A rat parotid gland cell line, Par-C10, exhibits neurotransmitter-regulated transepithelial anion secretion.

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USSING CHAMBER STUDIES with permanent cell lines of epithelial origin have contributed significantly to our understanding of ion secretory processes and their regulation. These cell lines include T84 (colon; Ref. 5) and Madin-Darby canine kidney (MDCK; Ref. 17) among others. However, no cell line of salivary gland origin, either acinar or ductal, has been reported to be useful in Ussing chamber studies of transepithelial ion movements and their regulation by neurotransmitters.

Recently, we reported the establishment of simian virus 40-transformed cell lines of rat parotid (15) and submandibular (14) gland acinar origin. Although not without some inconsistencies, these cell lines exhibit a substantial degree of fidelity to the cell type of origin, including morphological, biochemical, and functional characteristics. Among these is the expression of receptors for salivary gland-relevant neurotransmitters, including norepinephrine, acetylcholine, vasoactive intestinal peptide, and extracellular nucleotides. In this report, we describe experiments indicating the suitability of one of the parotid gland cell lines, Par-C10, in Ussing chamber studies, which revealed the presence in these cells of an anion-dependent short-circuit current (Isc) that is regulated by neurotransmitters.

METHODS

Cell culture. Par-C10 cells (5 x 10⁵) were plated on Falcon cell culture inserts (diameter 2.4 cm, pore size 0.4 µm; Becton Dickinson, Franklin Lakes, NJ) coated with 0.10 mg/ml bovine type I collagen. The cultures were grown to confluence in 3–4 days and developed transepithelial resistance values of ≥2,000 Ω·cm². Morphological examination revealed that Par-C10 cells grew as polarized monolayers exhibiting tripartite junctional complexes and the acinar cell-specific characteristic of secretory canaliculi. Par-C10 Isc was increased in response to muncaricin cholinergic and α- and β-adrenergic agonists on the basolateral aspect of the cultures and to ATP and UTP (through P2Y₂ nucleotide receptors) applied apically. Ion replacement and inhibitor studies indicated that anion secretion was the primary factor in agonist-stimulated Isc. RT-PCR, which confirmed the presence of P2Y₂ nucleotide receptor mRNA in Par-C10 cells, also revealed the presence of mRNA for the cystic fibrosis transmembrane conductance regulator and CIC-2 chloride channel proteins. These findings establish Par-C10 cells as the first cell line of salivary gland origin useful in transepithelial ion secretion studies in Ussing chambers.

parotid salivary gland; cell culture; Ussing chamber; ion secretion; P2Y₂ receptors; polarized epithelia
temperature at 37°C, and the medium in both reservoirs was mixed and oxygenated by bubbling with 95% O2-5% CO2. $I_{sc}$ was measured continuously, and transepithelial potential difference was measured intermittently, using a VCC-600 automatic voltage-clamp apparatus (Physiologic Instruments, San Diego, CA) and calomel electrodes connected to the chamber baths with 4% agar-KCl bridges. $I_{sc}$ and automatic fluid resistance compensation current were applied through Ag-AgCl electrodes connected to chamber baths with 4% agar-KCl bridges. Resistance measurements were made by occasionally clamping the potential difference to a known voltage and measuring the current required to establish the potential. Resistance and conductance were calculated using Ohm’s law. $I_{sc}$ values are expressed as the peak change obtained in response to agonist (µA/cm²) or alternatively as the area under the curve for the first 2 min following agonist addition (arbitrary units) to incorporate differences in the sustained phase of the response.

RT-PCR. Total RNA was prepared from confluent Par-C10 cultures in 100-mm-diameter dishes (passages 60) obtained in response to agonist (µA/cm²) or alternatively as indicated in Table 1. As a positive control for the P2Y6 primer set, cDNA was prepared from total RNA isolated from 1321N1 cells heterologously expressing the P2Y6 receptor and amplified as described above. Aliquots from the PCR reactions were electrophoresed on agarose gels. The remaining PCR products were purified directly from the reaction mixture using a Wizard PCR Prep DNA purification system (Promega, Madison, WI) and sequenced with specific internal primers and fluorescent chain terminator dNTPs on an Applied Biosystems sequencer (Perkin-Elmer, Foster City, CA).

Data analysis. The statistical significance of differences between mean values was determined by unpaired Student’s $t$-test or by one-way ANOVA and Student-Newman-Keuls post hoc test. Differences with $P \leq 0.05$ were considered significant.

Materials. The following reagents were purchased from the indicated sources: fetal bovine serum, GIBCO BRL; epidermal growth factor, Collaborative Research (Bedford, MA); gentamicin, Fujisawa (Deerfield, IL); glutaraldehyde and OsO4, EMCorp (Chestnut Hill, MA); DNase, Boehringer-Mannheim (Indianapolis, IN); and Vent and Vent(exo−) DNA polymerases, New England Biolabs (Beverly, MA). All other reagents were obtained from Sigma Chemical (St. Louis, MO).

RESULTS

Development of transcellular resistance in Par-C10 cell cultures grown on permeable supports. As shown in Fig. 1, transcellular resistance across Par-C10 cell cultures grown on collagen-coated permeable supports increases as a function of time, approaching a maximum by 4 days. This time course was similar to that for cell proliferation, wherein confluence was typically attained after 3 days in culture. Par-C10 cultures grown on supports not coated with collagen or coated with other matrix components exhibited similar transcellular resistances at confluence, as did another parotid cell line, Par-C5, immortalized by the same technique used for Par-C10 (15). However, our initial

![Graph showing transcellular resistance over time](image-url)
Fig. 2. Morphological aspects of Par-C10 cells cultured on permeable supports. Shown are transmission electron micrographs of Par-C10 cells cultured with (A, C, and D) or without (B) cholera toxin and processed for morphological evaluation as described in METHODS. Arrowhead, tight junction component of a tripartite junctional complex; arrows, membrane pores containing cell processes; c, secretory canaliculi; g, secretory granules; r, rough endoplasmic reticulum; w, terminal web. Magnifications: A, ×3,900; B, ×13,400; C, ×5,700; D, ×24,800.
investigations with these two parotid cell lines, as well as two immortalized submandibular gland cell lines, SMG-C6 and SMG-C10 (14), revealed that Par-C10 cells typically displayed the highest transcellular resistance values and exhibited the most polarized phenotype. As a result, we focused on the Par-C10 cell line for the studies described in this paper.

Morphological evaluation. By transmission electron microscopy, confluent Par-C10 cell cultures consisted of a monolayer of plump cells attached to the collagen layer on the upper surface of the microporous membrane insert (Fig. 2A). The cells contained scattered secretory granules with a substructure that was mostly scanty but occasionally dense (Fig. 2, A and B). None of these was observed to be in the process of exocytosis. Cell processes extended into the pores of the membranes, some all the way to the bottom (Fig. 2, A and C), but did not spread out on the bottom surface. The cells were joined along their apical plasmalemmas by tripartite junctional complexes, consisting of tight and intermediate junctions and several desmosomes (Fig. 2, A and D). A terminal web was associated with the tight junctions (Fig. 2D). Numerous intercellular crevices were observed that had lumina segregated by junctional complexes (Fig. 2, A and D) and thus could be distinguished from ordinary intercellular spaces as secretory canaliculi (22). The plasmalemmas on the apical surfaces and lining the canaliculi were studded with small microvilli. The cytoplasm was rich in free ribosomes and also contained numerous mitochondria and dilated profiles of rough endoplasmic reticulum (Fig. 2, B and D). The only difference observed between the cells cultured with and without cholera toxin was that, in the latter, secretory granules occurred in clusters more frequently.

Agonist-induced changes in $I_{sc}$. We reported previously that Par-C10 cells are responsive, in terms of mobilization of second messengers, to salivary gland-relevant receptor agonists, including the muscarinic cholinergic agonist carbachol, the $\beta$-adrenergic receptor agonist isoproterenol, the $\alpha$-adrenergic receptor agonist phenylephrine, and the P2 nucleotide receptor agonists ATP and UTP (15). Representative tracings of Par-C10 monolayer $I_{sc}$ responses to maximally effective concentrations of three Ca$^{2+}$-mobilizing agonists, UTP, carbachol, and phenylephrine, are presented in Fig. 3. As shown, UTP, when applied to the medium bathing the apical side of the monolayer, was the most efficacious of the three agents, whereas carbachol was more effective than phenylephrine when these latter two agents were applied basolaterally. The addition of UTP to the basolateral side or of carbachol or phenylephrine to the apical side was without effect on $I_{sc}$. The patterns and magnitudes of response shown in Fig. 3 for these three agonists were consistent across a wide range of passage numbers of Par-C10 cells. Conversely, although initial studies with the cAMP-mobilizing agonist isoproterenol (applied basolaterally) revealed a strong enhancement of $I_{sc}$, subsequent experiments revealed little or no $I_{sc}$ response to this $\beta$-adrenergic receptor agonist, despite the fact that cAMP production in response to isoproterenol was similar among the various cell preparations used. Thus, although many of the characteristics of Par-C10 cells appear to be maintained, others appear to be more variable.

The potency and efficacy of the naturally occurring nucleotide receptor agonists ATP and UTP for increasing Par-C10 cell monolayer $I_{sc}$ were similar following apical addition (Fig. 4). EC$^{50}$ values were $0.46 \pm 0.11$ and $1.2 \pm 0.4 \mu$M for ATP and UTP, respectively. These results, combined with the lack of effect of UTP basolaterally, suggest that the P2Y$_{2}$ subtype of nucleotide receptor is expressed in Par-C10 cells (see also Fig. 8), that this expression is limited to the apical membrane of these cells, and that this receptor can account for all of the effects of apically applied ATP and UTP on $I_{sc}$. It is important to note that ATP, in contrast to UTP, is also effective in increasing $I_{sc}$ following basolateral addition (data not shown), an effect mediated by other P2 receptor subtypes that we are currently characterizing. Finally, Fig. 4 also shows the concentration-response curve for carbachol stimulation of $I_{sc}$, which gave an EC$^{50}$ value of $52 \pm 29 \mu$M, similar to values obtained for...
this agonist in a variety of tissues, including other salivary gland preparations (e.g., Refs. 3, 18).

The ionic basis of the $I_{sc}$ and the role of Ca$^{2+}$ are as follows. As in many other cell types, P2Y$_2$ nucleotide, muscarinic cholinergic, and $\alpha_1$-adrenergic receptors in Par-C10 cells are coupled through phospholipase C to increases in the intracellular Ca$^{2+}$ concentration (15), a process that involves both the mobilization of intracellular stores and the influx of extracellular Ca$^{2+}$. We therefore examined the effect of removal of Ca$^{2+}$ from the basolateral and apical media on agonist-induced increases in $I_{sc}$. As shown in Fig. 5, the magnitude of the response to apically applied UTP (A) and basolaterally applied carbachol (B), expressed as the area under the curve for the first 2 min following agonist addition, was unaffected when the Ca$^{2+}$ concentration was lowered into the nanomolar range in the medium bathing the apical side of the monolayers. Conversely, lowering the basolateral medium Ca$^{2+}$ concentration dramatically decreased, by 50% or more, the ability of both agonists to produce the sustained increases in $I_{sc}$ observed in the presence of Ca$^{2+}$. The effect of lowering the Ca$^{2+}$ concentration in both baths simultaneously was not greater than that observed with the decrease in only the apical medium. This finding indicates that the response to both agonists is dependent on the availability of extracellular Ca$^{2+}$ and that this Ca$^{2+}$ is available to the cell only from the basolateral side.

The $I_{sc}$-enhancing effects of Ca$^{2+}$-mobilizing agonists in a variety of other epithelia are due to increases in apical anion secretion, i.e., Cl$^-$ and HCO$_3^-$ (20). A view toward defining the ionic nature of agonist-induced increases in Par-C10 cell $I_{sc}$ responses to apically applied UTP in media lacking Cl$^-$ or HCO$_3^-$ or both were compared with the effects observed in the presence of both anions in the apical and basolateral media. As shown in Fig. 6A, the removal of Cl$^-$ decreased both the maximum level and the duration of the UTP-induced increase in $I_{sc}$, compared with the response obtained in the presence of Cl$^-$ (Fig. 6A). Furthermore, the omission of HCO$_3^-$ from Cl$^-$-containing medium (Fig. 6B) also resulted in a marked decrease in the response to UTP, and the omission of both anions (Fig. 6B) resulted in only a slight, transient increase in $I_{sc}$. As summarized in Fig. 6C, $I_{sc}$ responses to UTP were decreased by $\sim$50% when either HCO$_3^-$ or Cl$^-$ was omitted and by $\sim$90% in the absence of both anions. Conversely, the replacement of Na$^+$ with N-methyl-D-glucamine or choline, or the inclusion of amiloride in the apical medium, had no effect on agonist-induced increases in $I_{sc}$ (data not shown).
To confirm the observations presented above, which suggest that Par-C10 I\textsubscript{sc} is dependent on HCO\textsubscript{3}{\textsuperscript{-2}} and Cl\textsuperscript{-2}, the effects of three inhibitors of anion transport on UTP- and carbachol-stimulated I\textsubscript{sc} were examined. As shown in Fig. 7A, apically applied diphenylamine-2-carboxylic acid, DIDS, and 5-nitro-2-(3-phenylpropylamino)benzoic acid were all effective in decreasing significantly the I\textsubscript{sc} response to UTP, whereas basolateral addition of these inhibitors was without effect. The same inhibitory pattern was observed when carbachol (applied basolaterally) was used as the agonist. Taken together, the results presented in Figs. 6 and 7 and the lack of effect of amiloride or Na\textsuperscript{+} removal on agonist-stimulated I\textsubscript{sc} strongly suggest that apical anion secretion underlies the changes in I\textsubscript{sc} elicited by UTP and carbachol.

RT-PCR analysis for P2Y receptor subtypes and anion transporters in Par-C10 cells. We have confirmed the presence of muscarinic cholinergic receptors in Par-C10 cells with radioligand binding assays and by demonstrating the effectiveness of atropine in blocking carbachol-stimulated Ca\textsuperscript{2+} mobilization and I\textsubscript{sc} (data not shown). Because there is no radioligand binding assay or high-affinity selective antagonist for the P2Y\textsubscript{2} receptor, we used RT-PCR detection of the mRNA for the P2Y\textsubscript{2} receptor to support the pharmacological identification (Fig. 4 and Ref. 15) of this subtype in Par-C10 cells. As shown in Fig. 8, RT-PCR with primers specific for the P2Y\textsubscript{2} receptor mRNA produced a product of the correct size (778 bp) that was >99% identical to the published rat P2Y\textsubscript{2} receptor sequence. A recent study has indicated that another uridine-nucleotide-preferring P2Y receptor subtype, P2Y\textsubscript{6}, is expressed, along with P2Y\textsubscript{2} receptors, in the apical membrane of another epithelial cell type (12). However, RT-PCR with primers specific for the P2Y\textsubscript{6} receptor mRNA amplified no appropriately sized (871 bp) product from cDNA prepared from Par-C10 cell total RNA (Fig. 8), whereas a product of appropriate size and sequence was obtained with cDNA prepared from 1321N1 cells heterologously expressing the P2Y\textsubscript{6} receptor. These results suggest that P2Y\textsubscript{6}...
receptors are not expressed in Par-C10 cells. A faint smaller band, amplified from Par-C10 cell RNA with the P2Y$_6$ primers in the presence of RT, is the same size as a more abundant band obtained with these primers in rat aortic smooth muscle cells. The sequence from the smooth muscle cells is unrelated to mRNAs encoding any known receptor proteins (data not shown). As is also shown in Fig. 8, RT-PCR with primers specific for two of the anion-transporting proteins identified previously in salivary gland acinar cells, the cystic fibrosis transmembrane conductance regulator (CFTR) (25) and the CIC-2 Cl$^-$ channel (1), gave products of the appropriate sizes (352 and 755 bp, respectively) and sequences, suggesting that these anion channels are also expressed in Par-C10 cells.

DISCUSSION

One of the goals of salivary gland research at the cellular level has been to understand the pathways involved in the ion transport processes essential to saliva formation and modification. The understanding of equivalent processes and their regulation in other epithelia has been facilitated by the availability of permanent cell lines that exhibit sufficient polarization, transepithelial resistance, and other features that make them suitable for bioelectric measurements in Ussing chambers. Two particularly useful examples are the T84 (colon; Refs. 5, 11, 23) and MDCK (kidney; Refs. 6, 17, 21) cell lines. The Par-C10 cell line is, to our knowledge, the first cell line of salivary origin that can be reliably utilized in Ussing chambers. Furthermore, Par-C10 cells (Ref. 24, but see Ref. 9) appear to express both CFTR and CIC-2 (Fig. 8) and possibly other anion transporting proteins relevant to normal salivary acinar cells (1, 25). In addition, Par-C10 cells appear to express both CFTR and CIC-2 (Fig. 8) and possibly other anion transporting proteins relevant to normal salivary acinar cells (1, 25). The results with anion transport inhibitors (Fig. 7) suggest an exclusively apical distribution for these proteins, consistent with observations made in normal salivary acinar cells. Conversely, Par-C10 cells are not diminished by inhibitors of Na$^+$-K$^+$-2Cl$^-$ cotransport such as bumetanide (Camden and Turner, unpublished observations), a finding considered more reflective of salivary duct cells than acinar cells (13). In addition, as reported previously (13), functional substance P receptors apparently are not expressed in Par-C10 cells, whereas evidence suggests that these receptors are found in normal salivary acinar but not ductal cells (4, 20). Finally, Par-C10 cell monolayers develop high transepithelial resistances, similar to values obtained with MDCK (11, 23) and T84 cells (6, 21), and thus do not fit the classical definition of salivary acini as a “leaky” epithelium. Nonetheless, the suitability of Par-C10 cultures for studies of transepithelial ion movement and its regulation promises to open new avenues for studying salivary gland secretion.

For the most part, agonist (UTP, carbachol, and phenylephrine) effects in terms of second messenger production (15) and increased $I_{sc}$ (Figs. 3–7) were found to be reasonably consistent across at least 30 cell passages. One marked exception to this observation was the effect of the $\beta$-adrenergic receptor agonist isoproterenol on $I_{sc}$, which varied from an increase similar to that obtained with maximally effective concentrations of UTP to no response at all, in various Par-C10 cultures (data not shown). This variability was paralleled by similar changes in the response to forsk-
lin, whereas all of the cultures exhibited robust cAMP responses to isoproterenol and forskolin, suggesting that the expression of a component in the pathway downstream of the β-adrenergic receptor, Gs, and adenylyl cyclase, possibly CFTR itself, may be altered. The decrease in the responsiveness to cAMP-mobilizing agents is not simply a function of passage number but appears to involve subtle differences in culture conditions or medium constituents, including cholera toxin, which we have used routinely in an attempt to promote a differentiated state of the Par-C10 cells. The basis for the differences in apparent CFTR activity requires investigation.

The above caveats notwithstanding, the Par-C10 parotid acinar cell line exhibits a number of acinar cell-like features as well as the characteristic, unique among salivary cell lines, of sufficient polarization and transepithelial resistance to be useful in Ussing chambers. It is hoped that this cell line will prove as beneficial to salivary gland research as similar cell lines have been in studies of other organ systems with important epithelial components.

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