The Type 8 Adenylyl Cyclase Is Critical for Ca\(^{2+}\) Stimulation of cAMP Accumulation in Mouse Parotid Acini*

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Capacitative Ca\(^{2+}\) entry stimulates cAMP synthesis in mouse parotid acini, suggesting that one of the Ca\(^{2+}\)-sensitive adenylyl cyclases (AC1 or AC8) may play an important role in the regulation of parotid function (Watson, E. L., Wu, Z., Jacobson, K. L., Storm, D. R., Singh, J. C., and Ott, S. M. (1998) Am. J. Physiol. 274, C557–C565). To evaluate the role of AC1 and AC8 in Ca\(^{2+}\) stimulation of cAMP synthesis in parotid cells, acini were isolated from AC1 mutant (AC1-KO) and AC8 mutant (AC8-KO) mice and analyzed for Ca\(^{2+}\) stimulation of intracellular cAMP levels. Although Ca\(^{2+}\) stimulation of intracellular cAMP levels in acini from AC1-KO mice was indistinguishable from wild type mice, acini from AC8-KO mice showed no Ca\(^{2+}\)-stimulated cAMP accumulation. This indicates that AC8, but not AC1, plays a major role in coupling Ca\(^{2+}\) signals to cAMP synthesis in parotid acini. Interestingly, treatment of acini from AC8-KO mice with agents, i.e. carbachol and thapsigargin that increase intracellular Ca\(^{2+}\), lowered cAMP levels. This decrease was dependent upon Ca\(^{2+}\) influx and independent of phosphodiesterase activation. Immunoblot analysis revealed that AC5/6 and AC3 are expressed in parotid glands. Inhibition of calmodulin (CaM) kinase II with KN-62, or inclusion of the CaM inhibitor, calmidazolium, did not prevent agonist-induced inhibition of stimulated cAMP accumulation. In vitro studies revealed that Ca\(^{2+}\), independently of CaM, inhibited isoproterenol-stimulated AC. Data suggest that agonist augmentation of stimulated cAMP levels is due to activation of AC8 in mouse parotid acini, and strongly support a role for AC5/6 in the inhibition of stimulated cAMP levels.

To date, 10 different ACs, each with distinct regulatory properties, have been cloned; their existence suggests that they may be differentially regulated. The enzymes exhibit type specific stimulatory and inhibitory regulation by G-protein α and βγ subunits, Ca\(^{2+}\), CaM, forskolin, P-site inhibitors, protein kinases A and C (PKC) (2–6), and calcineurin (7). A number of the members of the AC family can be regulated by alterations in [Ca\(^{2+}\)]. Of these, AC1, AC3, and AC8 are stimulated by Ca\(^{2+}\)/CaM in vitro (8–10). In vivo, AC1 and AC8 are stimulated and AC3 is inhibited by Ca\(^{2+}\)/CaM (8, 9, 11–13). Furthermore, transgenic mice deficient for both AC1 and AC8 demonstrate complete ablation of Ca\(^{2+}\)/CaM stimulated activity in brain (14).

An involvement of capacitative Ca\(^{2+}\) entry in cAMP metabolism has been reported for C6–2B glioma cells (15), SH-SY5Y human neuroblastoma cells (16), and HEK 293 cells transfected with AC1 and AC8 (8, 11, 12). In C6–2B glioma cells (15), neuroblastoma cells (16), pituitary-derived GH3 cells (17), and heart (18), capacitative Ca\(^{2+}\) entry was associated with inhibition of stimulated cAMP synthesis, whereas, in HEK 293 cells transfected with AC1 and AC8, capacitative Ca\(^{2+}\) entry was associated with augmentation of stimulated cAMP synthesis (11, 19). Muscarinic augmentation of stimulated cAMP accumulation, resulting in potentiation of amylase release (20), has also been shown to involve capacitative Ca\(^{2+}\) entry in mouse parotid acini (1), and data obtained demonstrated that Ca\(^{2+}\) entry plays an important role in promoting AC synthesis. These data, combined with findings that AC8 is expressed in mouse parotid acini (1) and that Ca\(^{2+}\)/CaM stimulates AC and augments the effects of forskolin on cyclase activity in membrane fractions and intact cells (21, 22), are consistent with results obtained in HEK 293 cells expressing AC8 (8, 11).

Interpretation of the mechanism(s) involved in the cross-talk that occurs between the Ca\(^{2+}\) and cAMP signaling pathways in cells is complex and requires not only identification of AC subtypes expressed, but also tools that provide definitive answers as to regulation of AC synthesis in specific cell types. Thus, the goal of the present study was to determine the involvement of AC8 in agonist-induced augmentation of stimulated cAMP levels in mouse parotid acini by examining the effects of carbachol and the microsomal Ca\(^{2+}\)-ATPase inhibitor, thapsigargin, on isoproterenol-induced cAMP accumulation in acini from AC8-KO mice. Our data show that carbachol and thapsigargin augmented stimulated cAMP accumulation in acini from wild type (WT) mice as previously reported (1), whereas these agents not only prevented augmentation, but inhibited isoproterenol-induced cAMP accumulation in AC8-KO mice. Augmentation of stimulated cAMP accumulation, however, was not affected in acini from AC1-KO mice. Agonist-induced inhibition of stimulated cAMP accumulation was reversed in a nominally Ca\(^{2+}\)-free buffer and in the presence of lanthanum (La\(^{3+}\)), but not by KN-62, an inhibitor of CaM kinase, or by the CaM antagonist, calmidazolium. Studies with isolated parotid membranes revealed that Ca\(^{2+}\), independently of CaM, inhibits AC activity in a concentration-dependent manner, consistent with the expression of the Ca\(^{2+}\)-inhibited AC5/6 isoforms in parotid gland. Results demonstrate that capacitative Ca\(^{2+}\) entry is associated with the activation of AC8 in mouse parotid acini and support an involvement of AC5/6 in the inhibition of cAMP synthesis.

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‡ The abbreviations used are: AC, adenylyl cyclase; KO, mutant; WT, wild type; PKC, protein kinase C; BSA, bovine serum albumin; MIX, 3-isobutyl-1-methylxanthine; PVDF, polyvinylidene difluoride; CaM, calmodulin; PDE, phosphodiesterase; KHB, Krebs-Henseleit bicarbonate; PAGE, polyacrylamide gel electrophoresis; kb, kilobase pair(s).

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**EXPERIMENTAL PROCEDURES**

Materials were obtained as follows: hyaluronidase, carbachol, isopropylmalonamide, lanthanum chloride (La\(^3+\)), bovine serum albumin (BSA), EGTA, HEPES, 3-isobutyl-1-methylxanthine (MIX), phosphocreatine, creatine phosphokinase, and GTP were from Sigma; cAMP radioimmunoassay kits were from DiaSorin (Stillwater, MN); collagenase type CLS2 were from Worthington; thapsigargin, KN-62, and calmidazolium were from Calbiochem (La Jolla, CA). All other reagents were of analytical grade.

**Generation of AC8-KO Mice—** Isogenic AC8 clones were isolated from a 129/Sv murine genomic library (Strategene, La Jolla, CA). Two overlapping clones that extend 5.0 kb upstream and 7.0 kb downstream of the translational start codon, pND21 and pND22, respectively, were used to construct the targeting vector. A 6.2-kb XbaI fragment of the AC8 gene, which includes DNA sequences residing 4.7 kb upstream and 1.8 kb downstream of the translational start codon, was replaced by a NEO cassette. The targeting vector (A12) consisted of sequences, flanking the 6.2-kb XbaI fragment on the 5’ end (3.75 kb) and the 3’ end (2.5 kb), that were ligated to the 3’ and 5’ ends of the neo cassette in pBluescript (Strategene). To enrich for homologous recombinants, a herpes simplex viral thymidine kinase gene (TK) cassette was ligated to the 3’ end (9.0 kb) and excision of the thymidine kinase (TK) gene was confirmed by digestion with XbaI and hybridization with a radiolabeled probe 22s identifying a 7.0-kb fragment representing the disrupted AC8 allele.

**Generation of AC1-KO Mice—** Mutant mice in which AC1 was inactivated by targeted mutagenesis were generated as reported previously by Wu et al. (24).

**Preparation of Porotid Acini—** Small groups of isolated mouse parotid cells (acini) were prepared as described previously by Watson et al. (25) with modification. Briefly, parotid glands from male AC8-KO, AC1-KO, WT B6129PF1 (Jackson Laboratories, Bar Harbor, ME) and Simonsen mice were removed quickly, trimmed, and minced in a siliconized dish in Krebs-Henseleit bicarbonate (KHB) buffer, pH 7.4, containing 0.9 mM MgCl\(_2\) and 1.28 mM CaCl\(_2\), 30 mM Hepes, 90 units/ml collagenase (CLS2), and 1 mg/ml hyaluronidase. Enzymatic digestion was conducted in a rotary water bath at 37 °C for 60 min under continuous CO\(_2\)/O\(_2\) (5%/95%) gassing. After the first 40 min of digestion, the suspension was pipetted up and down 12 times with a 10-ml plastic pipette. This was repeated two more times at approximately 5-min intervals. The pH during the dispersion was maintained at 7.2-7.4. Following digestion, the cells were centrifuged at 50 × g for 2 min, washed with buffer (KHB minus enzymes with 4% BSA, pH 7.4), filtered through two layers of nylon, and washed two additional times. Cells were suspended in KHB minus enzyme buffer containing 1% BSA and rested for 30 min at 37 °C with continuous gassing.

**Cyclic Nucleotide Measurements—** Cyclic AMP levels were measured in intact mouse parotid acini suspended 1:300 (w/v) in KHB, pH 7.4, containing 0.1% BSA as described previously (22). For experiments in which La\(^3+\) was used, a phosphate- and bicarbonate-free buffer was used (1). Cell suspensions (1.5 ml) were incubated with agonists for varying times up to 8 min. Incubations were terminated by addition of an equal volume of ice-cold 10% trichloroacetic acid. Cyclic AMP was determined by the radioimmunoassay procedure of Steiner et al. (26). Measurements were calculated as picomoles of cAMP/mg of protein.

**Measurement of [Ca\(^{2+}\)] in Intact Cells—** Acini were suspended 1:50 (w/v) in KHB containing 0.176 mg/ml ascorbic acid and 0.2% BSA, pH 7.4, and loaded with fura-2/AM at 3.3 µg/ml cell suspension for 30 min at 37 °C with continuous gassing (5% CO\(_2\), 95% O\(_2\)) and shaking. Fura-2/AM was prepared at 1 mg/ml in Me\(_2\)SO just prior to use. Loaded cells were washed three times in the 0.2% BSA/KHB containing ascorbic acid, resuspended at 1:50 (w/v), and maintained at 24 °C with gassing and shaking. After a 20-min incubation period, an aliquot was washed twice in the above buffer and placed in UV grade fluorometric cuvettes (Spectrocel) for [Ca\(^{2+}\)] measurements. [Ca\(^{2+}\)] was calculated using the equation of Grynkiewicz et al. (27), where \(K_{d} = 224 \text{ m}\). A Filterscan spectrofluorometer system equipped with a magnetic stirrer and constant temperature cuvette holder from Photon Technology International Inc. (South Brunswick, NJ) was used for the [Ca\(^{2+}\)] measurements.

**Gel Electrophoresis and Western Blot Analysis—** Brain and heart homogenates were removed as described previously (28). Membranes were prepared by homogenizing glands in ice-cold buffer containing 0.25 mM sucrose, 10 mM Tris-HCl, and 10 mM MgCl\(_2\), pH 7.5, with and without 2 mM EGTA. Homogenates were centrifuged at 20,000 × g at 4 °C for 20 min. Pellets were rehomogenized and washed twice with the above buffers, recentrifuged, and resuspended in 10 mM Tris-HCl and 10 mM MgCl\(_2\), pH 7.5. Fresh membrane fractions were used in all experiments.

**Adenylyl Cyclase Assay—** Membranes were isolated from mouse parotid acini, and the adenylyl cyclase assay was carried out as described by Ammer and Schulz (29) with modification. Adenylyl cyclase was determined in a reaction mixture (100 µl) containing 40 mM Tris-HCl, pH 7.4, 0.2 mM EGTA, 100 mM NaCl, 10 mM MgCl\(_2\), 0.5 mM ATP, 5 mM MgCl\(_2\), 5 mM GTP, 2 mM creatine phosphokinase, 10 µM MIX. Reactions were started by the addition of 15 µg of membrane protein, incubated for 10 min at 30 °C, and stopped with 100% trichloroacetic acid to a final concentration of 10%. When CaCl\(_2\) was included in the assay, the concentration of free Ca\(^{2+}\) was derived from the computer program BACD (30). Adenylyl cyclase was calculated as picomoles/mg of protein/10 min.

**Gel Electrophoresis and Western Blot Analysis—** Brain and heart homosmal membrane preparations, used as positive antibody controls, were prepared as described (31). Proteins of tissue fractions of mouse brain, heart, and parotid were resolved concomitantly with proteins of standard molecular weight at room temperature by 10% SDS-PAGE (32) using mini-gels (10-well, 1 mm thick), the Xcell II Mini-Cell electrophoresis system and protocols of Novex (San Diego, CA). Resolved proteins were transferred overnight at a constant voltage 30 V in transfer...
buffer (33) at 4 °C to polyvinylidene difluoride (PVDF) filters (Novex, San Diego, CA) using the Mini Trans-Blot system of Bio-Rad. Peptides immobilized to the PVDF filters were screened for AC isoforms by immunoblot analysis performed at room temperature using rabbit affinity purified polyclonal antibodies specific to the carboxyl-terminal domain of AC3 or AC56 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1 μg/ml and 1-h incubation. Primary antibody binding was detected using donkey horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) at 1:20,000 dilution and 1-h incubation with chemiluminescent (ECL) substrate and protocols of Amersham Pharmacia Biotech. Chemiluminograms of AC immunoblots were scanned into a computer, image data were captured and saved in TIFF format using Photoshop® software (Adobe Systems Inc., San Jose, CA), and blot densities and molecular weights were derived from TIFF files using Un-Scan-IT gel® (SilkScientific, Orem, UT).

Miscellaneous Procedures—Protein determinations were by the method of Lowry et al. (34).

Data Analysis—Cyclic AMP and AC data were calculated as the mean ± S.E. Statistical analysis was performed using Student's t test (p < 0.05).

RESULTS

Ca²⁺ Inhibits Isoproterenol-stimulated AC Activity in Membranes from AC8-KO Parotid Glands—To determine the consequence of disrupting the AC8 gene, AC sensitivity to Ca²⁺ in parotid membranes from AC8-KO mice was examined and compared with activity in membranes from WT mice. As shown in Fig. 2A, Ca²⁺ inhibited, in a concentration-dependent manner (0.01 μM–100 μM), isoproterenol (1 μM)-stimulated AC activity in membranes from AC8-KO mice. Further, Ca²⁺ inhibition of enzyme activity was also observed when membranes were washed with 2 mM EGTA to remove endogenous CaM (21) (Fig. 2B), suggesting that Ca²⁺ acts independently of CaM. In contrast to the observed inhibitory effects of Ca²⁺ in membranes from AC8-KO mice, Ca²⁺ stimulated AC activity in membranes from WT mice (Fig. 2A); at a 1 μM Ca²⁺ concentration, AC activity was increased by 80%, in agreement with data previously reported for the mouse parotid gland (21).

Carbachol and Thapsigargin Inhibit Isoproterenol-stimulated cAMP Accumulation in AC8-KO Mouse Parotid Acini—Since capacitative Ca²⁺ entry can regulate AC in mouse parotid acini, and the AC8 isoform is expressed in these cells (1), and known to be activated by Ca²⁺ entry (8, 11), we examined the influence of Ca²⁺ entry in agonist augmentation of stimulated cAMP accumulation in parotid acini from control and AC8-KO mice. As shown in Fig. 3A, incubation of acini with carbachol (10 μM) resulted in augmentation of isoproterenol (0.1 μM)-stimulated cAMP accumulation in a time-dependent manner in parotid acini from WT mice, as reported previously by Watson et al. (1). In parotid acini from AC8-KO mice, addition of carbachol not only prevented augmentation of isoproterenol-stimulated cAMP accumulation, supporting an involvement of AC8 in capacitative Ca²⁺ entry in a non-neuronal cell, but resulted in a significant inhibition of stimulated cAMP levels (Fig. 3B). Time-course studies showed that inhibition was detected as early as 0.5 min, peaked by 1 min, and remained constant for the remainder of the experiment. At 1 min, cAMP levels were reduced by approximately 47% in acini treated with carbachol (10 μM); cAMP values were 150 ± 15.6 and 68.6 ± 6 pmol/mg of protein in the absence and presence of carbachol, respectively.

In similar studies, the microsomal Ca²⁺-ATPase inhibitor, thapsigargin (2 μM), was considered a useful tool for examining the relationship between capacitative Ca²⁺ entry and stimulated cAMP accumulation, because it depletes intracellular Ca²⁺ pools independently of receptor activation and phosphinositide production (35). Any potential effects of receptor-generated PKC, and βγ subunits of G-proteins would thus be eliminated. As shown in Fig. 4A, addition of thapsigargin to acini from WT mice also increased stimulated cAMP accumulation in a time-dependent manner, as reported previously by Watson et al. (1). In acini from AC8-KO mice, isoproterenol-stimulated cAMP accumulation was inhibited by thapsigargin (Fig. 4B). Unlike the early inhibitory effects produced by carbachol, inhibition by thapsigargin was not observed until after 1 min. Cyclic AMP levels peaked by 5 min and remained constant for the remainder of the experiment. By 8 min, cAMP levels were reduced by 38%; cAMP values were 222.2 ± 19.7 and 137.8 ± 3.3 pmol/mg of protein in the absence and presence of thapsigargin, respectively. For both carbachol and thapsigargin, time-course studies were beneficial because they revealed a temporal relationship between inhibition of stimulated cAMP synthesis and capacitative Ca²⁺ entry (Figs. 3 and 4) similar to that previously described for mouse parotid acini (1).

Inhibition of Stimulated cAMP Accumulation Is Independent of Activation of a Phosphodiesterase (PDE) Enzyme—To rule out the possibility that inhibition of stimulated cAMP accumulation in parotid acini from AC8-KO mice was due to activation of a PDE isoenzyme, acini were incubated in the presence of MIX (500 μM) for 10 min prior to the addition of thapsigargin (2 μM). In time-course studies, MIX had no effect on the inhibition
of stimulated cAMP accumulation by thapsigargin (Fig. 5); by 8 min, cAMP values were reduced from 2212.7 ± 360 to 1072.3 ± 222 pmol/mg of protein in the absence and presence of MIX, respectively. Inhibition by thapsigargin was approximately 52%, consistent with results obtained in the absence of MIX (Fig. 4B).

**Ca**

**2+ Entry and Adenylyl Cyclase Activation**

**Ca**

2+ Is Required for Inhibition of Isoproterenol-stimulated cAMP Accumulation—Because of the temporal relationship observed between capacitative Ca2+ entry and inhibition of stimulated cAMP accumulation, we addressed the question of whether capacitative Ca2+ entry is, in fact, responsible for inhibition of stimulated cAMP accumulation. For these experiments, we tested the ability of thapsigargin to inhibit cAMP accumulation in the absence of extracellular Ca2+ and under conditions where Ca2+ entry was blocked. Acini were incubated in nominally Ca2+-free, and (100 μM) La3+-containing KHB buffers. Lanthanum, at 100 μM, was previously found to completely inhibit thapsigargin-induced capacitative Ca2+ entry and stimulated cAMP accumulation in mouse parotid acini (1). Incubation of acini in a nominally Ca2+-free buffer reduced isoproterenol (0.1 μM)-induced cAMP accumulation by 32% (Table I, Fig. 6A); by 8 min, cAMP levels were reduced from 270 ± 12 to 218.3 ± 14.5 pmol/mg of protein. In the absence of extracellular Ca2+, thapsigargin (2 μM)-induced inhibition of stimulated cAMP accumulation was prevented (Fig. 6A); cAMP levels were similar to those produced by isoproterenol in the absence of Ca2+. Incubation of acini in a La3+-containing buffer also reduced isoproterenol-stimulated cAMP levels, i.e. by 32%
Ca$^{2+}$ Entry and Adenyl Cyclase Activation

La$^3+$ buffer containing Ca$^{2+}$ and isoproterenol-stimulated cAMP accumulation in parotid acini from AC8-KO mice. Acini were incubated with MIX for 30 min in KHB buffer containing Ca$^{2+}$ (1.28 mM), prior to addition of isoproterenol (Iso, 0.1 μM) and isoproterenol + thapsigargin (Thaps, 2 μM). Results represent three experiments performed in duplicate. Values are the mean ± S.E. For symbols without error bars, the S.E. was similar to the symbol.

**TABLE 1**
Factors affecting isoproterenol-stimulated cAMP accumulation in acini from AC8-KO mice

| Condition                        | cAMP accumulation for AC8-KO (pmol/mg protein) |
|----------------------------------|-----------------------------------------------|
| +Ca$^{2+}$                       | 234.2 ± 12                                   |
| -Ca$^{2+}$                       | 159.5 ± 14.5                                 |
| -La$^3+$                         | 245.1 ± 33.6                                 |
| +La$^3+$                         | 161.5 ± 22.4                                 |
| -Calmidazolium                   | 172.3 ± 9.8                                  |
| +Calmidazolium                   | 93.5 ± 1.5                                   |
| -KN-62                           | 2125.9 ± 147.7                               |
| +KN-62                           | 1418.4 ± 144.6                               |

*p < 0.05 (comparison of cells incubated with and without Ca$^{2+}$, La$^3+$, calmidazolium, or KN-62).

**A** Presence of MIX.

(Table I, Fig. 6B); by 8 min, cAMP levels were 245.1 ± 21.7 and 161.5 ± 7.7 pmol/mg of protein in the absence and presence of La$^3+$, respectively. In the presence of La$^3+$, thapsigargin (2 μM) failed to inhibit stimulated cAMP accumulation; cAMP levels were not significantly different from those produced by isoproterenol in the presence of La$^3+$ (Fig. 6B). The decrease in isoproterenol-stimulated cAMP levels noted in nominally Ca$^{2+}$-free and La$^3+$-containing buffers was not unique to the AC8-KO mice, as we previously noted similar inhibitory effects on isoproterenol-stimulated cAMP levels in WT Simonsen mice (25) and suggested that Ca$^{2+}$ is required for full activation of AC.

Thapsigargin Augments Isoproterenol-stimulated CAMP Accumulation in AC1-KO Mouse Parotid Acini—Like the AC1 isoform, AC8 has been assumed to be expressed solely in the brain (8, 19). Recent studies with keratinocytes, however, suggest that, like the mouse parotid gland, AC8 is also expressed in non-neuronal tissues (36). Since the distribution of the AC8 message and its enzymatic properties are most closely related to AC1 (8, 9) and both isoforms are activated by capacitative Ca$^{2+}$ entry (11, 19), we further examined thapsigargin augmentation of stimulated cAMP levels in acini from the AC1-KO mice. Time-course studies revealed that thapsigargin-induced augmentation of stimulated cAMP accumulation in acini from AC1-KO mice was similar to that observed in WT mice (Fig. 7). By 8 min, thapsigargin-induced augmentation of stimulated cAMP levels were 219.7 ± 19 and 227.6 ± 6 pmol/mg of protein in WT and AC1-KO mice, respectively.

AC3 Is Not Involved in Agonist Inhibition of Isoproterenol-stimulated CAMP Accumulation—Several ACs have been linked to agonist-induced inhibition of stimulated cAMP accumulation in vivo, i.e. AC3, AC5, and AC6 isoforms (12, 15, 19, 37). In all cases, these isoforms are inhibited by capacitative Ca$^{2+}$ entry (12, 15). It is also known that AC3 is phosphorylated (13) and inhibited by CaM kinase II (12). Thus, we used a specific inhibitor of CaM kinase II, KN-62 (38), to determine the involvement of AC3 in thapsigargin-induced inhibition of stimulated cAMP levels. The conditions used were as reported by Wayman et al. (12) for HEK cells expressing AC3. If CaM kinase II-mediated inhibition of AC3 was responsible for the decrease in cAMP levels, then KN-62 would be expected to block the inhibition. Parotid acini were preincubated with KN-62 (100 μM) for 1 h prior to the addition of isoproterenol, and isoproterenol plus thapsigargin. MIX was present in the...
media to obviate any potential effects of KN-62 on PDE activity since a CaM-dependent PDE isoenzyme has been reported to be phosphorylated by Ca\textsuperscript{2+}/CaM-dependent protein kinase II (39, 40). As shown in Fig. 8, incubation of acini with KN-62 had the same inhibitory effect (33%) on isoproterenol-stimulated cAMP accumulation as was observed when acini were incubated in nominally Ca\textsuperscript{2+}-free and La\textsuperscript{3+}-containing buffers (Table I). KN-62, however, did not block thapsigargin-induced inhibition of stimulated cAMP levels. Inhibition was approximately 50% and 52% in the absence and presence of KN-62, respectively.

In other experiments, acini from AC8-KO mice were incubated with calmidazolium (10 \mu M), a CaM antagonist. As observed with acini incubated in nominally Ca\textsuperscript{2+}-free and La\textsuperscript{3+}-containing buffers, and KN-62, isoproterenol-stimulated cAMP levels were also reduced in the presence of calmidazolium; cAMP levels were reduced by approximately 46%. These results are summarized in Table I. Results also show that calmidazolium, like KN-62, failed to abolish thapsigargin-induced inhibition of AC (Fig. 9A); inhibition was 53% and 46% in the absence and presence of calmidazolium, respectively. Calmidazolium, however, did inhibit thapsigargin-induced augmentation of stimulated cAMP levels in acini from WT mice expressing AC8 (Fig. 9B), as expected for a cyclase that is CaM dependent (8, 41).

Mouse Parotid Acini Express AC5/6—Despite the fact that AC3 did not appear to contribute to thapsigargin-induced inhibition of stimulated cAMP accumulation, parotid membranes from acini of both WT and AC8-KO mice were found to express AC3 based on immunoblot analysis using antibodies specific to this isoform (Fig. 10). The finding that thapsigargin-induced inhibition of stimulated cAMP accumulation was not affected by the CaM inhibitor, calmidazolium (Fig. 9A), suggested that AC3/6 may also be expressed in the mouse parotid gland and contributes to the inhibition of stimulated cAMP accumulation by [Ca\textsuperscript{2+}]. As shown in Fig. 10, AC5/6 is expressed in both WT and KO parotid glands. AC3 and AC5/6 proteins of 124 and 135 kDa, respectively, from membranes of WT and KO mice migrated in 6% SDS-PAGE slightly faster than their respective proteins in brain (130 kDa) and heart (137 kDa). Differences in mass between parotid AC isoforms is apparent, whereas differences in mass between brain and heart AC isoforms and parotid isoforms may be the consequence of markedly different gel loads of protein between tissues controls and parotid. This was necessitated by the estimated greater than 15-fold higher levels of AC3 in brain and AC5/6 in heart than in the parotid. No apparent differences in abundance of parotid AC3 or AC5/6 isoforms was observed between WT and KO mice, suggesting no down- or up-regulation of these AC isoforms resulted from loss of AC8 expression.

Isoproterenol Activation of a PKC-stimulated AC Isoform—Data supporting a role for Ca\textsuperscript{2+} in isoproterenol-stimulated cAMP accumulation in acini from both WT and AC8-KO mice suggested that Ca\textsuperscript{2+} is involved in the regulation of an AC other than AC8. Since a Ca\textsuperscript{2+}-dependent PKC-regulated AC has been reported (42), and PKC potentiation of \beta-adrenergic-stimulated AC in mouse parotid membranes was found to be Ca\textsuperscript{2+}-dependent (43), further experiments evaluated the effects of the PKC inhibitor, calphostin C, on isoproterenol-stimulated cAMP accumulation in acini from AC8-KO mice. Time-course studies show that calphostin C inhibited isoproterenol-stimulated cAMP accumulation when acini were incubated in a 1.28 mM Ca\textsuperscript{2+}-containing buffer. Similar results were also observed with WT mice and when MIX was added to the incubation medium (data not shown). In the absence and presence of MIX, cAMP accumulation was inhibited by 45% and 42%, respectively, at 8 min (data not shown). Inhibition (34%) was also observed when acini were incubated in a nominally Ca\textsuperscript{2+}-free buffer (Fig. 11), suggesting that the primary source of Ca\textsuperscript{2+} required for PKC activation is derived from intracellular stores.

**DISCUSSION**

Although cross-talk between the Ca\textsuperscript{2+} and cAMP signaling pathways in exocrine cells has been documented (1, 22, 44–47), little information has been available regarding the expression and regulation of AC isoforms in these cells. In a recent study from our laboratory, we reported that capacitative Ca\textsuperscript{2+} entry increases the synthesis of cAMP in mouse parotid acini, and suggested an involvement of AC8 based on Northern blot analysis (1) and the known role of capacitative Ca\textsuperscript{2+} entry in activation of AC8 (11). Data presented in the present study clearly identify AC8 as the target with which Ca\textsuperscript{2+} interacts to augment stimulated cAMP levels in parotid acini, as augmentation...
of stimulated cAMP accumulation was abolished in acini from AC8-KO mice. Results are consistent with the ability of mouse parotid AC to be stimulated by Ca2\(^{2+}\) in vitro (21). To our knowledge, the present study represents only one of two reported studies showing that AC8 is expressed in non-neuronal tissues. In the other, AC8 was found to be expressed in skin keratinocytes (36). The present report also represents the first study to utilize AC8-KO mice for determining an involvement of this isoform in capacitative Ca2\(^{2+}\) entry-induced AC activation. Thus, contrary to a previous report (48), AC8 is expressed in non-neuronal cells and is stimulated by Ca2\(^{2+}\).

Studies also showed that in the absence of AC8, thapsigargin produced an inhibition of isoproterenol-stimulated cAMP accumulation. Inhibition was due to Ca2\(^{2+}\) entry, as incubating acini in a nominally Ca2\(^{2+}\)-free or La3\(^{3+}\)-containing KHB buffer blocked the response. Time-course studies revealed a temporal relationship between inhibition of stimulated CAMP synthesis and capacitative Ca2\(^{2+}\) entry as described previously for mouse parotid acini. Thus, by knocking out AC8, a Ca2\(^{2+}\)-inhibitable AC was unmasked, supporting a role for at least one other AC in the regulation of cAMP synthesis in mouse parotid acini. Our data support a role for the Ca2\(^{2+}\)-inhibitable AC6/5 in inhibition of stimulated CAMP accumulation based on immunoblot analysis with AC5/6 specific antisera, and in vitro studies showing that Ca2\(^{2+}\), in a concentration-dependent manner, inhibits AC in parotid membranes. Since antisera detected both AC5 and AC6, we cannot specify which isoform(s) is present in parotid cells. However, Ca2\(^{2+}\)-inhibitable AC6 has been reported to attenuate cAMP accumulation in other cell types including NCB-20 (49, 50), C6–2B (15, 51), GH3 rat pituitary tumor (17), and smooth muscle cells (52). Unlike AC8, there is evidence that Ca2\(^{2+}\), independently of CaM, inhibits AC6 (53). Our results support this finding; however, despite the finding that the dissociation of CaM by EGTA does not result in loss of inhibition, questions remain regarding how the Ca2\(^{2+}\) sensitivity of the Ca2\(^{2+}\)-inhibitable AC5/6 is achieved (19, 37).

Of interest was the finding that stimulation of cAMP accu-
ulation by isoproterenol alone was reduced significantly in acini from both WT and AC8-KO mice incubated in a nominally Ca\(^{2+}\)-free and La\(^{3+}\)-containing KHB buffers, and in the presence of calmidazolium and KN-62. Similar results have been obtained for control mouse parotid acini from Simonsen mice incubated in nominally Ca\(^{2+}\)-free or La\(^{3+}\)-containing buffers (25).\(^2\) Thus, it appears that Ca\(^{2+}\) influx is important for isoproterenol-induced AC activation. However, it is not clear whether Ca\(^{2+}\) released from intracellular stores enhances Ca\(^{2+}\) influx or whether isoproterenol affects a plasma membrane Ca\(^{2+}\) channel by a phosphorylation event as described for excitable cells (54). Reductions in isoproterenol-stimulated cAMP accumulation with calmidazolium and KN-62 also suggest that the inhibitory effects are dependent on CaM, but we cannot exclude an involvement of CaM itself in the regulation of AC.

Interestingly, KN-62 has been shown to antagonize the effects of dibutyryl cAMP in the regulation of hepatocytic autophagy, a process by which cells degrade their cytoplasmic macromolecules in response to the nutritional status of the cell (55), suggesting a role for CaM kinase II in this process. In excitable cells, a recent report shows that cAMP activation of \(\alpha\)-type Ca\(^{2+}\) channels, via Ca\(^{2+}\)/CaM-dependent protein kinase, is prevented by KN-62 (56). Since little is known about Ca\(^{2+}\) channels in nonexcitable cells, it is possible that isoproterenol-stimulated cAMP accumulation is dependent, in part, on a phosphorylation event involving a Ca\(^{2+}\)/CaM-dependent protein kinase II. A Ca\(^{2+}\)/CaM-dependent protein kinase II has been reported to be involved in Ca\(^{2+}\)-mediated regulation of capacitative Ca\(^{2+}\) entry in oocytes (57) and in CHO cells (58). If this were the case, however, it would be expected that thapsigargin-induced inhibition of stimulated cAMP accumulation, as well as isoproterenol-induced cAMP accumulation would have been blocked by KN-62. It is more likely that isoproterenol releases Ca\(^{2+}\) from an intracellular store as reported for rat parotid and submandibular cells (46, 59, 60). In a recent study with rat parotid microsomes, cAMP, acting through a cAMP-dependent kinase, induced Ca\(^{2+}\) release from ryanodine-sensitive stores (59). There is also evidence for the phosphorylation and regulation of ryanodine-sensitive channels (61–63) and inositol 1,4,5-trisphosphate receptors (64) by CaM kinase. Thus, the consequence of inhibiting Ca\(^{2+}\) release with KN-62 would result in decreased Ca\(^{2+}\) influx.

Although high concentrations of isoproterenol have been found to activate \(\alpha\)-adrenergic receptors (65, 66) producing the release of Ca\(^{2+}\) from intracellular stores, results from the mouse parotid acini showed that the effects of isoproterenol, at a concentration of 0.1 \(\mu\)M, on cAMP accumulation were independent of effects on \(\alpha\)-adrenergic receptors as the \(\alpha\)-adrenergic blocking agent, prazosin, failed to reverse these effects.\(^2\) Forskolin, which acts independently of the receptor, mimicked the effects of isoproterenol.\(^2\) Failure to observe changes in cytosolic Ca\(^{2+}\), in other reported studies, may be due to species differences or to a localized rise in Ca\(^{2+}\) at the apical region of the cell that is not detected using fluorescent Ca\(^{2+}\) indicators. Data obtained by Fagan et al. (11) support a co-localization of Ca\(^{2+}\)-stimulable AC with capacitative Ca\(^{2+}\) entry sites on the plasma membrane. Further studies will be required to determine the role of Ca\(^{2+}\) influx and release on isoproterenol-stimulated cAMP accumulation.

The additional finding, that calphostin C inhibits the effects of isoproterenol on cAMP both in the absence and presence of a PDE inhibitor, suggests an involvement of a PKC isofrom in stimulated AC. In the mouse parotid gland, phorbol ester was shown to enhance forskolin-stimulated cAMP accumulation, and the addition of purified PKC to parotid membranes enhanced both forskolin- and isoproterenol-stimulated cAMP synthesis (67). Further, purified PKC action was Ca\(^{2+}\)-dependent. Data would suggest that release of Ca\(^{2+}\) from intracellular stores by isoproterenol (45, 59, 60) may be required for activating a PKC-sensitive AC. An involvement of AC3 is not likely, as Ca\(^{2+}\) inhibition of stimulated cAMP accumulation was not affected by calphostin C (12). Also, Ca\(^{2+}\)-sensitive PKC activation has not been observed for AC6 or AC9 (68). On the other hand, Watson et al. (43) reported that, although the Ca\(^{2+}\) ionophore, A23187 alone, did not activate AC7, it was activated in the presence of isoproterenol and suppressed by staurosporine. It was suggested that AC7 is insensitive to the direct effects of Ca\(^{2+}\), but that Ca\(^{2+}\) may act through a kinase-related mechanism to affect the activity of the enzyme (69). Thus, in the parotid gland, the dual effect of isoproterenol, i.e. on AC activation and in increasing [Ca\(^{2+}\)]\(_i\), may be sufficient to mimic the synergism required for activation of a PKC-sensitive AC. A Ca\(^{2+}\)-sensitive PKC has been reported by Kawabe et al. (42).

In summary, the present results demonstrate that AC5 is expressed in the non-excitable mouse parotid cell and is predomantly involved in the regulation of muscarinic augmentation of cAMP synthesis. The fact that inhibition of stimulated cAMP accumulation in AC8-KO mice was observed supports the presence of another AC isoform that was found to be inhibited by capacitative Ca\(^{2+}\) entry. Both immunoblot analyses and in vitro AC assays indicated the AC isoform to be AC5. The inhibition of stimulated cAMP accumulation was not affected by calphostin C (12). Also, the inhibition of stimulated cAMP accumulation by isoproterenol (45, 59, 60) may be required for activating a PKC-sensitive AC. An involvement of AC3 is not likely, as Ca\(^{2+}\) inhibition of stimulated cAMP accumulation was not affected by calphostin C (12). Also, Ca\(^{2+}\)-sensitive PKC activation has not been observed for AC6 or AC9 (68). On the other hand, Watson et al. (43) reported that, although the Ca\(^{2+}\) ionophore, A23187 alone, did not activate AC7, it was activated in the presence of isoproterenol and suppressed by staurosporine. It was suggested that AC7 is insensitive to the direct effects of Ca\(^{2+}\), but that Ca\(^{2+}\) may act through a kinase-related mechanism to affect the activity of the enzyme (69). Thus, in the parotid gland, the dual effect of isoproterenol, i.e. on AC activation and in increasing [Ca\(^{2+}\)]\(_i\), may be sufficient to mimic the synergism required for activation of a PKC-sensitive AC. A Ca\(^{2+}\)-sensitive PKC has been reported by Kawabe et al. (42).
The Type 8 Adenylyl Cyclase Is Critical for Ca\(^{2+}\) Stimulation of cAMP Accumulation in Mouse Parotid Acini

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