Biochimica et Biophysica Acta 889 (1986) 216–224
Elsevier

BBA 11753

Muscarinic receptor-stimulated Ca^{2+} signaling and inositol lipid metabolism in avian salt gland cells

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(Received 3 April 1986)
(Revised manuscript received 1 August 1986)

Key words: Muscarinic cholinergic receptor; intracellular Ca^{2+}; Inositol lipid turnover; Stimulus-response coupling; (Avian salt gland cell)

Activation of muscarinic cholinergic receptors was studied by measuring agonist-stimulated inositol lipid turnover and changes in $[Ca^{2+}]_i$ in dissociated salt gland secretory cells. Carbachol stimulation of quin2-loaded cells results in a sustained 4-fold increase in $[Ca^{2+}]_i$, while incorporation of $[^{32}P]P_i$ into phosphatidylinositol (PI) and phosphatidate are similarly increased. [^{3}H]Inositol phosphates, measured in the presence of Li^+, increased 13-fold. The stimulated increment in $[Ca^{2+}]_i$ required extracellular Ca^{2+}, whereas [^{3}H]inositol phosphate accumulation was independent of external Ca^{2+}. Dose-response curves for carbachol-induced increments in $[Ca^{2+}]_i$, PI labeling, and labeled inositol phosphate release are similar, with EC_{50} values of 6, 4.5 and 8 μM, respectively. Dissociation constants for atropine vs. the quin2 and phospholipid responses are 0.59 ± 0.3 nM and 0.48 ± 0.28 nM, respectively. These cells thus provide a model system for the study of non-exocytotic secretion as a consequence of stimulated inositol lipid turnover.

Introduction

Studies on stimulus-response coupling in exocrine glands provide information concerning the mechanisms by which intracellular mediators link neurohormonal receptor activation to secretory function. In a wide variety of exocrine tissues, secretion of proteins and/or electrolytes and water depends on the elevation of the intracellular calcium concentration ($[Ca^{2+}]_i$ [1]). This Ca^{2+} response can be triggered by occupation of a variety of plasma membrane receptors, including the widely distributed muscarinic acetylcholine receptor (mAChR) [2,3]. Labeling studies have shown that activation of mAChRs can also result in stimulated turnover of membrane phosphoinositides [1,4–6].

Avian salt glands provide a useful model tissue for studying these events. In response to a dietary salt load or to direct cholinergic stimulation, these glands secrete a hypertonic fluid which is rich in NaCl and devoid of macromolecules such as proteins [7]. Study of stimulus-response mechanisms in this tissue, therefore, are not complicated by exocytotic function superimposed on fluid secre-

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Abbreviations: PI, phosphatidylinositol; P_i, inorganic phosphate.

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tion, features that are commonly shared by many exocrine glands. The mAChRs have been characterized in salt glands by ligand binding studies with homogenates and intact dissociated cells [8,9], and have been localized autoradiographically along basolateral membranes of the secretory epithelium [9]. In vitro studies with salt glands have demonstrated that stimulation of mAChRs elicits a variety of physiological events associated with ion secretion, including increases in ouabain-sensitive oxygen consumption [10–12], stimulation of Na^+ efflux [13], potentiation of Na^+ pump activity [14], elevation of cGMP levels [12] and induction of transmural short-circuit currents [15]. More proximal responses to mAChR activation have not been studied in detail, although both Ca^{2+} and phospholipid turnover have been implicated indirectly on the basis of Ca^{2+} dependence of stimulated ouabain-sensitive respiration [12] and increased turnover of phosphatidylinositol (PI) and phosphatidate following exposure of salt gland slices to cholinomimetic agents [16]. Additionally, little is known concerning the possibility that a muscarinic receptor subtype mediates these various responses in salt gland.

In the present study, we have characterized the functional activation of mAChR by cholinomimetic agents in dissociated salt gland cells. We show that unlike the response in exocrine glands such as the pancreas [17–19], agonist-induced Ca^{2+} mobilization in salt gland cells results in a sustained elevation of $[Ca^{2+}]_i$, and that the rise in $[Ca^{2+}]_i$ is dependent on extracellular Ca^{2+}. Stimulated breakdown of phosphoinositides in contrast is independent of external Ca^{2+}. A preliminary report of some of these results has been presented in abstract form [20].

Materials and Methods

Animals. Pekin ducklings were purchased from Ridgeway Hatcheries (La Rue, OH) and maintained on commercial poultry feed. Adaptation of the salt glands was induced by adding 1% NaCl to the drinking water for at least 1–2 weeks prior to experiments [11,21].

Materials. Carbachol, acetylcholine, methacholine, muscarine, bethanechol, arecoline, pilocarpine, atropine, type I collagenase, type I-S hyaluronidase, chymotrypsin, Hepes, ouabain, EDTA, EGTA and type I-S trypsin inhibitor were all purchased from Sigma Chemical Co. (St. Louis, MO). Eagle's minimal essential medium with Earle's salts was obtained in powdered form from Gibco (Grand Island, NY). Dowex 1-X8 (formate form, 100–200 mesh) was from Bio-Rad (Richmond, CA). Pirenzepine and McN-A-343 were generous gifts of Dr. Karl Thomae, GmbH (F.R.G.) and Dr. Stephen K. Fisher, respectively. Quin2 acetoxymethyl ester was purchased from Calbiochem (La Jolla, CA). [^{32}P]P_i (carrier-free) was a product of Amersham (Arlington Heights, IL), and *myo*[2-^{3}H]inositol (14 Ci/mmol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All other chemicals used were of reagent grade.

Preparation of dispersed salt gland cells. A dissociated suspension of duck salt gland cells was obtained as previously described [11]. Physiological and morphological characteristics of this cell preparation are described in detail elsewhere [9,11,14]. Cells were resuspended after isolation in Hepes-buffered Krebs Ringer (buffer 1) solution consisting of (mM): NaCl, 118.7; KCl, 4.8; $CaCl_2$, 1.3; $NaHCO_3$, 4.8; $MgSO_4$, 1.2; KH_2PO_4, 1.2; glucose, 6.0; and Na-Hepes, 10.0. The pH of the medium was adjusted to 7.4 with 1.0 M HCl.

Measurement of ^{32}P phospholipid labeling. Following isolation, the cells were gently centrifuged and resuspended in a phosphate-free physiological salt solution (buffer 2) consisting of (mM): NaCl, 142; KCl, 5.6; $CaCl_2$, 2.2; $NaHCO_3$, 3.6; $MgCl_2$, 1.0; glucose, 5.6; and Hepes, 30 (adjusted to pH 7.4). Aliquots of dissociated salt gland cells ($3–4 \cdot 10^6$ cells per 0.5 ml final vol.) were transferred in buffer 2 to tubes and incubated with [^{32}P]P_i (approx. 15 μCi per tube) in the presence or absence of various muscarinic agonists. Incubations were terminated after 30 min by the addition of 1.5 ml of chloroform/methanol (1 : 2). Tubes were stored on ice for 15–60 min, after which 0.5 ml of 2.4 M HCl and 1.0 ml of chloroform were added [22]. The tubes were vigorously vortexed (30 s) and the phases were separated by centrifugation. The chloroform layer was transferred to a separate tube and the aqueous layer and protein interface was re-extracted with an additional 1.0 ml of chloroform. The chloroform layers were combined and

extracted once with 2.25 ml of methanol/water/2.4 M HCl (1 : 1 : 0.25, v/v). After vortexing and centrifugation, the upper aqueous layer was removed by aspiration. A portion (200 μl) of the lower layer was removed and scintillation counted in order to establish recovery, while the remainder of the organic phase was taken to dryness under nitrogen. The phospholipids were reconstituted in 100 μl of chloroform/methanol (2 : 1, v/v) and the entire contents spotted on TLC plates (Merck, Darmstadt, Silica gel 60, 20 × 20 × 0.25 cm) which had previously been run in a solution containing 1.2 g of potassium oxalate in 40 ml of methanol and 60 ml of water, then activated immediately before use by heating for 10–20 min at 110°C. Lipids were separated, as previously described [22], by TLC in a solvent system containing chloroform/ acetone/ methanol/ acetic acid/ water (40 : 15 : 13 : 12 : 7). Lipid bands were visualized by autoradiography, scraped and scintillation counted.

[^{3}H]Inositol experiments. *Myo*-[2-^{3}H]inositol was passed over a Dowex-1 (formate) column to remove possible acidic contaminants before use. Release of inositol phosphates from cells prelabeled with [^{3}H]inositol in the presence of Li^+ was determined by a batch-absorption procedure on Dowex-1 (formate), essentially as described by Fisher et al. [23]. In brief, freshly isolated cells were centrifuged and resuspended ($1 \cdot 10^7$/ml) in buffer 2 containing 5–20 μCi/ml of *myo*-[2-^{3}H]inositol (14 Ci/mmol) and incubated at 37°C for 60 min. Following the preincubation period, 450 μl of the cell suspension was added to 50 μl of agonist and the reaction was allowed to proceed for an additional 30 min in the presence of Li^+ (10 mM). The cells were gassed with 95% O_2/5% CO_2 at 15–30 min intervals during the incubations. The reaction was stopped by the addition of 1.5 ml of chloroform/methanol (1 : 2 v/v) and the tubes were stored on ice for 15–60 min. After addition of 1 ml of chloroform and 0.5 ml of water, the tubes were vortexed vigorously and the phases were separated by centrifugation, A portion (1.4 ml) of the upper (aqueous) phase was removed and heated at 50°C for 15 min to remove traces of chloroform, brought to 3.0 ml with water, and 0.5 ml of a 50% (w/v) slurry of Dowex-1 (formate) was added. Following vigorous vortexing and centrifugation, the supernatant layer was aspirated and the resin was washed four times with 2.5 ml of 5 mM *myo*-inositol. Labeled inositol phosphates were eluted from the resin with 1 ml of 1 M ammonium formate/0.1 M formic acid of which a 0.8 ml portion was counted. For separation of individual [^{3}H]inositol metabolites, the aqueous layer of cell extracts was applied to Dowex-1 (formate form) ion-exchange columns and eluted as described by Berridge et al. [26].

$[Ca^{2+}]_i$ measurement. Dissociated cells were loaded with quin2 acetoxymethyl ester (quin2/AM) using a procedure similar to that described by Tsien et al. [24]. Cells at a concentration of $8–12 \cdot 10^6$ per ml were incubated at 37°C in buffer 1 at pH 7.4 with 50 μM quin2/AM. After 30 min the cells were diluted to approx. $3 \cdot 10^6$/ml and allowed to incubate 15 min longer, after which they were centrifuged and resuspended in fresh medium ($10 \cdot 10^6$ cells/ml) and maintained at room temperature up to 2 h. A 1 ml vol. containing $10 \cdot 10^6$ cells was placed in a cuvette and constantly stirred at 37°C throughout the fluorescence recording. Excitation and emission wavelengths were 339 ± 4 and 490 ± 10 nm, respectively. Quin2 fluorescence recordings were obtained on an Aminco-Bowman Spectrophotofluorometer interfaced with both a strip-chart recorder and an LED readout, from which numerical values were recorded at regular intervals and were used to quantitate $[Ca^{2+}]_i$ using the equations described by Tsien et al. [24,25].

Results

Changes in $[Ca^{2+}]_i$

Quin2-loaded cells challenged with carbachol displayed a dose-dependent, sustained increase in $[Ca^{2+}]_i$ which was antagonized by atropine in a similar, cumulative manner. The calculated resting cytosolic $[Ca^{2+}]_i$ was 197 ± 20 nM ($X \pm$ S.E., $n = 16$) and maximally effective carbachol concentrations (10^{-3} M) increased the $[Ca^{2+}]_i$ to 759 ± 84 nM. The agonist-induced rise in $[Ca^{2+}]_i$ was stable for at least 10 min, the longest time interval measured in these experiments.

Loading cells with quin2 allows quantitation of $[Ca^{2+}]_i$, but also may effect changes in Ca^{2+} homeostasis due to the buffering capacity of the

indicator. To examine such effects, cells were loaded with 10, 20 and 50 μM of quin2/AM, and carbachol-stimulated changes in $[Ca^{2+}]_i$ were measured. The major effect of varying the intracellular quin2 concentration was on the kinetics of the carbachol-stimulated alteration in $[Ca^{2+}]_i$. Specifically, the carbachol-induced (10^{-3} M) maximal change in $[Ca^{2+}]_i$ was reached in 32.5 ± 2.5, 67.8 ± 5.7, and 192.5 ± 17.5 s ($n = 2$–4) in cells loaded with 10, 20 and 50 μM quin2/AM, respectively. Loading cells with these various quin2 concentrations, however, did not significantly alter the calculated basal or stimulated $[Ca^{2+}]_i$, nor did it affect the carbachol concentration quin2 fluorescence response relationship.

^{32}P-lipid labeling

Muscarinic acetylcholine receptor (mAChR) activation stimulated the de novo incorporation of $[^{32}P]P_i$ into PI, with maximally effective carbachol concentrations (10^{-4}–10^{-3} M) eliciting a 3.4 ± 0.55-fold increase over basal ^{32}P labeling ($n = 11$). Carbachol caused similar increases in $[^{32}P]P_i$ incorporation into phosphatidate, while no significant effects on phosphatidylinositol 4-phosphate (PIP) or phosphatidylinositol 4,5-bisphosphate (PIP_2) were observed.

[^{3}H]Inositol phosphates

A convenient and sensitive assay for agonist-induced activation of the inositide cycle is by measurement of the release and accumulation of [^{3}H]inositol phosphates in the presence of Li^+, which prevents degradation of inositol 1-phosphate (IP_1) generated from phospholipid turnover [26,27]. In dissociated avian salt gland cells, carbachol stimulated labeled inositol phosphate release 13.4 ± 3.5-fold ($n = 3$) over basal values (see also legend to Fig. 1). The carbachol concentration-response relationships for increases in $[Ca^{2+}]_i$, [^{32}P]PI labeling and [^{3}H]inositol phosphate release fit a similar dose-response curve (Fig. 1). EC_{50} (concentration which stimulates 50% of maximum) values were 4.5, 6, and 8 μM for [^{32}P]PI, $[Ca^{2+}]_i$ and [^{3}H]inositol phosphate release, respectively (Fig. 1). The similarity of dose-responses for ^{32}P labeling of PI and for release of labeled inositol phosphates reflects the probability that they are both valid markers of receptor activation of the inositide cycle in salt gland cells.

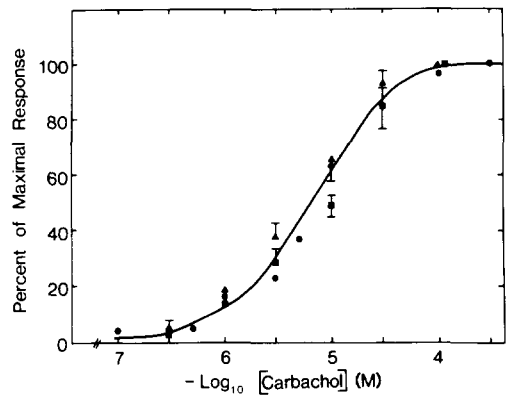

Fig. 1. Dose-response relationships for carbachol-induced changes in quin2 fluorescence, [^{32}P]PI and [^{3}H]inositol phosphate release. Data are plotted as a percentage of maximal for each response. The resting cytosolic $[Ca^{2+}]_i$ was 197 ± 20 nM, and a maximally effective carbachol concentration (10^{-3} M) increased the $[Ca^{2+}]_i$ to 759 ± 84 nmol ($n = 9$). Basal $^{32}P_i$ incorporation into PI was 3640 ± 660 dpm, while stimulated values for [^{32}P]PI were $13\,500 \pm 2360$ dpm ($n = 6$). Basal [^{3}H]inositol phosphate release was 890 ± 10 dpm and carbachol-stimulated release averaged 9910 ± 2580 dpm ($n = 3$). The absence of an error bar indicates that the S.E. was within the margins of the symbol. The EC_{50} values for quin2 (●, $n = 5$), [^{32}P]PI (▲, $n = 6$) and [^{3}H]IP (■, $n = 3$) were 6.0 ± 1.2 μM, 4.5 ± 0.7 μM and 8.0 ± 0.2 μM, respectively.

Analysis of individual [^{3}H]inositol phosphates was performed in cells labeled with [^{3}H]inositol (20 μCi/ml, 90 min), then stimulated with carbachol (10^{-3} M) for intervals ranging from 10 s to 30 min in the presence of 10 mM Li^+. The reactions were terminated by adding either 1.5 ml chloroform/methanol (1 : 2 v/v), or 550 μl of 1.0 M trichloroacetic acid (followed by diethyl ether extraction of the aqueous supernatant), and [^{3}H]inositol metabolites were separated by ion-exchange chromatography (Dowex-1, formate form) as previously described [26]. The major [^{3}H]inositol metabolite detected at both short (30 s) and long (30 min) incubation times was IP_1, which accounted for the largest part of the receptor response (89% of the total radioactivity). Labeled inositol bisphosphate (IP_2) increased approx. 2–4-fold upon mAChR stimulation and represented about 2–9% of the labeled metabolites, while labeled inositol trisphosphate (IP_3) was increased about 45–60% over basal levels and

constituted less than 4% of the total [^{3}H]inositol phosphates.

Ca^{2+} dependency of responses

To examine whether the carbachol-induced elevation in $[Ca^{2+}]_i$ is dependent on extracellular Ca^{2+}, quin2-loaded (50 μM quin2/AM) cells were resuspended in Ca^{2+}-free buffer 1, or in Ca^{2+}-free buffer 1 containing 0.1 mM EGTA. Under these conditions, carbachol failed to produce a change in $[Ca^{2+}]_i$. In order to avoid possible deleterious effects of relatively prolonged incubation (e.g., 30 min) of cells in Ca^{2+}-free media, quin2 loaded cells in normal Ca^{2+}-containing buffer 1 were treated with EGTA 30–45 s before the addition of agonist. Under these conditions, carbachol still failed to produce a detectable increase in $[Ca^{2+}]_i$ (Fig. 2). Readdition of 1.5 mM $[Ca^{2+}]$ to the incubation medium restored $[Ca^{2+}]_i$ to the elevated level seen in stimulated control cells (Fig. 2). Subsequent addition of atropine partially blocked this response, restoring $[Ca^{2+}]_i$ to the near normal resting levels generally measured in the absence of EGTA. In control experiments, cells were treated with EGTA as above, but carbachol and atropine were added prior to the readdition of Ca^{2+}, whereupon $[Ca^{2+}]_i$ returned to normal resting levels.

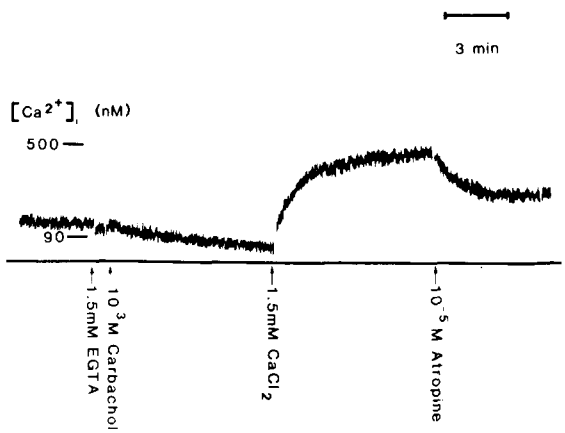

Fig. 2. Carbachol-stimulated changes in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. Quin2-loaded salt gland cells were assayed to determine changes in $[Ca^{2+}]_i$. Immediately prior to addition of carbachol, 1.5 mm EGTA was added to chelate medium Ca^{2+}. Under these conditions, carbachol failed to produce changes in $[Ca^{2+}]_i$ until the readdition of normal $[Ca^{2+}]$. This experiment was typical of four experiments. The same results were obtained in experiments in which the cells were suspended in calcium-free Krebs-Ringer-Hepes buffer without EGTA.

TABLE I

Ca^{2+} DEPENDENCY OF [^{3}H]INOSITOL PHOSPHATE RELEASE FROM CARBACHOL-STIMULATED SALT GLAND CELLS

Values represent $X \pm$ S.E. dpm for triplicate determinations normalized for total cellular radioactivity between groups, and are representative of results obtained in three separate experiments. Cells were prelabeled with [^{3}H]inositol in the absence of Li^+ for 60 min, after which the incubation volume was divided into three equal portions. Cells were pelleted by centrifugation and resuspended in the appropriate buffer. This procedure was repeated twice, at which time a 10 μl portion of the final suspension was removed and scintillation counted. The labeled cells were then distributed to tubes containing buffer or carbachol and Li^+, and further incubated for 30 min.

	[^{3}H]Inositol phosphate	
	Basal	Carbachol (1.0 mM)
Control	5480 ± 200	47200 ± 1500
$-Ca^{2+}$	2080 ± 320	42900 ± 2350
$-Ca^{2+}$ + 1 mM EGTA	1540 ± 120	30200 ± 1160

The Ca^{2+}-dependency of mAChR-stimulated labeled inositol phosphate release was examined to further elucidate the interrelationship between various salt gland responses. In contrast to the absolute dependency for extracellular Ca^{2+} on receptor-induced elevations in $[Ca^{2+}]_i$ (Fig. 2), the absence of external Ca^{2+} did not alter [^{3}H]inositol phosphate release (Table I). Moreover, a significant increase in labeled inositol phosphates was still evident in cells subjected to prolonged exposure to 1 mM EGTA, although the magnitude of this response was depressed somewhat as compared with control cells (Table I).

Muscarinic acetylcholine receptor (mAChR) subtype characterization

To further characterize the mAChR responses in avian salt gland cells, pharmacological antagonists and a series of muscarinic agonists were examined. Atropine and pirenzepine antagonized the [^{32}P]PI and Ca^{2+}-quin2 responses elicited by carbachol in a concentration-dependent manner (Figs. 3 and 4, respectively). The dissociation constants (K_i) for atropine and pirenzepine were

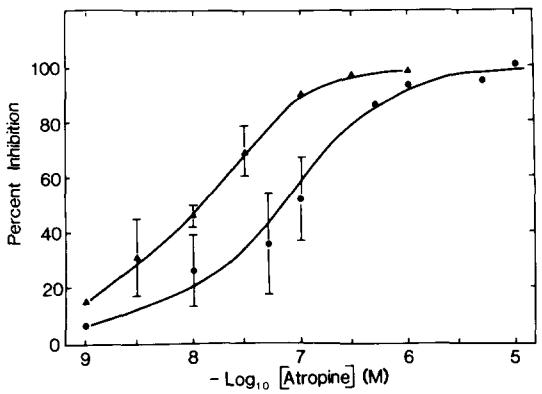

Fig. 3. Atropine inhibition of carbachol-stimulated changes in Ca^{2+} and [^{32}P]PI in dissociated avian salt gland cells. Each point is the mean (±S.E.) plotted as percent inhibition and is based on 3–5 separate experiments. Quin2 (●): $IC_{50} = 10^{-7}$ M, [carb] = 10^{-3} M; $K_i = 0.59 \pm 0.30$ nM. [^{32}P]PI (▲): IC_{50} 10^{-8} M, [carb] = 10^{-4} M; $K_i = 0.48 \pm 0.28$ nM.

calculated using IC_{50} values derived from dose-inhibition curves (Figs. 3 and 4) by the method of Cheng and Prusoff [28]. The K_i values for atropine vs. the quin2 and [^{32}P]PI responses were 0.59 ± 0.30 nM ($n = 3$) and 0.48 ± 0.28 nM ($n = 6$), respectively (Fig. 3). Pirenzepine antagonism of quin2 and [^{32}P]PI responses yielded K_i values of 227 ± 67 nM ($n = 5$) and 90.2 ± 2.1 nM ($n = 4$), respectively (Fig. 4).

Various muscarinic agonists were tested at a

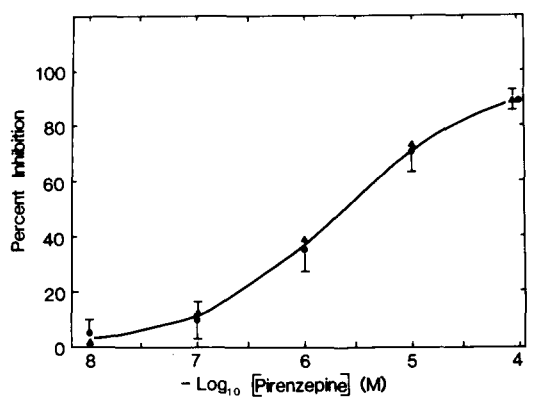

Fig. 4. Pirenzepine inhibition of carbachol-induced changes in quin2 fluorescence and [^{32}P]PI in dissociated avian salt gland cells. Each point represents the mean (±S.E.) percent inhibition based on 3–5 replicates of the experiment. Quin2 (●): $IC_{50} = 4.5 \cdot 10^{-6}$ M, [carb] = 10^{-4} M; $K_i = 227 \pm 67$nM. [^{32}P]PI (▲): $IC_{50} = 2.1 \cdot 10^{-6}$ M, [carb] = 10^{-4} M; $K_i = 90.2 \pm 2.1$ nM.

TABLE II

AGONIST EFFICACY FOR STIMULATION OF RESPONSES IN SALT GLAND CELLS

Data are expressed ($X \pm$S.E., $n = 3$) as percent of carbachol stimulation. In three experiments conducted with the agonist as described in Materials and Methods, basal and carbachol-stimulated ^{32}P incorporations into PI were, respectively, 9210 and 42400 dpm, 7990 and 24000 dpm; 1330 and 5430 dpm. Basal free $[Ca^{2+}]_i$ as measured by quin2 fluorescence was 197 ± 20 nM and carbachol increased $[Ca^{2+}]_i$ to 759 ± 84 nM ($n = 9$).

Agonist (10^{-3} M)	PI labeling (stimulation, %)	$[Ca^{2+}]_i$ (stimulation, %)
Carbachol	100	100
Acetylcholine	97 ± 2	128 ± 20
Muscarine	108 ± 4	97 ± 12
Methacholine	92 ± 6	71 ± 8
Arecoline	69 ± 7	44 ± 6
Bethanechol	61 ± 5	43 ± 6
Pilocarpine	12 ± 9	0
McM-A-343	0	0

maximally effective concentration (10^{-3} M) for their ability to enhance PI labeling and quin2 responses in dissociated salt gland cells (Table II). Addition of carbachol, acetylcholine, muscarine or methacholine resulted in larger responses than did arecoline or bethanechol. Pilocarpine and McN-A-343 were without effect in either system. With the exception of acetylcholine and muscarine, all other agonists tested were slightly more effective in stimulating the [^{32}P]PI labeling than in augmenting $[Ca^{2+}]_i$.

Discussion

Receptor activation of tissues by cholinomimetic agents is thought to be transduced into intracellular responses via breakdown of inositol lipids and mobilization of Ca^{2+} [1–5]. In tissue slices from salt glands, cholinergic stimulation of ouabain-sensitive respiration is dependent on the presence of Ca^{2+} in the medium [12]. In addition, muscarinic receptor activation in confluent cultures of salt gland cells [15] can be mimicked with the Ca^{2+} ionophore A23187 which also elicits a short-circuit current, consistent with electrogenic chloride secretion, that is blocked by ouabain and furosemide [29].

Early studies by Hokin and Hokin [16] indicated that cholinergic stimulation of salt gland tissue slices increased the turnover of phosphatidate and PI. Using ^{32}P-prelabeled dissociated cells, we showed that carbachol elicits a rapid atropine-sensitive loss of radiolabel from PIP and PIP_2 and an increase in labeling of phosphatidate within 1–2 min [30]. Stimulated labeling of PI was detectable only after 5 min. These data suggest that, as in other stimulated tissues [4–6], breakdown of polyphosphoinositides precedes stimulated labeling of phosphatidate and PI, the latter being regarded as the restorative phase of an inositol lipid labeling cycle which eventually resynthesizes PIP_2.

The present results extend these observations by directly relating changes in $[Ca^{2+}]_i$ and stimulated turnover of phosphoinositides to muscarinic receptor activation of dissociated cells. The correspondence between EC_{50} values for carbachol-induced increments in $[Ca^{2+}]_i$, [^{32}P]PI labeling, and release of [^{3}H]inositol phosphates (Fig. 1), and between K_i values for inhibition of these responses by atropine and pirenzepine (Figs. 3 and 4, respectively), provide compelling evidence that PI turnover and Ca^{2+} mobilization are mediated by the same receptor.

Studies with a number of tissues indicate that receptor-stimulated breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) leads to the formation of 1,4,5-IP_3 and diacylglycerol (Refs. 4,5,31). IP_3 has been shown to induce the release of intracellular bound Ca^{2+} from permeabilized cells [32,33] and, more specifically, from endoplasmic reticulum preparations obtained by subcellular fractionation of rat pancreas [33,34]. In salt glands, the demonstration of stimulated breakdown of polyphosphatidylinositides [30], combined with the generation of inositol phosphates, and the concomitant rise in $[Ca^{2+}]_i$ following cholinergic stimulation, are consistent with the notion that these responses are an early consequence of receptor-ligand interaction. However, some important differences exist between the responses seen in salt glands and those that have been described in other tissues, such as the pancreas.

The data presented in Fig. 2 indicate that the atropine-sensitive rise in $[Ca^{2+}]_i$ following carbachol stimulation reaches a stable plateau and that the initial elevation is dependent on the presence of extracellular Ca^{2+}. This mode of Ca^{2+} mobilization by muscarinic agonists differs from that seen in pancreatic acinar cells where the initial rise in $[Ca^{2+}]_i$ subsequently falls to a lower sustained level and is elicited even in the absence of extracellular Ca^{2+} with EGTA present [18,19]. A similar pattern to that of the pancreas, and other acinar cell types [35] has been described for cholinergic stimulation of a neurosecretory cell line (PC12 cells [36]). It is not likely that methodology accounts for differences between the observed pancreatic and salt gland Ca^{2+}-quin2 responses, since we also have been able to show that guinea pig pancreatic acinar cells, prepared in the same manner as salt gland cells, produce the expected Ca^{2+} pattern for pancreatic tissue (data not presented).

In pancreatic acinar cells [33,34], as well as other cell types [5], Ca^{2+} has been shown to be released from non-mitochondrial stores by IP_3, thereby providing a cellular mechanism for the initial Ca^{2+}-independent phase of receptor stimulation. The present data suggest, however, that the early phase of rapid rise in $[Ca^{2+}]_i$ in salt gland cells, as well as the sustained phase, is strictly dependent on extracellular Ca^{2+} (Fig. 2). In this regard, recent studies with cholinergic stimulation of parietal cells [37,38] show a similar quin2 fluorescence pattern to that presented here for salt gland cells, including a prolonged stable plateau without subsequent decay, and significantly, strict reliance of the response on extracellular Ca^{2+}. In these studies, intracellular Ca^{2+} mobilization was attributed to enhanced plasma membrane permeability to Ca^{2+} without release of Ca^{2+} from intracellular sources. It should be noted in this context that in contrast to pancreatic acinar cells, salt gland cells lack the abundant endoplasmic reticulum [21] which serves as the source for Ca^{2+} release in some tissues [33,34].

Although a variety of mechanisms have been proposed which could relate phosphoinositide metabolism to increased plasma membrane permeability to Ca^{2+}, none have, as yet, been directly demonstrated [4,5]. In the present study, some data are presented that raise the question as to whether IP_3 release and mobilization of Ca^{2+} are

always causally related or whether in some instances they can be parallel consequences of mAChR activation. In salt gland cells, stimulated alterations in $[Ca^{2+}]_i$ were dependent on the presence of extracellular Ca^{2+} (Fig. 2), whereas labeled inositol phosphate accumulation was essentially independent of Ca^{2+} (Table I). Accordingly, these studies confirm that receptor-activated inositide breakdown is a proximal event and, as has been previously demonstrated [39], is not Ca^{2+}-regulated. However, there is as yet no evidence that IP_3 is produced in significant quantities or that Ca^{2+} is released from intracellular stores in the avian salt gland as a consequence of receptor activation. It is possible that efficacious amounts of IP_3 are released and degraded too rapidly to measure by current assay procedures. An alternative explanation may be that the labeled IP_1 is formed primarily by breakdown of PI, largely bypassing IP_3 formation. For the present, the data in both the avian salt gland and parietal cells lead us to conclude that extracellular Ca^{2+} entry is effected by an as yet unidentified mechanism as a direct result of receptor activation. One possible mechanism for this Ca^{2+} entry may be via a GTP-binding protein acting on an ion channel as has recently been demonstrated in dorsal root ganglion neurons [40].

The muscarinic receptor in salt glands may be classified on the basis of functional criteria (e.g., Ca^{2+} mobilization, stimulated PI turnover) as belonging to the M_1 subclass. Pharmacological subclassification of muscarinic receptors [41] has been based primarily on the actions of the selective agonist McN-A343 and the antagonist pirenzepine. The putative M_1 receptor exhibits high affinity for pirenzepine ($K_i < 30$ nM), is stimulated by McN-A343, and is generally regarded to be coupled to PI turnover and Ca^{2+}-mediated responses [41]. Conversely, the M_2 receptor should be relatively insensitive to pirenzepine inhibition or to stimulation by McN-A343, and should not stimulate inositide turnover, but rather inhibit adenylate cyclase [41]. In the dissociated salt gland cells, pirenzepine blocked both PI turnover and Ca^{2+} responses, with K_i values ranging from 90 to 227 nM, while McN-A343 was ineffective as an agonist. Thus, this receptor appears to be of the M_2 subtype based on the pharmacological classification, but M_1 based on the fact that it stimulates inositide turnover. It may prove more meaningful to categorize the salt gland muscarinic acetylcholine receptor according to the classification described by Fisher et al. [42] for brain cholinergic receptors. The "group A" or full agonists are acetylcholine, carbachol, methacholine and muscarine, while "group B" or weak agonists include arecholine, pilocarpine and bethanechol as we have shown in salt gland cells (Table II).

We have shown that while the mAChR-induced breakdown of cellular phosphoinositides is independent of extracellular Ca^{2+}, the change in $[Ca^{2+}]_i$ requires external Ca^{2+}. The concentration-response relationships for carbachol-induced inositol phosphate release, $[Ca^{2+}]_i$ signaling, and $[^{32}P]$PI labeling suggest in addition to a causal relationship with mAChR activation, the absence of amplification steps between these responses.

Acknowledgments

The technical assistance of Linda O'Dell and James Schreiber is gratefully acknowledged. We also thank Dr. Stephen K. Fisher and John Olson for help with the inositol phosphate experiments and Stephanie Dunham for excellent secretarial assistance. This study was supported by NIH grants AM 27559 (S.A.E.), NS 20920 (R.M.S.), NS 15143 (B.W.A.). Dr. Lowy is a recipient of National Research Service Award AM07487.

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