Gap junction proteins and cell–cell communication in the three functional zones of the adrenal gland

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Abstract

Mouse and monkey adrenal glands were used to study the relationships between gap junction protein expression, intercellular communication and adrenal zonation. Dye communication patterns were determined by incubating freshly excised and hemisected adrenal glands in Lucifer yellow, a gap junction permeable fluorescent dye. Immunohistochemical techniques were used to localize adrenal gap junction proteins. The combination of these two techniques permitted the correlation of gap junction proteins with dye transfer and hormone responses in specialized regions of the adrenal cortex. Lucifer yellow dye communication was most pronounced in the inner glucocorticoid/androgen-producing regions (zona fasciculata/zona reticularis), but was virtually absent in the outer mainly mineralocorticoid-producing region (zona glomerulosa). This pattern of dye communication was coincident with immunohistochemical localization of the gap junction protein, \( \alpha_1 \text{Cx43} \). The variations in communication and \( \alpha_1 \text{Cx43} \) expression within the adrenal cortex are thought to be relevant to normal physiological regulation of the adrenal gland.

Introduction

It has been amply demonstrated that gap junctions provide pathways for the direct intercellular exchange of small molecules (Goodenough 1978, Trautmann 1988) and it has been postulated that these small molecules include ‘signals’ that modulate growth, differentiation and function in recipient cells (Larsen et al. 1977, Loewenstein 1979, Hellmann et al. 1999, Trosko et al. 2000). In the case of the adrenal gland, it has been proposed that such communication also facilitates and amplifies hormonally stimulated cell responses (Decker et al. 1978, Murray & Fletcher 1984, Usadel et al. 1993, Murray et al. 1995a, Munari-Silem & Rousset 1996, Oyoyo et al. 1997).

As demonstrated in vitro, procedures which compromise gap junction function consistently decrease adrenocorticotropin (ACTH)-induced steroidogenesis (Oyoyo et al. 1997). This is true for bovine adrenal cells either transfected with \( \alpha_1 \text{Cx43} \) antisense cDNA or treated with the gap junction communication inhibitor, 18-\( \alpha \)-glycerrhetinic acid (Munari-Silem et al. 1995, Shah & Murray 2001) and also applies to a mouse adrenal tumor cell line maintained in culture in which loss of gap junction functionality parallels loss of ACTH-stimulated steroid production (Decker et al. 1978). Conversely, ACTH treatment increases both gap junction protein number and steroidogenesis in adrenal cell cultures (Decker 1976, Decker et al. 1978, Oyoyo et al. 1997, Shah & Murray 2001).

As outlined above, direct evidence of cell–cell communication in the adrenal gland, as in most organs, has been limited to cells in culture and has not dealt with the fact that, in the intact gland, adrenal cortical cells are segregated into separate zones with distinct functions (McNicol 1992). This unique zonation of the adrenal gland has allowed us to demonstrate a differential distribution of gap junctions corresponding to differing functions. Cells in the outermost and more rapidly growing adrenal cortical zone, the zona glomerulosa, produce aldosterone (Malendowicz et al. 2001) but have few or no gap junctions (Murray et al. 1995a, b). Cells in the inner two zones, the zonae fasciculata and reticularis produce cortisol and sex hormones respectively (McNicol 1992), and both the zones exhibit numerous gap junctions (Murray et al. 1995a). Thus, although all three adrenal cortical zones may have ACTH receptors (Xia & Wikberg 1996, Chorvatova et al. 1999, Kau et al. 1999), cells with abundant gap junctions (zonae fasciculata and reticularis) are those which are most responsive to ACTH, while cells which lack gap junctions (zona glomerulosa) are characterized by their vigorous response to angiotensin II and to a lesser degree Na\(^+\), K\(^+\) and ACTH (Biglieri et al. 1987, McNicol 1992, Rocco et al. 1994).
The clear morphological, architectural and functional differences between adrenal cortical zones have permitted us to undertake studies designed to demonstrate the distribution of gap junctions as they relate to functional communication within this organ. We demonstrate a close correlation between patterns of dye communication and α1/Cx43 gap junction distribution within the mouse adrenal gland and thus confirm our hypothesis that dye communication would be limited in the zona glomerulosa, where very few α1/Cx43 gap junctions were found. We also conclude that other members of the gap junction family of proteins either are not present in the zona glomerulosa or are not functional under these conditions. As predicted from the results of our previous studies, we now report that in the freshly excised mouse adrenal gland, morphological structures (gap junction number) can be closely correlated with function (extensive dye communication and ACTH-stimulated response). In addition, we provide data on cell–cell communication of Lucifer yellow cation and ACTH-stimulated response. In order to undertake studies designed to demonstrate the communication assay, the adrenal tissues were fixed in 4% paraformaldehyde for 1–2 h at room temperature, embedded in optimum cutting temperature tissue freezing medium (Triangle Biomedical Sciences, Durham, NC, USA), flash frozen in liquid nitrogen, and stored at –80 °C. Adrenal gland sections (8–10 µm thick) were cut and collected on glass slides in preparation for analysis of Lucifer yellow dye communication (Tsien & Weingart 1976), gap junction immunohistochemistry (Oyoyo et al. 1997) or lipid content determination by Oil Red O staining (Troyer 1980).

**Materials and Methods**

**Animals**

Adult female C57Bl6 mice were obtained from the Charles Rivers Laboratory (Wilmington, MA, USA). Adult ovariectomized female rhesus monkeys (Macaca mulatta) were obtained from the Center for Research in Reproductive Physiology, University of Pittsburgh (Pittsburgh, PA, USA). The mice were fed Purina laboratory chow and the monkeys were fed Purina monkey chow (Ralston Purina, St Louis, MO, USA). Water was provided ad libitum. Animals were weighed, killed, and the adrenal glands were harvested.

**Adrenal gland dye coupling assay**

Lucifer yellow dye loading for demonstration of cell–cell coupling was performed with a modified procedure (Tsien & Weingart 1976). The adrenal glands were quickly removed from the animals, cut into segments and incubated in 0.5% Lucifer yellow CH (Molecular Probes, Eugene, OR, USA, or Sigma, St Louis, MO, USA) in phosphate-buffered saline (PBS) (pH 7.4) and/or in 0.5% rhodamine dextran at 37 °C for 10 min. In some studies, the excised adrenal gland segments were preincubated in oleamide (100–200 µM), a gap junction communication inhibitor molecule (Boger et al. 1998) for 5 min. The tissues were then incubated for 10 min in 0.5% Lucifer yellow assay mixture containing oleamide. Fourteen half gland, nine oleamide-treated and three whole gland adrenal preparations were assayed. Following the dye communication assay, the adrenal tissues were fixed in 4% paraformaldehyde for 1–2 h at room temperature, embedded in optimum cutting temperature tissue freezing medium (Triangle Biomedical Sciences, Durham, NC, USA), flash frozen in liquid nitrogen, and stored at –80 °C. Adrenal gland sections (8–10 µm thick) were cut and collected on glass slides in preparation for analysis of Lucifer yellow dye communication (Tsien & Weingart 1976), gap junction immunohistochemistry (Oyoyo et al. 1997) or lipid content determination by Oil Red O staining (Troyer 1980).

**Gap junction antibodies and probes**

Affinity-purified polyclonal rabbit antibodies (IgG) were gifts from Drs Norton B Gilula and Nalin Kumar. Preparation and characterization of these antibodies have been described previously (Kumar & Gilula 1992). These antibodies were prepared against synthetic peptides corresponding to the cytoplasmic domains of α1/Cx43 peptide extending from residue 370 to 381 and corresponding to the carboxyl terminus of the α1/Cx43.

**Immunohistochemistry of gap junction protein**

The tissues in this study were prepared and stained for connexin immunohistochemical analysis as previously described (Murray et al. 1995a, Oyoyo et al. 1997). Frozen sections were collected on gelatinized slides and incubated for 5 min in PBS before the addition of a blocking solution (3% bovine serum albumin (BSA), 3% normal goat serum (Sigma-Aldrich, St Louis, MO, USA) in PBS). The α1/Cx43 antibody or pre-immune rabbit IgG (5–10 µg/ml in 3% normal goat serum with 3% BSA in PBS) was added for 1 h. After rinsing with PBS, the sections were incubated in secondary antibody (Cy3-conjugated goat anti-rabbit IgG; Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 45 min at 37 °C. Sections were washed thoroughly in PBS, stained with Hoechst nuclear dye and mounted in Fluoromount-G anti-quench mounting media (Southern Biotechnical Laboratory, Birmingham, AL, USA). Gap junction distribution in adrenal preparations was visualized with a Zeiss-LSM confocal microscope or Nikon Microphot FXA fluorescence phase microscopes. Images were collected from the microscope interfaced to an Optimus Image Analysis (Media Cybernetics, Silver Springs, MD, USA) program run on an IBM computer.

**Histochemistry**

For light microscopy, the tissue was sectioned on a cryostat (Minotome; International Equipment Co., Boston, MA, USA). Sections were collected on gelatinized slides and stained with either hematoxylin–eosin or Oil Red O staining (Troyer 1980).
hematoxylin (Troyer 1980). Images were captured with an Optimas Image Analysis program interfaced to an IBM computer.

**Figure 1** Demonstration of dye communication in an adrenal cell culture monolayer. Cells in culture communicate Lucifer yellow dye four to five cells from the cut edge.

**Figure 2** Immunohistochemical localization of α1-Cx43 gap junction antigen (A and C) and Lucifer yellow dye (green in B and D) in two representative mouse adrenal gland sections. Extensive expression of α1-Cx43 is observed primarily in the zonae fasciculata/reticularis (ZF/ZR), ZG, zona glomerulosa; arrowheads, connective tissue capsule; arrows, trabeculae; blue stain, nucleus. (A and B) bar = 50 µm; (C and D) bar = 30 µm.

**Figure 3** Lucifer yellow dye coupling in adrenal cortex. The dye is excluded from the cells of the zona glomerulosa but is evident in the connective tissue capsule (arrowheads) and trabeculae (arrow). Note that numerous individual cells and packages of cells are not coupled to neighboring cells (as indicated by asterisks in B). (A) bar = 10 µm; (B) bar = 20 µm.

**Stereological methods**

The number of gap junctions was determined by computer-assisted image analysis of the immunolabeled adrenal glands. Gap junction number in immunohistochemically prepared samples was expressed either as number of plaques per measured area or as the levels of α1-Cx43 fluorescence per measured area. To quantitate the dye within the adrenal cortical zones, computer-assisted image analysis of the Lucifer yellow dye fluorescence was measured with the Optimas program area morphometric function. Statistical comparisons of mean values were carried out with a one-way analysis of variance and Duncan’s multiple comparison procedure. To determine the significance of differences, Student’s two-tailed t-test was also employed.

**Results**

Proceeding from the observations that cells in monolayer culture communicate Lucifer yellow dye from the cut edge (Fig. 1), we have modified the technique of Tsien & Weingart (1976) in order to study the relationship between gap junction protein expression and cell–cell communication patterns in intact adrenal tissue from two
samples. As assessed in this ‘gland dye coupling’ assay, Lucifer yellow dye was found to be communicated extensively in the inner two cortical zones (zonae fasciculata and reticularis) (Figs 2–4). A similar distribution of gap junctions was revealed with immunohistochemical analysis (Figs 2, 4 and 5). Remarkably, both dye coupling and gap junction plaques were virtually absent among cells of the outermost cortex (zona glomerulosa) (Figs 2 and 4). At the interface of the zona fasciculata and zona glomerulosa, we observed cells and/or packets of cells which lacked dye and therefore were not thought to be functionally coupled to other cells in the region (Fig. 3). The number of gap junction plaques was counted by computer-assisted image analysis and, in addition, the extent of the staining was also measured as a function of fluorescence intensity per area in order to determine gap junctional distribution. When compared, the fluorescence indices of Lucifer yellow dye communication and that of the gap junction plaques were almost directly proportional throughout the three cortical zones (Figs 2 and 4).

The presence of Lucifer yellow within the ovoid clusters of zona glomerulosa cells was rare and, when evident, it was typically confined to a single cell within a given packet of cells (Fig. 3). Although dye was clearly evident at the cut edge of injured zona glomerulosa cells, dye did not enter neighboring cells (Figs 6 and 7). Under identical experimental conditions, extensive dye communication occurred between cells of the inner two cortical zones.

As anticipated, incubation of excised adrenal glands in oleamide, a potent gap junction communication inhibitor (Boger et al. 1998), eliminated most appreciable dye movement except in small foci of tissue injury (Fig. 8). Occasional dye communication could be seen within the inner two adrenal cortical zones of oleamide-incubated adrenal glands (Fig. 8C). Tissue next to injury sites in the presence of oleamide did not contain dye or appeared to contain less dye then that observed in non-oleamide-treated controls.

In the adrenal capsule, an abundance of gap junction plaques was evident, as was the transfer of Lucifer yellow dye (Figs 2 and 3). However, neither the connective tissue cells of the capsule nor the penetrating trabeculae appear to communicate dye to cells of the zona glomerulosa (Fig. 3). Dye in the adrenal connective tissue capsule or penetrating trabeculae was rarely transferred to cells within the cortex, and the zona glomerulosa cells were consistently excluded from the extensive coupling of cells in the zona fasciculata. This was most apparent in the monkey adrenal cortex (Fig. 9). Although not shown here, it should be noted that medullary staining with Lucifer yellow was highly inconsistent and, when present, seemed to be within blood vessels or non-specific staining.

The observed close correlation between the zonal distribution of gap junctions and dye communication, observed in the mouse, was also evident in the freshly excised monkey adrenal gland (Fig. 9). In the monkey, as in the mouse, dye communication was observed between cells located in the zona fasciculata and reticularis.
However, the zona glomerulosa cells neither communicate with one another nor with cells of the connective tissue capsule (Fig. 9). Occasionally, individual cells within an ovoid cluster of zona glomerulosa cells contained Lucifer yellow dye (Fig. 9). Thus, the similarity of the dye distribution and gap junctions observed in the monkey tissue validates our findings in the mouse adrenal glands.

Discussion

The studies presented here were designed to monitor the movement of a gap junction-permeable molecule between cells of the adrenal gland and to assess the presumptive role of gap junctions in making such movement possible. Using immunohistochemistry for gap junction visualization and Lucifer yellow dye coupling assay, we have assessed these processes in the intact cells of the freshly excised adrenal glands. The techniques applied here have preserved the adrenal anatomical zonation so critical to secretory function and have permitted all cell types within the adrenal cortex to be exposed to identical conditions of preservation, staining and analysis, thus providing direct comparisons of dye transfer and gap junction expression.

A considerable body of evidence has been presented to demonstrate that gap junctions are indeed the conduits for intercellular transfer of small molecules (Goodenough 1975, 1978, Peracchia 1980, Hertzberg et al. 1981, Willecke et al. 1983, TenBroek et al. 1994, Nagy et al. 1996). Most of this evidence comes from in vitro studies of primary cell cultures or cell lines (Murray & Fletcher 1984, Bouille et al. 1991, Baldwin & Calabrese 1994, Munari-Silem et al. 1995, Veenstra 1996, Willecke & Haubrich 1996, Tomai et al. 1999). Because cell functions in cultures of immortalized cell lines are, by definition, significantly altered as are the three-dimensional relationships of primary cells in culture compared with the intact gland, assessment of in vivo conditions is often compromised.

By utilizing the organ system, we have been able to compare directly the patterns of dye distribution and α1,CX43 gap junction location. We found that there is a regional specificity of dye transfer in the adrenal cortex such that there is little or no dye transfer in the zona glomerulosa and extensive transfer in the inner two zones. Furthermore, although the connective tissue cells have α1,CX43 gap junction plaques and appear to readily communicate with one another, they lack a comparable communication with the cells of the zona glomerulosa. In the inner zones, the relationship was less clear, given the fact that virtually all cells were clearly labeled and communication of dye between connective tissue cells or between connective tissue cells and the cortical parenchyma could not be distinguished.

The areas of the adrenal gland that we find to efficiently transmit dye and to exhibit numerous α1,CX43 gap junctions are characterized by their production of glucocorticoids and androgens in response to ACTH (Garren et al. 1971, Le Roy et al. 2000). This is of particular interest given the observations that ACTH treatment increased α1,CX43 gap junction plaques in primary cell cultures (enriched for the two inner cortical adrenal zones), and in established adrenal cell lines, as well as in adrenal glands of hypophysectomized mice (Murray et al. 2000b). The ACTH-induced increase in α1,CX43 gap junction protein levels has been correlated with a decrease in proliferation in adrenal cell lines (Oyoyo et al. 1997, Shah & Murray 2001), and it is suggested that the increased communication of regulatory molecules through gap junctions following ACTH stimulation plays a pivotal role in hormone responsiveness, differentiation and cytogenesis (Decker 1976, Usadel et al. 1993, Murray et al. 2000a,b).

Cytogenesis in the adrenal is generally thought to involve the migration and differentiation of cells from the outer to the inner cortex (Salmon & Zwemer 1941,
Bertholet 1980, Zajicek et al. 1986). A functional transition zone has been described in the rat adrenal gland in which cells differ both morphologically and physiologically from either zona fasciculata or zona glomerulosa cell type (Mitani et al. 1994). Certainly, if such a migration does occur a transition zone should exist in which both, or neither, zona glomerulosa and zona fasciculata type cells would be found. In our study, such a transition zone was also evident to the extent that intercellular dye transfer in the outermost zona fasciculata was inconsistent. The majority of cells in this area were coupled while other single cells or packets of cells were uncoupled. The significance of such a zone is not known but we now report that gap junction plaques in this transition area are less abundant than those located in deeper adrenal cortical zones and that intercellular communication within this area was more variable. The relationships between endocrine function, cytogenetic fate and dye communication of these uncoupled cells remains to be elucidated. The observation that some of the cells of the outer zona fasciculata were uncoupled, however, would suggest that these cells do not exchange low molecular weight molecules with their neighbors. These cells may be zona fasciculata cells that are devoid of gap junctions, zona

Figure 7 Lucifer yellow dye coupling in the adrenal cortex. Lucifer yellow dye diffused into damaged cells at the cut edge but did not transfer to cells deep within the zona glomerulosa (ZG) (A and B). In contrast, Lucifer yellow dye was observed deep within the inner zones (zonae fasciculata/reticularis, ZF/ZR). (C) This illustrates that rhodamine (Rhod)-dextran, which exceeds gap junctional pore size, is limited to damaged cells at the cut edge. The nuclei of cells seen in (B) and (C) were stained with Hoescht dye (D). Bar=50 µm.

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glomerulosa cells devoid of gap junctions, or cells that possess gap junction channels which are in a closed conformation. The latter hypothesis seems more likely since it has been demonstrated in thyroid cell populations that communication was established in a sub-population of thyroid cells following hormone treatment (Munari-Silem et al. 1991).

We have previously proposed that cell–cell communication may be of particular importance for hormonally responsive glandular tissues (Murray & Fletcher 1984, Oyoyo et al. 1997). In such tissues, individual cells with a decreased number of cell surface receptors might be less compromised if neighboring cells were able to communicate the presence of hormonal stimulation and initiate the process of amplification. In addition, the selective communication of molecules through gap junctions may inhibit some cellular activities, i.e. proliferation (Loewenstein & Rose 1992, Tsuda et al. 1995, Shah & Murray 2001), and thus direct cellular activities toward those metabolic pathways used in steroidogenesis and/or cellular differentiation. It appears that the cells of the inner zones have a greater capacity for, and presumably a greater functional dependence on, intercellular communication than cells of the zona glomerulosa. The specific benefits derived from the presence of such communication is not totally clear, but might include the ability to control proliferation and the capacity to transmit hormonal signaling between cells.

From a more technical standpoint, the importance of these studies may relate to the fact that we have demonstrated the specificity of dye movement within a single ‘block’ of tissue. The distribution of dye movement appears to be precisely correlated with the presence of gap junctions, a pattern which we interpret as a demonstration that cell damage at the cut edge of the tissue block alone is not adequate to promote cell–cell transfer of Lucifer yellow dye. The fact that this relationship holds true in a primate adrenal cortex, although limited to a small sample,
suggests that the technique provides the basis for a more comprehensive study in primates as an antecedent to possible clinical applications.

Based on our demonstrations of zone-specific patterns of dye communication and gap junction distribution in the adrenal cortex, it follows that there exists a regional potential for communication of regulatory material. The observed variations within the adrenal cortex may prove to be relevant to normal physiological regulation of the adrenal gland, especially as it relates to regulation of proliferation, regeneration and ACTH-stimulated steroidogenesis. We anticipate that the continual application of the methods reported here may increase our insight into both physiological and pathological processes.

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