrical action potential echoes observed under +50 mV depolarization are junctional currents from coupled secondary chromaffin cells.

Gender dimorphism of adrenal medullary electrical coupling.

In rats, females in general express a greater increase in plasma catecholamine in response to stress (28, 48). In Fig. 1, we reported a gender dimorphism in the extent of electrical coupling in the adrenal medulla. We considered the possibility that female mouse gap junctions are able to carry more charge than male mouse chromaffin cells. To investigate this possibility, a male mouse chromaffin cell in situ was voltage-clamped at −130 mV: electrical echoes were evoked from a voltage-
clamped cell as in Fig. 1 over a 20-min time course to determine stability of the response (t = 0, 10, and 20 min). Region shaded in grey represents the integral of the echo, which is quantified in Ai and in subsequent figures. Aii: quantified data from each time point were plotted as means ± SE (n = 10 echoes/time point; P = 0.996 by ANOVA). Bii: effect of CBX on echo charge was tested. Cells were categorized as coupled if AP echoes were present at t = 0 min. An adrenal slice containing a coupled cell pair was bath-treated with 100 μm CBX. Echo charge was determined by current integration as above. Bii: echo integral (Int) data from all CBX time points (0, 10, and 20 min) were pooled, quantified, and plotted as means ± SE (n = 16 echoes/time point; *statistical significance of 0- and 20-min time points with P = 0.007 by Student’s t-test). C: input resistance (R_{memb}) was calculated using the same CBX treatment time course as in Bi. Cells were hyperpolarized to −130 mV from the holding potential of −80 mV to evoke a passive current to calculate membrane resistance (R_{memb}) of the cell. Representative current traces for the CBX t = 0-min and CBX t = 20-min time points are provided. Bii: this procedure was repeated at the 0-, 10-, and 20-min time points, and the R_{memb} was calculated, pooled, and presented as means ± SE (n = 25, 9, and 9 cells for 0-, 10-, and 20-min time points, respectively; *statistical significance of 0- and 20-min time points at P = 0.002).

Greater Cx43 and Cx36 expression in the female mouse adrenal medulla. We considered that the gender dimorphism in electrical coupling may be due to a differential expression of gap junction-forming connexins. Although high-conductance Cx43 and low-conductance Cx36 have both been shown to be present in the mouse adrenal medulla (15, 34), their gender-
Moreover, we also tested if male and female mice expressed similar levels of PACAP receptor (PACR₁) to determine if the females are more reactive to stress due to enhanced PACAP excitation (a point to be expanded upon below). Counter to our hypothesis, males exhibited increased expression level for PACR₁ (Fig. 3Cii; Table 2), indicating that if anything, males would be more sensitive to stress if this were a factor. Take together, these data support the hypothesis that female mice express a greater message for the high conductance Cx43, as well as the low conductance Cx36, than males and that this difference may contribute to observed differences in electrical coupling behavior.

**PACAP stimulation increases electrical coupling.** Studies (28, 48) describe gender-specific responses to acute sympathetic stress at the organismal level. Yet, little is known about the gender-specific roles of adrenal medullary gap junctions in PACAP-evoked acute stress. We investigated the possibility that PACAP stimulation leads to functional gender-specific gap junction remodeling in the adrenal medulla. We measured both echo integrals and input resistance as above, before and after focal perfusion. Sample raw records for each protocol are provided in Fig. 4, Ai and Bi, and show an increased echo amplitude and decreased input resistance with 10-min PACAP excitation, respectively. The electrical protocols were repeated in male and female mouse chromaffin cells, with resulting evoked membrane currents are shown. Ai: this protocol was repeated in 15 cells from male mouse and 27 cells from female mouse. Pooled input resistance values are presented as mean ± SE (statistical significance at P = 0.0003). Bi: in male and female mouse chromaffin cells, input resistance was calculated following the procedure in Fig. 2. Representative traces of resulting evoked membrane currents are shown. Bi: this protocol was repeated in 15 cells from male mouse and 27 cells from female mouse. Pooled input resistance values are presented as mean ± SE (statistical significance at P = 0.0003). C: real-time quantitative PCR was performed for connexin-43 (Cx43; solid line) and connexin-36 (dotted line) in male (●) and female mice (○). Representative amplification curves were normalized to GAPDH and plotted. Rn, normalized reporter dye fluorescence. Cii: Western blot analysis of Cx43, Cx36, and pituitary adenylate cyclase-activating peptide (PACAP) receptor (PACR₁) in the male and female mouse adrenal medulla. β-Actin or β-tubulin was used as a loading control. A representative blot from one experiment is presented in Cii, with averaged actin or tubulin-normalized gene expression ratios from multiple experiments presented in Table 2.

**Table 2. Western blot detection of connexins and PACR₁**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Male:Female Gene Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx36</td>
<td>0.59 ± 0.03:1*</td>
</tr>
<tr>
<td>Cx43</td>
<td>0.46 ± 0.05:1*</td>
</tr>
<tr>
<td>PACR₁</td>
<td>2.43 ± 1.05:1</td>
</tr>
</tbody>
</table>

Values are means ± SE. Western blot detection of Cx36, Cx43, and pituitary adenylate cyclase-activating peptide (PACAP) receptor (PACR₁) protein expression. β-Actin or β-tubulin normalized intensities from 3 experiments were pooled by gender and antibody type. Gene expression ratio (male:female) taken from normalized intensity values is reported. *Statistical significance was determined by unpaired Student’s t-test with P value <0.001.
mouse chromaffin and plotted as means ± SE. In both genders, PACAP treatment significantly decreased input resistance values. Taken together, both protocols report that acute PACAP excitation increases junctional communication between in situ chromaffin cells. Cells from female mice express greater coupling, but both genders’ electrical coupling is proportionally increased by PACAP.

**PACAP acts through PKC to increase electrical coupling.** Previous studies have demonstrated acute PACAP to signal through a requisite activation of PKC-dependent signaling (26, 39, 50). We investigated the possibility that PKC activation mirrors the effect of PACAP treatment and upregulates electrical coupling in the adrenal medulla. We again turned to the integrated action potential echo analysis as well as input resistance to test the effects of the phorbol ester PMA on adrenal medullary electrical coupling in male and female mice. Sample records for the action potential echo protocol recorded from a female medulla are provided in Fig. 5A(i) and show that 10 min of PMA stimulation (100 nM, focally and bulk perfused) increased action potential echo amplitude. Echo inte-

---

**Fig. 4. PACAP increases electrical coupling.** Ai: effect of PACAP stimulation on electrical coupling was tested and a representative recording is provided. Cell was treated with 1 μM PACAP by focal perfusion for 10 min, and the electrical echo protocol was repeated, showing an increase in echo magnitude. Ai: protocol was repeated in male mouse chromaffin cells (n = 21 echoes) and female mouse chromaffin cells (n = 27 echoes). Echo integral data from each PACAP-stimulation time point were separated by gender and plotted as means ± SE. Male: *statistical significance at P = 0.0001; female: *statistical significance at P = 0.011. Bi: input resistance in response to PACAP stimulation was measured, as in Fig. 2 from a chromaffin cell. Representative traces show an increase in inward I_{K1} after 10 min PACAP treatment at −130 mV, indicating a decreased input resistance. Bi: this protocol was repeated in 15 cells from male mice and in 27 cells from female mice. Input resistance data were separated by gender and treatment condition and plotted as means ± SE. Male: *statistical significance at P = 0.005; female: *statistical significance at P = 0.048.

**Fig. 5. PACAP enhances electrical coupling through a PKC-dependent mechanism.** Ai: electrical echoes were measured in chromaffin cells, as in Figs. 1–4, before (t = 0 min) and after (t = 10 min) focal and bulk perfusion of 100 nm PMA. Representative electrical echoes from each time point are shown. Ai: protocol was repeated in male mouse chromaffin cells (n = 10 echoes) and female mouse chromaffin cells (n = 22 echoes). Data from each PMA-stimulation time point were separated by gender and plotted as means ± SE. Male: *statistical significance at P = 0.025; female: *statistical significance at P = 0.048. Bi: an adrenal tissue slice was pretreated for 5 min with focal and bulk-perfused Gö6983 (100 nM) and then stimulated with focally perfused 1 μM PACAP for 10 min. Representative electrical echoes from the 0- and 10-min time point of PACAP stimulation are shown. Bi: protocol was repeated in male mouse chromaffin cells (n = 10 echoes) and female mouse chromaffin cells (n = 7 echoes). Data from each PACAP stimulation time point were separated by gender and plotted as means ± SE. Male: *statistical significance at P = 0.344; female: *statistical significance at P = 0.306.
grals were measured, pooled, and plotted as means ± SE in Fig. 5Aii. In the negative condition, we also confirmed that acute PACAP stimulation modulates gap junction connectivity through a PKC-sensitive step by pretreating adrenal slices with general PKC inhibitor, Go6983 (100 nM) and then treating cells with PACAP. Again, sample raw records for this condition were recorded from an adrenal slice taken from female mice and pooled and are presented in Fig. 5Bi, showing that Go6983 blocked the increased echo amplitude seen with PACAP stimulation. Pooled data in Fig. 5Bii confirm this effect. Input resistance was calculated under both PMA and PACAP + Go6983 conditions, and these values are presented and quantified, by gender, in Table 3. Input resistance significantly decreases in both genders under PMA stimulation. These data suggest that acute PACAP excitation increases adrenal medullary electrical coupling by a signaling mechanism that shares a PKC-dependent step, just as acute PACAP-evoked catecholamine secretion.

DISCUSSION

The sympathetic nervous system fires at a low tonic level in unstressed animals, setting them into the overall homeostatic state of energy storage. In this state, modest catecholamine release is mediated through splanchnic release of acetylcholine to trigger action potential-evoked $\text{Ca}^{2+}$-dependent catecholamine exocytosis from the adrenal medulla (1, 7, 13, 18). Under basal sympathetic tone, catecholamine release acts in concert with the parasympathetic nervous system to set integral physiological functions such as heart rate, insulin secretion, and shunting of blood to the viscera. Studies (32) have also reported that the cholinergic system exerts tonic inhibitory control over gap junction coupling. It seems likely that cholinergic inhibitory control of gap junctions under sympathetic tone works to fine-tune catecholamine secretion by limiting excitation during the “rest and digest” state of energy storage. Misregulation of catecholamine secretion can lead to hypertension, cardiac arrythmia, diabetes, hyperventilation, and paroxysmal inflammation.

Activation of the sympathoadrenal stress reflex fundamentally alters splanchnic-adrenal excitation. It has been documented that, under chronic stress, the adrenal medulla undergoes functional remodeling to increase cell-cell coupling (12), which can spread excitation to evoke catecholamine release from clusters of cells (31). While specific nicotinic acetylcholine receptor activation decreases cell coupling (11, 32), studies (32, 36) have shown that general cholinergic agonists enhance gap junctional communication or promote the spread of excitation. In the mouse hemi-sectioned adrenal gland, acetylcholine decreased the input resistance of chromaffin cells. The authors (36) proposed that the actions of ACh on input resistance likely result from ACh binding to muscarinic receptors and a subsequent decrease of junctional resistance through a muscarinic signaling pathway. Thus muscarinic ACh receptors, the activation of which lasts longer than rapidly inactivating nicotinic receptors (37), may work in concert with stress-evoked PACAP stimulation to positively modulate medullary electrical communication. This is an attractive possibility in that nicotinic receptors are clustered at the synapse while muscarinic receptors are broadly distributed (45) and may only be activated under elevated or sustained stimulation causing ACh synaptic spillover, a condition that would be expected under sympathetic stress. Another possibility is that under long-term PACAP exposure connexin gene expression increases via a PKA- or PKC-dependent mechanism, effectively remodeling the adrenal medulla to support sustained secretion. Indeed, PACAP has been shown to activate a number of stress-related genes on the hour timescale (38, 41, 49). Although a significant enhancement of connexin gene transcription is unlikely to account for the substantial and rapid enhancement in electrical coupling observed in our study, it seems likely that PACAP stimulates gene expression of connexins under a longer time scale to electrically remodel the adrenal medulla.

Gap junctions have been widely implicated in the secretion process in other tissues, as well. In the other tissues such as the pancreas, Benninger et al. (3) showed that insulin secretion is minimal under basal (low glucose) conditions in the neuroendocrine β-cells of the pancreatic islet of Langerhans. However, when the islet is dissociated, isolated β-cells respond to low glucose conditions with heightened insulin secretion, suggesting that gap junctions suppress insulin secretion in the intact islet. Interestingly, Head et al. (23) showed that regulation of insulin secretion occurs through Cx36 containing gap junction channels. However, it may be that in some systems gap junctional coupling does not necessarily relate to secretory function. In the lacrimal gland, Walcott et al. (46) reported a gender dimorphism in the distribution of Cx26 and Cx32, despite similar secretory responses. Our data indicate a differential expression of connexins within the male and female adrenal medullae. Despite statistically similar transcript levels, females exhibit a 2.3-fold increase in Cx43 protein expression and a 1.7-fold increase in Cx36 protein expression over males. This difference in Cx43 expression may be dictated by differences in circulating female sex hormones (21). In Fig. 1, we report that female mouse chromaffin cells exhibit more LY dye diffusion than do male mouse chromaffin cells. Several studies indicate that the connexin composition of gap junctions has a significant impact on the extent of LY dye diffusion. For example, Cx43-containing gap junctions allow the diffusion of LY much more readily than do Cx36-containing gap junctions (8). We provide data showing that gap junctions in female mouse adrenal medullae carry more electrical charge than male mouse adrenal medullae, as evidenced by higher echo integral and decreased input resistance in female mice. However, the observations that females have larger echoes, lower input resistance, and more extensive dye spreading argue that the gap...

Table 3. Membrane input resistance values

<table>
<thead>
<tr>
<th>Condition</th>
<th>Male Go Ω ± SE (n)</th>
<th>Female Go Ω ± SE (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>In situ HEPES</td>
<td>3.46 ± 0.31 (15)</td>
<td>2.06 ± 0.28 (27)†</td>
</tr>
<tr>
<td>+ Carbenoxolone (20 min)</td>
<td>7.22 ± 2.77 (5)*</td>
<td>3.81 ± 0.92 (6)**†</td>
</tr>
<tr>
<td>+ PACAP</td>
<td>1.67 ± 0.13 (5)*</td>
<td>0.685 ± 0.21 (5)**†</td>
</tr>
<tr>
<td>+ PACAP + Go6983</td>
<td>3.31 ± 0.49 (5)</td>
<td>2.15 ± 0.64 (5)</td>
</tr>
<tr>
<td>+ PMA</td>
<td>1.94 ± 0.15 (5)*</td>
<td>0.514 ± 0.14 (8)**†</td>
</tr>
<tr>
<td>Isolated HEPES</td>
<td>12.6 ± 4.85 (4)*†</td>
<td>12.7 ± 2.97 (6)*†</td>
</tr>
</tbody>
</table>

Average membrane resistance values ± SE for male and female mice are reported for all conditions. Sample size is reported in parentheses with significance for all data sets determined by an ANOVA test (P < 0.001). Paired (HEPES and treatment condition) and unpaired (between genders) Student’s t-tests were performed with significance at *P < 0.05 for paired t-tests; †P < 0.05 for unpaired t-tests.
PACAP enhances electrical coupling in the adrenal medulla

junctons linking females cells are of a higher conductance form. This is consistent with our finding that female mice express a higher proportion of Cx43-containing gap junctions than do male mice, which may account for functional differences. We also show that female mice have a higher total protein level for both connexin isofoms, which likely translates to more functional gap junctions. Interestingly, echo analysis revealed that PACAP excitation significantly increases junctional communication in both male and female mice. PACAP decreased input resistance in male and female mice, suggesting that PACAP increased junctional communication effectively in both genders.

Thus studies (48) conducted at the whole animal level have shown that females secrete more epinephrine than males in response to certain stressors. This gender-dimorphic response to stress is not necessarily occurring at the level of the electrically coupled adrenal unit and may lie in differences in gland morphology (i.e., different proportion of epinephrine- vs. nor-epinephrine-secreting cells, differential splanchnic innervation, etc.). Nevertheless, PACAP represents a critical component of the acute sympathetic stress response. The ability of PACAP to remodel gap junction coupling in the adrenal medulla may represent a point of regulation for secretion during acute sympathetic stress for both males and females.

ACKNOWLEDGMENTS

We thank Dr. Shyue-An Chan and Angelique Do for technical assistance in adrenal slice preparation and RT-quantitative PCR detection of Cxs, respectively.

GRANTS

J. Hill was supported by the National Heart, Lung, and Blood Institute Grant T32-HL-07887 and American Heart Association Grant 10PRE4100002. S.-K. Lee was supported by the National Institute of Diabetes and Digestive and Kidney Diseases Grant R37 DK30344 to Walter F. Boron.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


AJP-Cell Physiol • doi:10.1152/ajpcell.00119.2012 • www.ajpcell.org


