INTRODUCTION

Salivary glands are important organs of the upper gastrointestinal system and saliva is critical to the maintenance of oral health. Salivary gland hypofunction enhances the risk of oral and systemic disease and results in a deterioration in the ability to chew, swallow and speak (1). The secretion of saliva helps to maintain oral health by lubricating and hydrating the mucosal surfaces and by protecting the oral cavity from mechanical and chemical stress and microbial invasion. The quality of life for millions of people is adversely affected by salivary gland dysfunction associated with radiotherapy, drug therapies, Sjogren's syndrome, cystic fibrosis, diabetes mellitus or smoking (2-7). The development of interventions to improve salivary gland function for these individuals requires a thorough understanding of the molecular physiology of salivary secretion.

Salivary secretion couples the osmotic flow of water to the active transport of electrolytes, mainly Cl⁻ and bicarbonate. Specific ion channels, cotransporters, exchangers and water channels have been identified as key transporters in the secretion and reabsorption of Cl⁻ (8-10) but their involvement in salivary HCO₃⁻ secretion is not well understood. HCO₃⁻ ions in saliva play a vital role in buffering the acid generated from dietary sugars by oral bacteria, thus helping to minimise tooth decay. Salivary glands are capable of secreting HCO₃⁻ at concentrations up to 60 mM (8, 9, 11). Although it is known that secretion involves both acinar cells and ductal cells, and that various acid and base transporters are expressed by these cells, it is not clear how they work together to achieve net HCO₃⁻ secretion nor how the process is regulated (8, 9).

Our ultimate goal is therefore to determine these mechanisms using cellular physiological studies to evaluate their contribution to secretion. Because of the difficulty of obtaining normal human tissue, we have to evaluate and develop experimental models based on cell lines (12-14). Of those available, the well-differentiated Par-C10 cell line appears to offer many of the functional characteristics of normal parotid acinar cells. The cell line is derived from rat parotid acini, immortalized with Simian virus 40 (15). It has been shown to form well-differentiated epithelia (15), to express muscarinic and adrenergic receptors and to perform active anion secretion (16). The specific objectives of the present work were 1) to use physiological assays to determine the molecular mechanism of pH regulation and HCO₃⁻ secretion in Par-C10 cells, and 2) to characterize the intracellular signalling pathways that regulate these processes.

METHODS

Materials

Culture medium (Dulbecco's Minimal Essential Medium (DMEM):Ham's F12 1:1 mixture), fetal bovine serum, trypsin-EDTA, penicillin-streptomycin, retinoic acid, triiodothyronine,
hydrocortisone, 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS), amiloride, 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), nigericin, bumetanide, forskolin and ATP were from Sigma. Dulbecco's phosphate-buffered saline, 2',7-bis-(2-carboxyethyl)-5- and-6)-carboxyfluorescein-acetoxy methyl ester (BCECF-AM), 4,4'-dinitrostilbene-2,2'-disulphonic acid (DNDS), dihydro-4,4'-diisothiocyanostilbene-2,2'-disulfonate H_2DIDS were from Invitrogen.

**Cell culture**

The rat parotid cell line Par-C10 was a generous gift from Dr. David Quissel (School of Dentistry, University of Colorado Health Sciences Center, Denver, CO, USA). The experiments were performed on cells of passage number 3-15. Cells were grown in DMEM: Ham's F12 medium supplemented with 10% fetal bovine serum, 50 U ml^{-1} penicillin, 50 µg ml^{-1} streptomycin, 0.1 µM retinoic acid, 2 mM triiodothyronine and 0.4 µg ml^{-1} hydrocortisone. Cells were plated on permeable polyester supports (Transwell Clear, Costar, Corning Inc., NY, USA) with an area of 1.0 cm² and 0.4 µm pore size, and incubated at 37°C with 5% CO_2 in air for 8-13 days until the monolayers reached confluence. Confluence was assessed by measuring the transepithelial electrical resistance (TER) with an epithelial voltohmeter (EVOM, World Precision Instruments, Hamden, CT, USA).

**Solutions**

The HCO_3^- free, Heps-buffered solution contained (in mM): 137 NaCl, 5 KCl, 1 CaCl_2, 1 MgCl_2, 10 D-glucose and 10 HEPES, equilibrated with 100% O_2. The standard HCO_3^- buffered solution contained (in mM): 116 NaCl, 25 NaHCO_3, 5 KCl, 1 CaCl_2, 1 MgCl_2, 10 D-glucose and 5 HEPES, equilibrated with 5% CO_2/95% O_2. The Na^+ free and Cl^- free solutions contained equimolar NMDG and gluconate in place of Na^+ and Cl^- respectively. In solutions containing Na^+_HCO_3^-, the concentration of Na^+ was reduced to maintain constant osmolarity. All solutions were adjusted to pH 7.4 at 37°C.

**Measurement of short-circuit current**

Confluent cultures of Par-C10 cells on permeable supports were mounted in purpose-built Ussing chambers for measurement of agonist-induced changes in short-circuit current (I_SC). The apical and basolateral surfaces of the monolayers were superfused at 1.5 ml min^{-1} with pre-warmed, oxygenated buffer solutions. Transepithelial potential difference was measured with Ag-AgCl electrodes, connected to chamber baths via 4% agar-KCl bridges, using an epithelial voltage clamp amplifier (EC-825, Warner Instruments, Hamden, CT, USA). I_SC was measured continuously by voltage clamp using Ag/AgCl-wire current electrodes, and TER was measured intermittently in the Ussing chamber by voltage clamping to small positive and negative potentials.

**Measurement of intracellular pH**

Intracellular pH was measured by microfluorometry as described in detail elsewhere (17). Briefly, polarized monolayers of Par-C10 cells were mounted in a temperature-controlled perfusion chamber on the stage of a Nikon Eclipse TE200 inverted microscope. The cells were preloaded with the pH-sensitive fluoroprobe BCECF by incubation for 30 min in HCO_3^- buffered solution containing the acetoxymethyl ester BCECF-AM (2 µM) at 37°C. The chamber was bilaterally perfused at 2 ml min^{-1} with either Heps- or HCO_3^- buffered solution at 37°C. A small region of epithelium, of fixed size and containing a few hundred cells, was illuminated alternately at 440 and 490 nm, and the fluorescence intensities (F_{440} and F_{490}) were measured at 530 nm. Intracellular pH was calculated from the F_{440}/F_{490} ratio using calibration data that were obtained using the nigericin-K^+ method (18).

**Statistics**

Data are presented as means±SEM. For statistical comparisons Student's t-tests were performed, or ANOVA followed by Bonferroni test, as appropriate. Rates of change in pH were determined by fitting a 4th-order polynomial to the data and calculating the initial gradient (19).

**Abbreviations**

BCECF: 2',7'-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein; CFTR: cystic fibrosis transmembrane conductance regulator; EIPA: 5-(N-ethyl-N-isopropyl)-amiloride; H_2DIDS: dihydro-4,4'-diisothiocyanostilbene-2,2'-disulphonic acid; NMDG: N-methyl-D-glucamine; NBC: Na^+-HCO_3^- cotransporter; NHE: Na^+/H^+ exchanger; NKCC: Na^+-K^-2Cl^- cotransporter; pH_i intracellular pH.

**RESULTS**

**Short-circuit current measurements**

The mean TER of the Par-C10 cell monolayers under control conditions was 2552±59 Ωcm² (n=38). In the HCO_3^- buffered solution, only a small I_SC was detectable in the absence of stimulation (0.88±0.11 µA cm², n=26, range -0.82 to 5.07 µA cm²). However, apical administration of ATP (50 µM) caused a biphasic increase in I_SC with a mean peak value of 3.31±0.21 µA cm⁻² (Figs. 1B and 1C). This change in I_SC is consistent with the previously reported increase in anion secretion evoked by nucleotides and other Ca²⁺-mobilizing agonists (16).

Apical application of forskolin (10 µM), which stimulates adenylyl cyclase and thus elevates intracellular cAMP, caused a similar response: I_SC increased rapidly to a peak, and then declined to a lower, sustained level until forskolin was withdrawn. The pattern was similar in the absence and presence of HCO_3^- (Figs. 1B and 1C respectively). However, the increase in I_SC (ΔI_SC) evoked by forskolin was significantly greater in the presence of HCO_3^- (Fig. 1F). These results indicate the presence of a cAMP-stimulated anion secretory mechanism in Par-C10 cells and they suggest that HCO_3^- has an important role, either as a secreted anion itself or by supporting HCl^- secretion.

An increase in I_SC could be due to an increase in cation absorption rather than, or in addition to, an increase in anion secretion. Therefore, in separate experiments, ATP- and forskolin-evoked changes in I_SC were measured in the presence and absence of a low concentration of amiloride (10 µM) which is known to selectively block the epithelial sodium channel ENaC. If present, this would be the most likely pathway for cation absorption. At this concentration, apical amiloride did not significantly affect the basal I_SC (ΔI_SC = 0.017±0.021 µA cm⁻²) or changes in I_SC evoked by ATP or by forskolin. These data suggest that, both in the presence and absence of HCO_3^- the increases in I_SC evoked by these stimuli can be largely attributed to anion secretion across the Par-C10 monolayers.
In the absence of HCO$_3^-$ we also applied bumetanide (100 μM) to the basolateral membrane (Fig. 1D) to evaluate the possible contribution of the Na$^+$-K$^+$-2Cl$^-$ cotransporter (NKCC1) to the secretion of Cl$^-$ in the absence of HCO$_3^-$; Pretreatment with bumetanide resulted in a 48±15% (n=6) decrease in the initial $\Delta$ISC evoked by forskolin (Fig. 1F) and almost complete abolition of the sustained phase of the response.

Recovery of intracellular pH following an acid load

In order to identify the transporters responsible for HCO$_3^-$ uptake and H$^+$ extrusion in Par-C10 monolayers, the cells were acid loaded by bilateral application of 20 mM NH$_4^+$ for 3 min. The recovery of pH$_i$ was then recorded by microfluorometry in the presence and absence of Na$^+$ (Fig. 2). In the HCO$_3^-$-free, Hepes-buffered perfusate, there was no recovery of pH$_i$ following the NH$_4^+$ pulse when Na$^+$ was absent from both sides of the monolayer (Fig. 2A). Restoration of apical Na$^+$ resulted in only a small change in pH$_i$, whereas restoration of Na$^+$ to the basolateral side caused a rapid increase in pH$_i$ towards its normal, resting value.

The most likely explanation for the Na$^+$-dependent recovery of pH$_i$ under these conditions is the extrusion of H$^+$ by a basolateral Na$^+/H^+$ exchanger, probably NHE1. To test this hypothesis the experiment was repeated with the NHE1 inhibitor EIPA (30 μM) applied to the basolateral membrane during the recovery phase. As shown in Fig. 2B, EIPA largely abolished the recovery previously observed upon restoration of basolateral Na$^+$. Upon withdrawal of EIPA, pH$_i$ recovered quickly to its resting value. The recovery rates measured in these experiments are summarised in Fig. 2D and they clearly indicate the presence of a Na$^+$-dependent, HCO$_3^-$-independent acid extruder, most probably NHE1, at the basolateral membrane.

When these experiments were repeated in the presence of HCO$_3^-$, similar results were obtained (Fig. 2C). As before, there was no recovery of pH$_i$ from an acid load in the absence of extracellular Na$^+$, nor when Na$^+$ was restored to the apical side. However, when Na$^+$ was restored to the basolateral side, the recovery of pH$_i$ was extremely rapid, and occurred at a rate that was more than twice that observed in the absence of HCO$_3^-$ (Fig. 2D).

To identify the transporters responsible for acid extrusion in the presence of HCO$_3^-$, we examined the effects of inhibitors on pH$_i$ recovery rate in paired NH$_4^+$ pulse experiments (Fig. 3). In these experiments, where extracellular Na$^+$ was present throughout, the recovery from the initial NH$_4^+$ pulse served as a reference for comparing the effects of inhibitors on the recovery that followed the second pulse. Recovery rates following two identical NH$_4^+$ pulses in the absence of inhibitors were very similar (Fig. 3A). In contrast to the HCO$_3^-$-free experiments, EIPA had no significant effect on the recovery from an acid load.
in the presence of HCO₃⁻ (Fig. 3B). However, when EIPA was applied together with H₂DIDS (500 µM), an inhibitor of HCO₃⁻ uptake by the Na⁺-2HCO₃⁻ cotransporter (NBC1) (20), the recovery rate was reduced by 70±10% (n=6, Fig. 3C). Although
the remaining flux could be due to an additional transporter, our previous experience suggests that it more likely to be due to incomplete inhibition of NBC1 by H2DIDS.

Taken together, the data in Figs 2 and 3 show that the acid extrusion mechanisms in Par-C10 cells are Na+-dependent and entirely confined to the basolateral membrane. In other words, acid/base transport in these cells is strongly polarized to favour HCO3- secretion. It also seems likely that the main acid extruders at the basolateral membrane are NHE1 and NBC1.

Evidence for transepithelial HCO3- secretion

In order to examine whether Par-C10 cells secrete HCO3- ions, or whether the role of HCO3- is simply to support Cl- secretion, we used a protocol in which basolateral HCO3- influx and H+ efflux are suddenly blocked by the application of specific inhibitors (17, 21). This is achieved by simultaneous application of EIPA (30 µM) and H2DIDS (500 µM) to the basolateral side. (B) Changes in pH, induced by basolateral EIPA and H2DIDS during application of 10 µM forskolin to the apical side. (C) Changes in pH, induced by EIPA and H2DIDS during application of 50 µM ATP to the apical side. (D) Changes in pH, induced by EIPA and H2DIDS during application of 10 µM forskolin and 5 µM CFTRinh-172 to the apical side. (E) Mean initial acidification rates at the time points indicated. * P<0.05 compared with a; # P<0.05 compared with b.

Fig. 4. Measurements of the intracellular acidification evoked in Par-C10 cell monolayers by blocking the basolateral HCO3- uptake mechanisms in the presence or absence of ATP and forskolin. (A) Changes in pH, induced by application of 30 µM EIPA and 500 µM H2DIDS to the basolateral side. (B) Changes in pH, induced by basolateral EIPA and H2DIDS during application of 10 µM forskolin to the apical side. (C) Changes in pH, induced by EIPA and H2DIDS during application of 50 µM ATP to the apical side. (D) Changes in pH, induced by EIPA and H2DIDS during application of 10 µM forskolin and 5 µM CFTRinh-172 to the apical side. (E) Mean initial acidification rates at the time points indicated. * P<0.05 compared with a; # P<0.05 compared with b.

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Basolateral application of H2DIDS and EIPA to unstimulated Par-C10 cells caused only a slow decrease in pH, (Fig. 4A). Application of forskolin (10 µM) did not itself result in any change in pH, but when the basolateral inhibitors were applied in the continued presence of forskolin (Fig. 4B), the rate of decrease of pH was significantly faster than in the unstimulated control (Fig. 4E). Likewise, apical application of ATP (50 µM) did not itself have any effect on pH, but the acidification evoked by subsequent application of H2DIDS and EIPA (Fig. 4C) was strongly enhanced (Fig. 4E). These results suggest that both cAMP- and Ca2+-mediated stimuli may evoke HCO3- secretion by Par-C10 cells.

Because CFTR is known to be expressed by Par-C10 cells, we next examined the possibility that CFTR contributes to a secretory flux of HCO3- across the apical membrane, as it does in other HCO3- secreting epithelia. We therefore examined the effect of CFTRinh-172, a specific CFTR channel blocker, on the efflux of
HCO₃⁻ as measured by the basolateral inhibitor method. When CFTRinh-172 (5 µM) was applied with forskolin to the apical membrane, the acidification rate following block of the basolateral transporters with EIPA and H₂DIDS was significantly reduced (Fig. 4D). The reduction in acidification rate, expressed as a fraction of the increase evoked by forskolin alone, was 72±4.9% (n=6) (Fig. 4E). When applied to the basolateral membrane, CFTRinh-172 had no effect on the acidification rate (data not shown). We therefore conclude that the increase in base efflux from Par-C10 cells following stimulation occurs mainly at the apical membrane and involves CFTR, either directly or indirectly.

**Evidence for the presence of anion exchangers**

Since anion secretion by salivary acinar cells also involves Cl⁻/HCO₃⁻ exchange at the basolateral membrane (8), the next series of experiments was designed to assess the activity and location of anion exchangers in the Par-C10 cells. The approach was to substitute extracellular Cl⁻ with a non-transported anion, gluconate, and to record the resulting change in pHᵢ. If an exchanger is present in the membrane, substitution of Cl⁻ will reverse the normal concentration gradient for Cl⁻ and the consequent efflux of Cl⁻ will be coupled to a rapid uptake of HCO₃⁻ and a measurable increase in pHᵢ.

When the basolateral membrane of Par-C10 cells was exposed to a Cl⁻-free solution, pHᵢ showed a sustained increase of 0.16±0.015 pH units (n=38). It then returned to its normal value when extracellular Cl⁻ was restored (Fig. 5A). When the experiment was repeated in the presence of a Cl⁻/HCO₃⁻ exchange inhibitor, DNDS (3 mM), the increase in pHᵢ was significantly reduced (0.056±0.013 pH units, n=12, p<0.05) (Fig. 5B). Measurements of the initial rate of increase in pHᵢ induced by basolateral Cl⁻ substitution in the absence or presence of DNDS also strongly support the conclusion that a Cl⁻/HCO₃⁻ exchanger is present at the basolateral membrane of Par-C10 cells (0.074±0.008 ΔpH min⁻¹ vs. 0.039±0.008 ΔpH min⁻¹, respectively). When compared with the unstimulated state, pre-exposure to ATP (50 µM, Fig. 5C) or forskolin (10 µM, Fig. 5D) had no effect on either the magnitude or the rate of increase in pHᵢ. We therefore conclude that a basolateral Cl⁻/HCO₃⁻ exchanger is present and active in unstimulated Par-C10 cells and that its activity is not increased by intracellular Ca²⁺ or by cAMP.

**DISCUSSION**

Previous studies have shown that cultured epithelial cell lines with acinar or ductal characteristics can form confluent monolayers on permeable filters and are useful models for studying the molecular mechanisms of electrolyte transport in exocrine glands (17, 22). The Par-C10 cell line was developed a decade ago and found to grow well as a polarized epithelium (15). Par-C10 cells were shown to secrete electrolytes, to respond to muscarinic and adrenergic stimuli, and to express acinar ion transporter mRNAs (16, 23). However, it was not known whether anion transport was confined to Cl⁻ secretion alone or whether both Cl⁻ and HCO₃⁻ were secreted. Our present study provides evidence that these cells do indeed have the capacity to secrete HCO₃⁻.

In our experiments, transepithelial anion secretion was detected by short-circuit current measurements. We observed that not only ATP but also the cAMP-elevating agent forskolin stimulated anion secretion. In previous studies forskolin had been found to have varying effects (16) probably because the culture medium used in those studies contained cholera toxin which may have exhausted the cAMP-dependent pathway. To
avoid this, we omitted cholera toxin during culture. Our results with forskolin stimulation are consistent with the presence of functional CFTR in the membrane of Par-C10 cells. The expression of CFTR mRNA in salivary acinar and ductal cells is generally accepted, although the contribution of CFTR to anion secretion seems to be smaller than that evoked by Ca²⁺-mediated agonists (8). The present study indicates that Cl⁻ transport in Par-C10 cells can be activated by both Ca²⁺ and cAMP-dependent mechanisms. We also observed that the forskolin-evoked Iₘ was inhibited by bumetanide, an inhibitor of NKCC1. This finding is consistent with previous observations that parotid CFTR secretion is largely dependent on basolateral NKCC1 activity (8, 24).

In our work, the presence of HCO₃⁻ increased the forskolin-evoked Iₘ by about 100 percent, as observed previously (16). However, from the Iₘ, measurements alone, it is difficult to determine whether HCO₃⁻ is secreted in parallel with Cl⁻ or whether HCO₃⁻ facilitates Cl⁻ secretion. To address this question we used microfluorometric pH measurements.

To identify the transporters responsible for HCO₃⁻ accumulation in the cytosol during secretion, we examined the recovery of pH, following an acid load. In the absence of HCO₃⁻, this was dependent on Na⁺ and completely blocked by basolateral EIPA, suggesting the presence of NHE1 activity at the basolateral membrane of the cells. This is consistent with the assumption for ubiquitous presence of NHE1 in epithelial cells of the gastrointestinal tract (25). In the presence of HCO₃⁻/CO₂ the recovery of pH was dependent on Na⁺ and strongly inhibited by the simultaneous application of EIPA and H₂DIDS, suggesting that a basolateral NBC1 also contributes to the supply of HCO₃⁻. Our results are therefore in agreement with a recent paper reporting the presence of both electroneutral and electroneutral NBCs on the basolateral surface of polarized Par-C5 cells, which originated from the same laboratory as the Par-C10 cells (26). Furthermore, the presence of NBC1, based on RT-PCR, immunoblotting and pH studies has also been reported in guinea-pig, mouse, rat and human salivary glands (27-29). In isolated, non-polarised human parotid cells, however, only a minor role, if any, has been attributed to NBC1 in pH regulation (30). Nonetheless, our data clearly show that HCO₃⁻ accumulation is Na⁺-dependent and involves basolateral Na⁺/H⁺ exchange and Na⁺-HCO₃⁻ cotransport in Par-C10 cells.

Our previous studies on pancreatic ductal HCO₃⁻ secretion made use of the fact that secretion depends upon a tight coupling between HCO₃⁻ uptake across the basolateral membrane and HCO₃⁻ exit across the apical membrane (17, 21). Therefore, when basolateral HCO₃⁻ uptake is prevented, by blocking Na⁺/H⁺ exchange with EIPA and Na⁺-HCO₃⁻ cotransport with H₂DIDS, pH, initially declines as HCO₃⁻ continues to leave the cell across the apical membrane. We can therefore estimate instantaneous HCO₃⁻ secretion from the initial rate of fall in pH, measured by microfluorometry. In Par-C10 cells, basolateral application of EIPA and H₂DIDS caused a significant fall in pH, which was accelerated by pretreatment of the cells with forskolin, demonstrating that elevation of cAMP in the cells accelerates the eflux of HCO₃⁻ ions. Our data strongly suggest that Par-C10 cells perform transepithelial HCO₃⁻ secretion which can be stimulated by cAMP-dependent mechanisms. The inhibition of forskolin-induced acidification caused by the apical application of CFTRinh-172 strongly suggests a role for CFTR in HCO₃⁻ secretion. The fact that only the apically applied CFTR blocker was effective further confirms that the CFTR channel is located exclusively in the apical membrane of the cells, and that HCO₃⁻ eflux is directed towards the apical side. These findings suggest that Par-C10 cells transport HCO₃⁻ in a similar manner to pancreatic ductal cells (17, 22).

HCO₃⁻ secretion was also enhanced when Par-C10 cells were treated apically with the Ca²⁺-elevating agent ATP. An ATP-dependent Cl⁻ conductance has been described in mouse parotid acinar cells, possibly a novel ATP-gated Cl⁻ channel (31). In Par-C10 cells ATP raises intracellular Ca²⁺ when applied to the apical, but not the basolateral, membrane (32). These findings together offer an attractive mechanism to explain the synergism often seen when both Ca²⁺ and cAMP signaling systems are activated. ATP is released from secretory granules, and if the ATP-activated Cl⁻ channel is expressed on the apical surface of the cells, then activation of this channel in parallel with other Cl⁻ channels could result in enhanced secretion (32). This may lead to increased fluid secretion to wash out the proteins discharged from the secretory granules through the ductal system (8).

According to our Cl⁻ substitution experiments, a Cl⁻/HCO₃⁻ exchanger is present at the basolateral membrane of Par-C10 cells. It is active under unstimulated conditions and not sensitive to cAMP or to the Ca²⁺-elevating agent ATP. Its identity was confirmed by the inhibitory effect of DNDS and it is most likely to be the AE2 exchanger, which is expressed at the basolateral membranes of most epithelial cells (33). In salivary acinar cells, the basolateral Cl⁻/HCO₃⁻ exchanger provides an important additional pathway for the accumulation of intracellular Cl⁻ against its electrochemical gradient (8).

In conclusion, our study demonstrates that Par-C10 cells grown to confluence on permeable supports offer a good experimental model for studies of salivary HCO₃⁻ secretion. These cells achieve vectorial HCO₃⁻ transport from the basolateral to apical side in a regulated manner. Basolateral NHE1 and NBC1 seem to be the primary transporters involved in HCO₃⁻ uptake while Cl⁻ enters the cells via NKCC1 and AE2 at the basolateral membrane. Secretion can be stimulated by both cAMP- and Ca²⁺-elevating agents.

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