Regulation of Ca^{2+}-activated chloride channels by cAMP and CFTR in parotid acinar cells

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Abstract

The effect of intracellular cAMP and cystic fibrosis conductance regulator (CFTR) protein on the calcium-activated chloride current (I_{CaCl}) present in parotid acinar cells was studied using the patch clamp technique. Application of 1 mM of 8-(4-chloro-phenylthio)adenosine 3′:5′-cyclic monophosphate (CPT-cAMP), a permeable analog of cAMP, inhibited I_{CaCl} only at positive potentials. This inhibition was partially abolished in cells dialyzed with 20 nM PKI 6–22 amide, a potent peptide that specifically inhibits PKA. Because cAMP is an activator of the CFTR Cl^- channel, a known regulator of I_{CaCl}, we also investigated if the inhibition of I_{CaCl} was mediated by activation of CFTR. To test this idea, we added 1 mM CPT-cAMP to acinar cells isolated from knockout animals that do not express the CFTR channel. In these cells the cAMP effect was totally abolished. Thus, our data provide evidence that cAMP regulates I_{CaCl} by a dual mechanism involving PKA and CFTR.
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Fluid secretion by salivary glands is a Ca-dependent process [1]. It begins with activation of muscarinic receptors that result in generation of IP_3. This second messenger is then responsible for releasing Ca^{2+} from intracellular stores, which triggers the fluid secretion process [2]. Acinar cells from parotid glands are mainly responsible for the production of primary saliva. These cells express a variety of Cl^- channels, including Ca^{2+}-activated [3], swelling-activated [3,4], cAMP-activated [5], voltage-activated [3], and ATP-activated channels [6]. Of these, only the Ca^{2+}-activated Cl^- channel (I_{CaCl}) appears to play a significant role in fluid secretion [7,8].

The mechanism of I_{CaCl} activation by Ca^{2+} has been extensively studied in several preparations including rat and mice parotid acinar cells [7,9], lachrymal acinar cells [10], *Xenopus* oocytes [11], kidney cell line [12], endothelial cells [13], neutrophils [14], human colonic cell line [15], etc. Data show that in some cell types Ca^{2+} ions activate the channel by directly interacting with the protein, and this interaction is regulated by voltage

[7,11]. However, in other cell types this channel is gated by activation of the Ca- and CaM-dependent protein kinase II [9,16]. In parotid acinar cells I_{CaCl} is activated by the first mechanism [9].

Given the physiological role of this channel in the fluid secretion process of salivary glands it would be expected that its modulation by endogenous mechanisms be of obvious significance to the fluid secreting capability of the cell. Recently, it has been shown that cAMP inhibits I_{CaCl} in cultured Sertoli cells [17]. However, no mechanistic explanation for this phenomenon has been provided.

In secretory tissue, like rat submandibular glands cAMP is produced by β-adrenergic stimulation [18]. In addition, cAMP potentiates Ca^{2+} signals resulting from muscarinic stimulation in parotid acinar cells from mice [19]. However, the direct regulation of I_{CaCl} by cAMP-dependent mechanisms is unknown.

To date, there are several reports that show inhibition of I_{CaCl} by cystic fibrosis transmembrane regulator (CFTR) protein in other preparations [20,21]. The CFTR protein is a cAMP-regulated Cl^- channel present in both acinar and duct cells of salivary glands [5].

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Opening of CFTR Cl^- channel requires phosphorylation by the cAMP-dependent protein kinase A (PKA), and subsequent binding and hydrolysis of ATP [22]. Thus, cAMP could exert an inhibitory effect on I_{CaCl} by activating CFTR. Alternatively, I_{CaCl} could be directly regulated by cAMP through activation of PKA. To gain further insights into possible regulatory mechanisms of fluid secretion by cAMP, we investigated the modulation of I_{CaCl} in acinar cells of mouse parotid gland.

Materials and methods

Single cell dissociation

Single acinar cells were isolated from mouse parotid glands as previously reported [23]. Briefly, glands were dissected from exsanguinated mice (BlackSwissx129/SvJ hybrid mice) after CO_2 anesthesia. Glands were minced in Ca^{2+}-free minimum essential medium (MEM) (GIBCO) supplemented with 1% bovine serum albumin (BSA) (Fraction V, Sigma). Tissue was then treated for 20 min (37 °C) with a 0.02% trypsin solution (MEM-Ca^{2+}-free + 1 mM EDTA + 2 mM glutamine + 1% BSA). Digestion was stopped with 2 mg/ml of soybean trypsin inhibitor (Sigma) and the tissue further dispersed by two sequential treatments of 60 min each with collagenase (100 U/ml Type CLSPA, Worthington Biochemical) in MEM-Ca^{2+}-free + 2 mM glutamine + 1% BSA. The dispersed cells were centrifuged and washed with basal medium Eagle (BME) (GIBCO)/BSA-free. The final pellet was resuspended in BME/BSA-free + 2 mM glutamine, and cells were plated onto poly-L-lysine-coated glass coverslips (5 mm diameter) for electrophysiological recordings.

CFTR-knockout mice were obtained from Dr. James E. Melvin, Center for Oral Biology of the School of Medicine of the University of Rochester.

Electrophysiological recordings

Solutions. Calcium-activated Cl^- currents were recorded from cells bathed in a solution containing (in mM): 139 TEA-Cl, 20 Hepes, 0.5 $CaCl_2$, and 100 D-mannitol (pH 7.3). The pipette solution contained (in mM): 97.4 TEA-Cl, 20 Hepes, 21 $CaCl_2$, and 30 EGTA (pH 7.3). This solution has an estimated free $[Ca^{2+}]$ of 250 nM to activate I_{CaCl} [7].

Stock solutions for 8-(4-chlorophenylthio)adenosine 3′:5′-cyclic monophosphate (CPT-cAMP), the acid form of cAMP, and the PKA inhibitor (protein kinase A inhibitor amide, fragment 6–22) were prepared in water, aliquoted, and frozen. The day of the experiment aliquots of stock solutions were diluted in either the standard extracellular solution or the pipette solution at the desired concentration. All chemicals used were purchased from Sigma (St. Louis, MO).

Whole-cell recordings. Ca^{2+}-activated Cl^- currents were recorded at room temperature using the whole-cell configuration of the patch clamp technique with a PC-501A patch clamp amplifier (Warner Instruments, Holliston, MA). Glass pipettes were fabricated with Corning glass (Warner Instruments, Holliston, MA) and had a resistance of 3 and 4 MΩ when filled with the intracellular solution. Cells were held at 0 mV and the membrane potential was varied between −80 and +100 mV in 20 mV increments using 2 s square pulses. Currents were filtered at 1 kHz and data points were acquired at 2 kHz using the pCLAMP software without series resistance or leak current compensation.

Data analysis. Data analysis was performed using the Clampfit software of the pClamp suite version 8 and Origin (Origin Software, Northampton, MA). Current amplitudes measured at the end of each pulse were used to construct current–voltage relationships. Current

values were then normalized to values obtained at +100 mV. All data are given as mean values ± SE. Statistical analysis of current inhibition was performed using a paired *t* test.

Results

Inhibition of I_{CaCl} by cAMP

Intracellular cAMP enhances Ca^{2+} signals resulting from muscarinic stimulation [19] thereby cAMP could modulate I_{CaCl}. In addition, cAMP could regulate I_{CaCl} by alternative pathways independent of variations on $[Ca^{2+}]_i$ such as activation of PKA. To test this hypothesis, we applied CPT-cAMP, a permeable analog of cAMP, to patch-clamped single acinar cells. The intracellular solution had approximately 250 nM free $[Ca^{2+}]_i$ buffered with 30 mM EGTA to abolish possible changes in $[Ca^{2+}]_i$. Fig. 1A shows control raw I_{CaCl} traces obtained from a representative cell in the absence of CPT-cAMP. At positive voltages, I_{CaCl} displays an instantaneous current step representing channels already open at the holding potential, followed by a slow
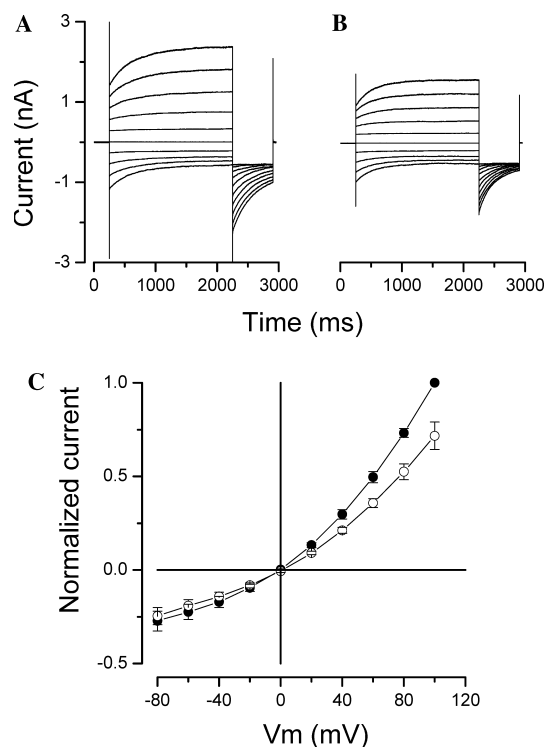

Fig. 1. CPT-cAMP inhibits I_{CaCl}. Whole-cell currents obtained from the same cell before (A), and after (B) treatment with 1 mM CPT-cAMP. Currents were recorded from −80 to +100 mV using 2000 ms test pulses applied every 20 s. Recordings started 5–10 min after reaching the whole cell configuration. (C) Average normalized I–V curves ($n = 7$) obtained under control conditions (●) and in the presence (○) of 1 mM CPT-cAMP are shown to summarize the effects of CPT-cAMP. Current amplitudes were normalized to that obtained under control conditions at +100 mV.
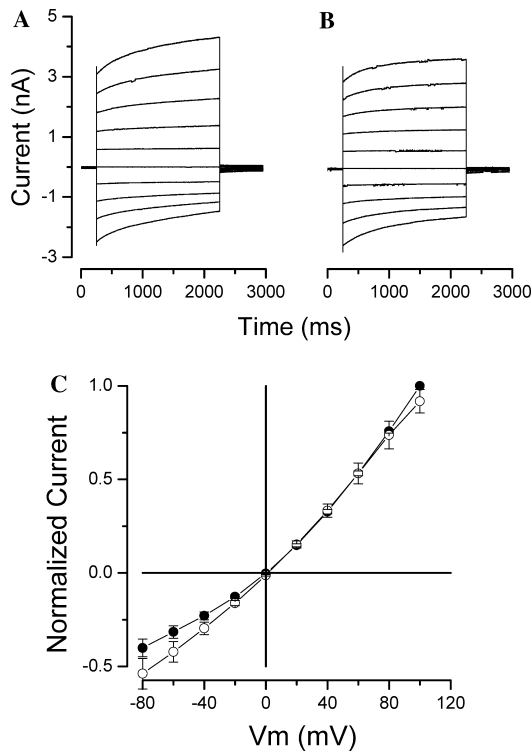
Fig. 2. Extracellular cAMP does not inhibit I_{CaCl}. Upper panel: whole-cell currents obtained from the same cell before (A) and after (B) exposure to 1 mM non-permeable cAMP (free acid). Currents were elicited by voltage pulses from −80 to +100 mV in 20 mV steps. (C) Average I–V curves ($n = 9$) obtained in the absence (●) and presence (○) of 1 mM cAMP applied to the bath.

time-dependent current that represents recruitment of additional channels. At negative voltages, the current record shows a transient component followed by a time-independent part. Upon repolarization at negative

voltages, a large tail current was observed. This current pattern is in agreement with previously reported data for parotid acinar cells [7]. Fig. 1B shows families of current traces obtained from the same cell, 10 min after addition of 1 mM CPT-cAMP to the bath solution. It can be observed that application of CPT-cAMP resulted in reduction of the current magnitude without significant effects on kinetics. Fig. 1C shows current–voltage (I–V) relationships that summarize the effects of CPT-cAMP at all voltages. Control I–V (filled circles) shows the outward rectifying behavior reported previously for these channels. In the presence of CPT-cAMP (open circles) the I–V curve shows a significant decrease at positive voltages, indicating current inhibition. The percentage of current inhibited by CPT-cAMP at +100 mV was $28 \pm 8\%$ ($n = 7$).

Unlike CPT-cAMP, the acid form of cAMP is membrane impermeable and was used as a negative control. Upper panel of Fig. 2 shows whole cell Cl⁻ currents obtained from an acinar cell before (A) and after (B) application of 1 mM cAMP to the bath solution. Little alterations in current amplitude and kinetics were observed after application of cAMP. Fig. 2C summarizes the effect of cAMP on the current measured at different voltages in the absence (filled circles) and in the presence (open circles) of cAMP. Taken together Figs. 1 and 2 demonstrate that intracellular cAMP acts on I_{CaCl} to reduce the current at positive potentials.

Role of PKA

Inhibitory effects of CPT-cAMP could be mediated by activation of cAMP-dependent protein kinase, which may in turn regulate I_{CaCl}. To test this possibility, acinar cells were dialyzed with 20 nM PKI 6–22 amide, a potent
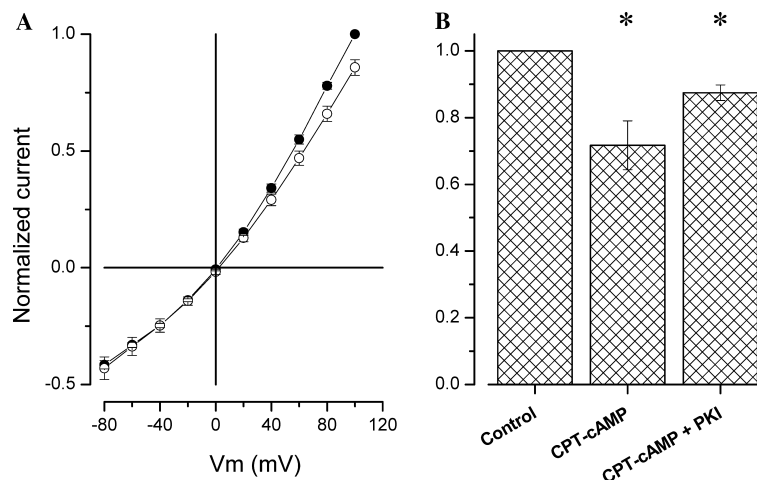

Fig. 3. Inhibition of I_{CaCl} by CPT-cAMP is partially prevented by PKI 6–22 amide. (A) Normalized average I–V curves ($n = 5$) before (●) and after (○) exposure to 1 mM CPT-cAMP obtained from cells dialyzed with 20 nM PKI 6–22 amide, a PKA inhibitor. (B) Statistical analysis of the effects of 1 mM CPT-cAMP on cells dialyzed with 0 (CPT-cAMP) or 20 nM PKI 6–22 amide (CPT-cAMP + PKI). Data obtained at +100 mV were used. Asterisks indicate statistical differences using a paired t test with $p < 0.05$.
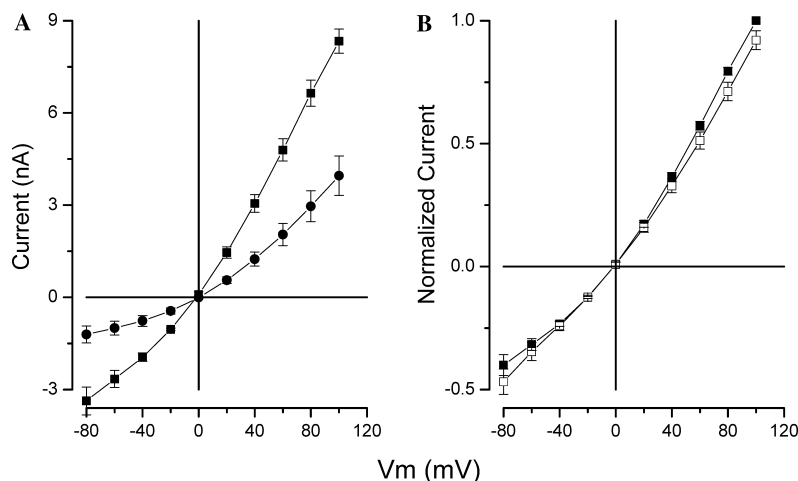
Fig. 4. Regulation of I_{CaCl} by CFTR. (A) Average I–V curves obtained from acinar cells isolated from wild type ($\bullet$; $n = 7$) and CFTR$^{-/-}$ mice ($\blacksquare$; $n = 4$). (B) Normalized I–V relationships ($n = 4$) obtained from CFTR$^{-/-}$ acinar cells in the absence ($\blacksquare$) and presence ($\square$) of 1 mM CPT-cAMP.

and specific PKA inhibitor. PKI 6–22 amide was equilibrated during 10 min before application of 1 mM CPT-cAMP. Fig. 3A shows I–V curves obtained from cells dialyzed with the inhibitor before (filled circles) and after (open circles) exposure to CPT-cAMP. The inhibitory effects of CPT-cAMP were reduced from 28 ± 8 to $14 \pm 3\%$ at +100 mV in the presence of PKI 6–22 amide. This result suggests that PKA is partially responsible for the inhibition of I_{CaCl} induced by CPT-cAMP. Panel B summarizes the inhibitory effect of 1 mM CPT-cAMP recorded from cells in the absence or presence of the inhibitor PKI 6–22 amide. Data were analyzed using a paired t test for each set of control/treatment columns. Asterisks represent significative differences between treated and control groups. Thus, the response to CPT-cAMP is reduced by the PKA inhibitor but is not completely prevented, suggesting a minor role for PKA in channel modulation.

Role of CFTR

The observation that inhibition of PKA by PKI 6–22 amide only abolished about 50% of the effect of CPT-cAMP suggests that additional regulatory elements are involved in the inhibition of I_{CaCl}. CFTR, a cAMP-activated Cl$^-$ channel, is present in acinar cells of salivary glands [24] and negatively regulates I_{CaCl} in other preparations. Thus, we explored the possibility that CFTR was involved in the inhibitory effects of CPT-cAMP. To directly test this idea, we used a CFTR knockout model mice that express the S489X mutation, which results in a nonfunctional truncated channel protein [24]. Fig. 4A shows average I–V curves obtained from acinar cells isolated from wild type (circles) and CFTR$^{-/-}$ (squares) mice. At all potentials, the average current amplitude in cells from knockout animals was 2-fold larger than in cells from wild type animals. This observation is in agreement with other reports that show that CFTR

downregulates I_{CaCl}. Fig. 4B illustrates normalized average I–V curves recorded from CFTR knockout cells before (filled squares) and after (open squares) addition of 1 mM CPT-cAMP to the bath. The current in the presence of CPT-cAMP was not different from control (paired t test). Thus, the lack of CFTR expression abolished the ability of CPT-cAMP to inhibit I_{CaCl} at positive voltages.

Discussion

Inhibition of I_{CaCl} by cAMP had previously been reported in cultured Sertoli cells [17]. In those cells 1 mM CPT-cAMP inhibited the channels at potentials more positive than +50 mV. However, no mechanistic explanation was provided for this inhibitory effect. Similarly, our data show that in parotid acinar cells I_{CaCl} is inhibited by 1 mM CPT-cAMP. Thus, cAMP exerts its effect in a voltage-dependent manner. However, our observation that impermeable cAMP had no effect suggests that the inhibition of I_{CaCl} was mediated by intracellular cAMP and was not due to voltage-dependent blockade of the channel pore. Our data using a specific PKA inhibitor (PKI 6–22 amide) demonstrate that about 50% of the inhibition of I_{CaCl} by cAMP was mediated by PKA activation.

Previous reports have documented an interaction between I_{CaCl} channels and CFTR Cl$^-$ channels in *Xenopus* oocytes and cultured bovine pulmonary artery endothelial (CPAE) cells [20,21]. In both cell types expression of CFTR channels inhibits the endogenous I_{CaCl} channels probably by a protein–protein interaction via the carboxy-terminal part of the CFTR regulatory domain [25]. In salivary glands CFTR is present in acinar and duct cells [5]. Our data show that I_{CaCl} channels from parotid glands are strongly modulated by CFTR. Cells isolated from CFTR$^{-/-}$ mice displayed current

magnitudes that were more than 2-fold larger than those recorded from wild type cells. Moreover, the inhibition of I_{CaCl} by cAMP was nearly abolished in cells isolated from CFTR$^{-/-}$ mice, which suggests that cAMP is inhibiting I_{CaCl} through CFTR. A plausible mechanism could be a protein–protein interaction, as suggested from the work on endothelial cells [25]. This idea is in agreement with our results using the CFTR$^{-/-}$ mice that express a channel protein truncated at residue 489. The truncated protein lacks the regulatory domain and therefore it cannot interact with I_{CaCl}. Alternatively, CFTR could reduce the protein channel level expressed in wild type cells. Unfortunately, our experiments did not address this question and at this time we are unable to distinguish between those two mechanisms.

Total saliva secretion is not affected by removing CFTR, which would imply that CFTR does not participate in fluid secretion (J.E. Melvin, personal communication). Yet our data show that CFTR tonically downregulates I_{CaCl}. If I_{CaCl} is controlling fluid secretion, its downregulation must result in a decrease of primary saliva production. However, total saliva production is a complex process. It results from primary saliva produced by acinar cells, which is later modified in its composition by duct cells. In this complex process several channels and transporters participate, many of which are regulated by CFTR—including ENaC present in duct cells [26]. Hence to unravel the physiological relevance of I_{CaCl} regulation by cAMP via CFTR requires further experiments.

Acknowledgment

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