Calcium-sensitivity of inositol 1,4,5-trisphosphate metabolism in exocrine cells from the avian salt gland

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The generation of inositol phosphates upon muscarinic-receptor activation was studied in [^3H]inositol-loaded exocrine cells from the nasal salt glands of the duck *Anas platyrhynchos*, and the metabolism of different inositol phosphates *in vitro* was studied in tissue homogenates, with particular reference to the possible interaction of changes in intracellular $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_i$) with the metabolic processes. In intact cells, there was a rapid (within 15 s) generation of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$, followed by an accumulation of their breakdown products, $\text{Ins}(1,3,4)\text{P}_3$ and inositol bis- and mono-phosphates. Ca^{2+} -sensitivity of the $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase was demonstrated in tissue homogenates, with the rate of phosphorylation increasing 2-fold at free Ca^{2+} concentrations $> 1 \mu\text{M}$. However, addition of calmodulin or the presence of the calmodulin inhibitor W-7 (up to $100 \mu\text{M}$) had no effect. 3-Kinase activity increased proportionally with the initial $\text{Ins}(1,4,5)\text{P}_3$ concentration up to $1 \mu\text{M}$, but a 10-fold higher substrate concentration produced only a doubling in the phosphorylation rate. $\text{Ins}(1,3,4,5)\text{P}_4$ was dephosphorylated to $\text{Ins}(1,3,4)\text{P}_3$, which accumulated in the homogenate assays as well as in intact cells. Depending on its concentration, $\text{Ins}(1,3,4)\text{P}_3$ was phosphorylated [in part to $\text{Ins}(1,3,4,6)\text{P}_4$] or dephosphorylated. To investigate the Ca^{2+} -sensitivity of the 3-kinase in intact cells, excess quin2 was used to buffer the receptor-mediated transient changes in $[\text{Ca}^{2+}]_i$ in [^3H]inositol-loaded cells. These experiments revealed that increasing $[\text{Ca}^{2+}]_i$ from < 100 to approx. 400 nM (i.e. within the physiological range) has no effect on the partitioning of $\text{Ins}(1,4,5)\text{P}_3$ metabolism (phosphorylation versus dephosphorylation) and on the accumulation of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$. This indicates that activation of the 3-kinase by physiologically relevant Ca^{2+} concentrations may not play a major role in the generation of $\text{Ins}(1,3,4,5)\text{P}_4$ signals upon receptor activation in these cells. The latter are mainly achieved by the receptor-mediated increase in $\text{Ins}(1,4,5)\text{P}_3$ in the cell and its phosphorylation by the 3-kinase in a substrate-concentration-dependent manner.

INTRODUCTION

It is now well established that salt secretion in the avian nasal gland is stimulated by muscarinic-acetylcholine-receptor activation (Borut & Schmidt-Nielsen, 1963; Hootman & Ernst, 1981; Lowy *et al.*, 1985; Shuttleworth & Thompson, 1987). Receptor activation in isolated salt-gland cells results in enhanced phosphatidylinositol turnover, inositol phosphate accumulation and characteristic patterns of intracellular Ca^{2+} signals involving receptor-mediated release of Ca^{2+} from intracellular stores and Ca^{2+} entry from the extracellular space (Fisher *et al.*, 1983; Snider *et al.*, 1986; Shuttleworth & Thompson, 1989; Shuttleworth, 1990).

Although the precise mechanism of receptor-mediated Ca^{2+} entry into the cytoplasm of non-excitable cells is still unresolved (Putney *et al.*, 1989; Berridge & Irvine, 1989), at least one current model proposes that $\text{Ins}(1,3,4,5)\text{P}_4$ acts as a cofactor mediating Ca^{2+} entry upon release of Ca^{2+} from intracellular stores by $\text{Ins}(1,4,5)\text{P}_3$ (Irvine, 1990). This proposed role for $\text{Ins}(1,3,4,5)\text{P}_4$ is supported by biochemical studies of the $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase from various tissues, showing that $\text{Ins}(1,3,4,5)\text{P}_4$ production increases with increasing Ca^{2+} concentrations (Biden & Wollheim, 1986; Balla *et al.*, 1988) in a calmodulin-dependent manner (Biden *et al.*, 1987; Daniel *et al.*, 1988; Heacock *et al.*, 1990; Takazawa *et al.*, 1990; Lee *et al.*, 1990). However, the Ca^{2+} concentrations used in those studies to elicit a significant activation of the 3-kinase were higher (micromolar) than those generally seen in stimulated cells, and several studies have reported a basic 3-kinase activity (equivalent to 20–50% of maximal activity) which is not dependent on the presence of Ca^{2+} (Johanson *et al.*, 1988; Biden *et al.*, 1988; Conigrave & Roufogalis, 1989; Heacock *et al.*, 1990). Furthermore, calmodulin

inhibitors in concentration ranges which proved to be effective in other Ca^{2+} /calmodulin-dependent reactions failed to inhibit $\text{Ins}(1,4,5)\text{P}_3$ phosphorylation by the 3-kinase (Biden *et al.*, 1987; Li *et al.*, 1989). Therefore, although a Ca^{2+} -sensitivity can clearly be demonstrated for the 3-kinase activity, its physiological significance remains questionable.

This study was undertaken to investigate the metabolism of $\text{Ins}(1,4,5)\text{P}_3$ and its products, especially $\text{Ins}(1,3,4,5)\text{P}_4$, in avian salt-gland cells, with the specific aim of determining the physiological significance of possible regulatory effects of Ca^{2+} on the 3-kinase and the relevance of this regulatory potential for the concentration of $\text{Ins}(1,3,4,5)\text{P}_4$ and its signalling role in the cell. Experiments were carried out with intact isolated cells and tissue homogenates prepared from the nasal salt glands of salt-stressed ducklings (Shuttleworth & Thompson, 1989).

EXPERIMENTAL

Materials

^3H -labelled inositol was purchased from ARC or NEN–DuPont (sp. radioactivity 15.6 Ci/mmol), and ^3H -labelled inositol phosphates were obtained from NEN–DuPont (sp. radioactivity 17 Ci/mmol). [^3H] $\text{Ins}(1,3,4,6)\text{P}_4$ was kindly given by Dr. S. Shears, NIEHS, U.S.A. $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(3,4,5,6)\text{P}_4$ and the proteinase inhibitors were from Boehringer Mannheim. Calmodulin, W-7 and quin2 (acetoxymethyl ester) were obtained from Calbiochem.

Cells

Maintenance of the ducklings (*Anas platyrhynchos*) and the methods for obtaining suspensions of isolated cells from the nasal salt gland were as described previously (Shuttleworth &

Fig. 1. Accumulation of radiolabelled inositol phosphates in [^3H]inositol-loaded cells from the nasal salt gland of ducklings upon muscarinic-receptor activation with 0.5 mM-carbachol at zero time

Cells were either pretreated with 10 mM-LiCl for 15 min (●) or Li $^+$ -free (▲). Results are presented as radioactivity in the relevant inositol phosphate fraction, expressed as a percentage of the total radioactivity in the membrane-bound phosphoinositides (PI). Each

Thompson, 1989). All experimental animals were between 4 and 10 days old and had been maintained on 1% NaCl drinking water *ad lib.* for 48 h before use, as previously described.

Inositol phosphate turnover measurements

Loading of the isolated cells with [^3H]inositol, the stimulation of those cells with carbamoylcholine chloride (carbachol), extraction and analysis of the water-soluble inositol phosphates and determination of the radioactivity in the membrane-bound phosphoinositide fraction were performed as described previously (Hildebrandt & Shuttleworth, 1991).

Preparation of nasal salt-gland homogenates

Birds were killed by decapitation, and the nasal salt glands were exposed. The glands were injected *in situ* with homogenizing buffer to remove the blood from the gland, rapidly dissected free, weighed and minced into 1 mm 3 cubes, followed by homogenization in 2.5 ml of ice-cold buffer containing (in mM): 100 KCl, 20 NaCl, 2 MgCl $_2$, 0.96 NaH $_2$ PO $_4$, 0.84 CaCl $_2$, 1 EGTA, 25 Hepes, 5 phosphocreatine, 1.48 ATP, 50 mg of creatine kinase/l, 2 g of BSA/l, proteinase-inhibitor cocktail (aprotinin, antipain, pepstatin and leupeptin, 2 mg/l each), pH 7.4. After storage on ice for 5 min, homogenates were diluted with buffer solution to give a tissue-fresh-weight/buffer-volume ratio of approx. 5 g/l. The free Ca $^{2+}$ concentration in these homogenates was measured with a Ca $^{2+}$ -sensitive MI-600 electrode (Micro-electrodes Inc., Londonderry, NH, U.S.A.) and adjusted by adding CaCl $_2$ solution or EGTA. The free Ca $^{2+}$ concentration in homogenates prepared with the above solution was approx. 170 nM. Portions of this diluted homogenate were warmed to 38 °C and the reactions started by adding the radiolabelled inositol phosphates. Reactions were terminated by addition of equal volumes of 1 M-trichloroacetic acid containing 2.7 mM-sodium phytate (Hughes *et al.*, 1988). Sample preparation and h.p.l.c. analysis of the inositol phosphates were performed as described previously (Hildebrandt & Shuttleworth, 1991).

Ca $^{2+}$ -dependency of the Ins(1,4,5)P $_3$ 3-kinase

Isolated salt-gland cells were suspended in Hepes-buffered saline (Shuttleworth & Thompson, 1982) and incubated with [^3H]inositol (200 mCi/l) and the fluorescent Ca $^{2+}$ indicator quin2 (acetoxymethyl ester) (approx. 50 μM) for 1 h at 38 °C. The cells were washed twice and resuspended in Hepes-buffered saline. To monitor intracellular free Ca $^{2+}$ concentration ([Ca $^{2+}$] $_i$) in these cells under the experimental conditions, a portion of the cell suspension was washed three times in a low-Ca $^{2+}$ (40 μM) Hepes-buffered saline and the cells were transferred to a stirred cuvette in the spectrofluorimeter (Perkin-Elmer LS-5B) containing low-Ca $^{2+}$ saline (38 °C). The fluorescence of the unstimulated cells was recorded (excitation wavelength 339 nm, emission wavelength 492 nm). The cells were then stimulated by adding carbachol (final concn. 0.5 mM), and the resulting fluorescence was recorded for 2 min before the normal extracellular Ca $^{2+}$ concentration (1.3 mM) was reestablished, after which the fluorescence was recorded for a further 3 min. Fluorescence signals were calibrated as previously described (Shuttleworth & Thompson, 1989), and [Ca $^{2+}$] $_i$ values were calculated by using a quin2/Ca $^{2+}$ dissociation constant, K_d , of 115 nM (at 38 °C; Tsien *et al.*,

data point represents the mean of 2 or 3 independent experiments. The absolute range of results from the individual measurements is indicated by the vertical bars. The data for Ins(1,4,5)P $_3$ and Ins(1,3,4,5)P $_4$ under Li $^+$ -free conditions were taken from Hildebrandt & Shuttleworth (1991) and are included for the purpose of comparison.

1982). The remaining cell suspension was divided into two portions; the cells were washed and resuspended in low-Ca²⁺ saline. Carbachol (0.5 mM) was added to both tubes, followed 2 min later by addition of either sufficient Ca²⁺ to raise extracellular Ca²⁺ concentration to 1.3 mM (experimental tube) or the same volume of low-Ca²⁺ saline (control tube). The reactions in both tubes were terminated 2 min later by addition of an equal volume of ice-cold trichloroacetic acid (1 M) containing 2.7 mM-sodium phytate. Sample preparation and h.p.l.c. analysis of the inositol phosphates were performed as described previously (Hildebrandt & Shuttleworth, 1991).

RESULTS

Inositol phosphate turnover in nasal salt-gland cells

Isolated salt-gland cells loaded with [³H]inositol showed a time-dependent increase in the generation of ³H-labelled inositol phosphates upon stimulation with carbachol (0.5 mM). Labelled Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ increased 2–3-fold from their control values within 15 s after receptor activation, and thereafter remained constant for up to 30 min (results not shown), a response that was not significantly affected by pretreatment of the cells with Li⁺ (Fig. 1). The dephosphorylation of Ins(1,4,5)P₃ led to an increase in labelled Ins(1,4)P₂ with a time course, in Li⁺-free medium, that resembled that of Ins(1,4,5)P₃. However, in the presence of Li⁺, Ins(1,4)P₂ accumulated at an approximately constant rate (Fig. 1), an effect probably due to the inhibition by Li⁺ of the inositol phosphate 1-phosphatase (Inhorn & Majerus, 1988). An essentially constant accumulation of [³H]Ins(1,3,4)P₃, the dephosphorylation product of [³H]Ins(1,3,4,5)P₄, was also observed (Fig. 1). No difference in the accumulation of Ins(1,3,4)P₃ between Li⁺-treated and untreated cells could be detected over the first 2 min of carbachol stimulation but, after stimulation with carbachol for 30 min, Ins(1,3,4)P₃ had accumulated approx. 3 times higher in the Li⁺-treated cells (results not shown).

Ins(1,4,5)P₃ metabolism in homogenates

After incubation of homogenates with [³H]Ins(1,4,5)P₃ (1.6 nM) for 45 min, only 5% of the total radioactivity remained as Ins(1,4,5)P₃ (Fig. 2). Most of the label was found in an inositol polyphosphate co-eluted with Ins(1,3,4,5)P₄, with slightly lesser amounts in the Ins(1,3,4)P₃ and inositol monophosphate fractions. With a different chromatographic technique (adapted from Shears, 1989b), we were able to resolve the InsP₄s produced in salt-gland homogenates spiked with [³H]Ins(1,4,5)P₃ into two peaks. One co-eluted with [³H]Ins(1,3,4,5)P₄; the other was generally larger (approx. 70% of total radioactivity in the InsP₄ fractions at 45 min) and co-eluted with a sample of [³H]-Ins(1,3,4,6)P₄ kindly provided by Dr. S. Shears (NIEHS). Only very small amounts of radioactivity (2%) could be detected in Ins(1,4)P₂ and Ins(3,4)P₂ fractions, and 5% in the inositol fraction (Fig. 2a). Buffering of the free Ca²⁺ concentration to < 100 nM by addition of 0.15 g of EGTA/l decreased the accumulation of Ins(1,3,4)P₃, but increased the amount of labelled inositol monophosphates in the same period of time (Fig. 2b). In contrast, increasing [Ca²⁺] to > 1 μM enhanced Ins(1,3,4)P₃ accumulation and suppressed the monophosphate production (Fig. 2c).

Because the free Ca²⁺ concentration seemed to influence the ratio of Ins(1,4,5)P₃ phosphorylation and dephosphorylation, the apparent Ca²⁺-dependency of the Ins(1,4,5)P₃ 3-kinase was studied by incubating Ins(1,4,5)P₃ (25 μM) in homogenates at different Ca²⁺ concentrations with the addition of 2,3-bisphosphoglycerate (1 g/l) to block the dephosphorylation reaction (Downes *et al.*, 1982; Shears *et al.*, 1987; Guillemette *et al.*,

1990). The phosphorylation rates were not affected by free Ca²⁺ concentrations in the physiological range (170–400 nM), but increased approx. 2-fold at 2.7 μM-Ca²⁺ (Table 1), a pattern that was unaffected by the addition of 10 μM-calmodulin. Preincubation of homogenates with the calmodulin antagonist W-7 at concentrations up to 100 μM also failed to affect the phosphorylation rate of Ins(1,4,5)P₃ (1 μM) when tested in the

Fig. 2. Distribution of radioactivity in the metabolites of 1.6 nM-[³H]Ins(1,4,5)P₃ after 45 min incubation in diluted salt-gland homogenates

(a) In intracellular buffer solution as described in the Experimental section; (b) in buffer solution with [Ca²⁺] < 100 nM; (c) in buffer solution with [Ca²⁺] > 1 μM. Each bar represents the mean of two determinations of the accumulated inositol phosphate moiety as a percentage of the originally introduced radioactivity in Ins(1,4,5)P₃ (arrow). The vertical bars indicate the variation in the absolute values of individual determinations from the mean value. Key: A, inositol; B, InsP; C, Ins(1,3)P₂; D, Ins(1,4)P₂; E, Ins(3,4)P₂; F, Ins(1,3,4)P₃; G, Ins(1,4,5)P₃; H, InsP₄.

Table 1. Phosphorylation of [^3H]Ins(1,4,5) P_3 (25 μM) by diluted salt-gland homogenate in the absence (–CaM) or in the presence of additional 10 μM -calmodulin (+CaM)

Values represent the means of four determinations $\pm$ variation of individual values from the means (incubation period 3 min).

Free Ca^{2+} concn. (μM)	Ins(1,4,5) P_3 phosphorylation rate (pmol/min per mg of protein)	
	–CaM	+CaM
0.17	25 $\pm$ 1	22 $\pm$ 1
0.40	22 $\pm$ 1	24 $\pm$ 1
2.70	41 $\pm$ 2	43 $\pm$ 2

Fig. 3. Effect of initial substrate concentration on phosphorylation and dephosphorylation rates of Ins(1,4,5) P_3 in diluted salt-gland homogenates

Homogenates containing 1 μM - Ca^{2+} and 10 mM-LiCl were incubated with various Ins(1,4,5) P_3 concentrations for 7 min (the [^3H]Ins(1,4,5) P_3 -related radioactivity in the assay with the lowest initial concentration decreased by less than 25% during this time period). Reactions were terminated by adding an equal volume of 1 M-trichloroacetic acid containing 2.7 mM-sodium pyruvate. Results presented are the total conversion rate of Ins(1,4,5) P_3 (■), the phosphorylation rate (▲) calculated from the accumulation of Ins(1,3,4,5) P_4 and Ins(1,3,4) P_3 , and the dephosphorylation rate of Ins(1,4,5) P_3 (●) calculated from the accumulation of Ins(1,4) P_2 and Ins4P. No [^3H]inositol was detected, owing to inhibition of the inositol monophosphatase activity by Li^+ . Each data point represents the mean of two determinations. The variation of the individual values from the mean was less than 6% for all different Ins(1,4,5) P_3 concentrations. The inset shows the same data on linear scales, demonstrating more clearly the limited phosphorylation rate and the preference for dephosphorylation at higher substrate concentrations.

presence of 1 μM - Ca^{2+} and 1 g of 2,3-bisphosphoglycerate/1 (results not shown). Only at a W-7 concentration of 250 μM was any inhibition of Ins(1,3,4,5) P_4 production observed (approx. 40%).

At a free Ca^{2+} concentration of 1 μM , the rates of Ins(1,4,5) P_3 phosphorylation and dephosphorylation were also influenced by its concentration in the range 10 nM–10 μM . At low substrate concentrations the phosphorylation of Ins(1,4,5) P_3 to Ins(1,3,4,5) P_4 predominated, but at concentrations above 1 μM the

Fig. 4. Distribution of radioactivity in the metabolites of 1.6 nM-[^3H]Ins(1,3,4,5) P_4 after 45 min incubation in diluted salt-gland homogenates

(a) In intracellular buffer solution as described in the Experimental section; (b) in the same buffer system without ATP and the ATP-generating system. Each bar (A–H, defined in Fig. 2 legend) represents the mean of two determinations of the accumulated inositol phosphate moiety as a percentage of the originally introduced radioactivity in Ins(1,3,4,5) P_4 (arrow). The vertical bars indicate the variation in the individual values from the mean value.

dephosphorylation rate exceeded that of phosphorylation (Fig. 3). The phosphorylation rate appeared to reach a plateau at substrate concentrations between 1 and 10 μM (Fig. 3, inset).

Ins(1,3,4,5) P_4 and Ins(1,3,4) P_3 metabolism in homogenates

The metabolism of Ins(1,3,4,5) P_4 was studied in diluted homogenates containing 1.6 nM ^3H -labelled substrate. After 45 min, 60% of the initial radioactivity was found in an Ins P_4 , 38% in Ins(1,3,4) P_3 and 1.4% in Ins(3,4) P_2 (Fig. 4a). In the absence of both ATP and the ATP-generating system, 88% of the label was found in Ins(1,3,4) P_3 , only 3% in Ins P_4 , 5% in Ins(3,4) P_2 , 3% in Ins(1,3) P_2 and 1% in the monophosphates (Fig. 4b).

Incubation of Ins(1,3,4) P_3 (0.9 nM) for 45 min resulted in a similar pattern of label in the inositol phosphates to that in the Ins(1,3,4,5) P_4 experiments (Fig. 5). Only 29% of the initial amount of radioactivity remained in the Ins(1,3,4) P_3 fraction, with 69% found in the Ins P_4 fraction and a negligible amount (2%) in the Ins(3,4) P_2 fraction (Fig. 5a). In the absence of ATP and the ATP-generating system, 92% of the initial radioactivity remained as Ins(1,3,4) P_3 and no radioactivity was found in the Ins P_4 fraction. Only minor portions (4% and 3% respectively) were found in Ins(3,4) P_2 and Ins(1,3) P_2 (Fig. 5b). In the normal buffer system (i.e. with ATP and the ATP-generating system), addition of 10 μM -Ins(3,4,5,6) P_4 , which has been reported to inhibit Ins(1,3,4) P_3 kinase activity (Hughes *et al.*, 1989), caused

Fig. 5. Distribution of radioactivity in the metabolites of 0.9 nM-[³H]Ins(1,3,4) P_3 after 45 min incubation in diluted salt-gland homogenates

(a) In intracellular buffer solution as described in the Experimental section; (b) in the same buffer system without ATP and the ATP-generating system; (c) in normal intracellular buffer solution containing 10 μ M-Ins(3,4,5,6) P_4 . Each bar (A-H, defined in Fig. 2 legend) represents the mean of two determinations of the accumulated inositol phosphate moiety as a percentage of the originally introduced radioactivity in Ins(1,3,4) P_3 (arrow). The vertical bars indicate the variation in the values of individual determinations from the mean value.

an almost complete suppression of the phosphorylation of Ins(1,3,4) P_3 (Fig. 5c).

Because the dephosphorylation of Ins(1,3,4) P_3 appeared to be very much slower than the phosphorylation reaction, experiments were carried out to determine whether this was due to the low initial concentration of Ins(1,3,4) P_3 (0.9 nM) in the incubation buffer. In these experiments unlabelled Ins(1,3,4) P_3 was added to elevate its initial concentration in the homogenates to 1 or 5 μ M.

Fig. 6. Effect of initial substrate concentration on phosphorylation and dephosphorylation rates of Ins(1,3,4) P_3 in diluted salt-gland homogenates

Homogenates were incubated with various Ins(1,3,4) P_3 concentrations for 45 min (the [³H]Ins(1,3,4) P_3 -related radioactivity in the assay with the lowest initial concentration decreased by less than 35% during this time period). Reactions were terminated by adding an equal volume of 1 M-trichloroacetic acid containing 2.7 mM-sodium phytate. Results presented are the total conversion rate of Ins(1,3,4) P_3 (■), the phosphorylation rate (▲) calculated from the accumulation of Ins P_4 , and the dephosphorylation rate (●) calculated from the accumulation of Ins(3,4) P_2 , Ins(1,3) P_2 , inositol monophosphates and inositol. Each data point represents the mean of two determinations. The variation of the individual values from the mean was less than 6% for all different Ins(1,3,4) P_3 concentrations.

The results obtained showed that the total conversion rate of Ins(1,3,4) P_3 increased with its concentration (Fig. 6). However, whereas the phosphorylation reaction predominated at 0.9 nM-Ins P_3 , the dephosphorylation reaction, mainly to Ins(3,4) P_2 , became increasingly important as the concentration of Ins(1,3,4) P_3 was increased to the micromolar range (Fig. 6).

Ca²⁺-dependency of the Ins(1,4,5) P_3 3-kinase in intact cells

Experiments were carried out to determine the [³H]inositol phosphate generation in [³H]inositol-loaded cells under conditions of sustained polyphosphoinositide hydrolysis and controlled variation of [Ca²⁺]_i. Transient changes in [Ca²⁺]_i, e.g. those due to the receptor-mediated release of Ca²⁺ from intracellular stores, were buffered by loading the cells with excess quin2 (Tsien *et al.*, 1982; Rink, 1988), which also enabled us to monitor the [Ca²⁺]_i in a sample of the experimental cell suspension. Receptor activation with carbachol (0.5 mM) in these cells suspended in low-Ca²⁺ (40 μ M) saline resulted in a small increase in [Ca²⁺]_i from the control value of 60 nM to a plateau of 85 nM (Fig. 7a, A) without generating the normally observed rapid transient increase in [Ca²⁺]_i (see Shuttleworth & Thompson, 1989). Restoration of the normal extracellular Ca²⁺ concentration (1.3 mM) caused an increase in [Ca²⁺]_i to approx. 400 nM (Fig. 7a, B), which is similar to the value of [Ca²⁺]_i normally seen in these cells during the sustained portion of the Ca²⁺ signal following receptor activation (Shuttleworth & Thompson, 1989; Hildebrandt & Shuttleworth, 1991). The buffering action of quin2 therefore successfully suppressed the transient increase in [Ca²⁺]_i due to release of Ca²⁺ from intracellular stores and slowed down the increase of [Ca²⁺]_i after adding back the extracellular Ca²⁺ but did not affect the final equilibrium value of [Ca²⁺]_i.

Measurements of [³H]inositol phosphates in samples of the same cell suspensions 4 min after addition of carbachol revealed that, although [Ca²⁺]_i in the experimental cells was maintained

Fig. 7. Ca^{2+} signals and inositol phosphate generation upon receptor activation with carbachol in quin2- and $[^3H]$ inositol-loaded intact salt-gland cells suspended in saline with a low Ca^{2+} concentration (40 μM)

(a) The transient as well as the sustained Ca^{2+} signal upon addition of 0.5 mM-carbachol (A) was attenuated to < 100 nM by the buffering action of quin2 and the low extracellular Ca^{2+} concentration respectively. Raising the extracellular Ca^{2+} concentration to 1.3 mM (B) caused an increase in $[Ca^{2+}]_i$ and a sustained Ca^{2+} signal of 350–450 nM. The trace presented represents the mean curve of six independent experiments. (b) Accumulation of $[^3H]$ inositol phosphates upon carbachol stimulation of salt-gland cells (measured in samples of the same cell suspensions used for the monitoring of $[Ca^{2+}]_i$). The extracellular Ca^{2+} concentration either had been kept at 40 μM for the entire experimental period (□) or had been raised to 1.3 mM 2 min after the carbachol stimulation (▨). The reactions were terminated 4 min after receptor activation with carbachol. The data are expressed as a percentage of the total radioactivity detected in the membrane-bound phosphoinositide fraction (PI). Values given here are means $\pm$ S.E.M. of six independent experiments. No significant differences were found in the $Ins(1,3,4,5)P_4$, $Ins(1,4,5)P_3$ or the $Ins(1,3,4)P_3$ accumulation, as a result of the difference in $[Ca^{2+}]_i$ (Student's *t* test, $P > 0.05$). The $Ins(1,4)P_2$ generation was significantly enhanced in cells with high $[Ca^{2+}]_i$ ($P < 0.05$). For purposes of comparison, the mean values $\pm$ S.E.M. ($n = 3$) of the inositol phosphate accumulation in quin2-free cells loaded with $[^3H]$ inositol and stimulated with 0.5 mM-carbachol for 2 min are included (▩), showing that the presence of quin2 in these cells does not affect the receptor-mediated inositol phosphate generation.

much higher (350–450 nM) than in the control cells (< 100 nM) for at least 1 min, there were no significant differences in the accumulation rates of labelled $Ins(1,4,5)P_3$, $Ins(1,3,4,5)P_4$ or $Ins(1,3,4)P_3$ (Fig. 7b). Changes in $[Ca^{2+}]_i$ in the physiological range seem not to affect the phosphorylation rate of $Ins(1,4,5)P_3$ by the 3-kinase in intact cells. Although no significant amounts

of radioactivity were found in the $Ins(1,3)P_2$ and the $Ins(3,4)P_2$ fractions, the accumulation of $[^3H]Ins(1,4)P_2$ was higher in cells with an increased $[Ca^{2+}]_i$ (Student's *t* test, $P < 0.05$, $n = 6$ independent experiments).

DISCUSSION

Muscarinic-receptor activation in intact isolated cells from the nasal salt gland of salt-stressed ducklings resulted in rapid 2–3-fold increases in radiolabelled $Ins(1,4,5)P_3$ and its phosphorylation product $Ins(1,3,4,5)P_4$ (Fig. 1), a response essentially similar to that reported in a variety of comparable cell systems such as hepatocytes (Burgess *et al.*, 1985) and parotid acinar cells (Hughes & Putney, 1989). Subsequently, both compounds were dephosphorylated to $Ins(1,4)P_2$ or $Ins(1,3,4)P_3$ respectively. More detailed analysis of the metabolic pathways involved, by using incubations of exogenous radiolabelled inositol phosphates with tissue homogenates, showed that, at low substrate concentrations, $[^3H]Ins(1,4,5)P_3$ was phosphorylated as well as dephosphorylated, and the phosphorylation rate seemed to be dependent on the free Ca^{2+} concentration in the homogenate (Fig. 2). In the presence of 2,3-bisphosphoglycerate and at a higher concentration of $Ins(1,4,5)P_3$ (25 μM ; Majerus *et al.*, 1988; Horstman *et al.*, 1988), the basal phosphorylation rate was constant over the $[Ca^{2+}]$ range 170–400 nM, but was almost doubled at 2.7 μM - Ca^{2+} (Table 1). This is in agreement with reports of a Ca^{2+} /calmodulin-dependency of purified $Ins(1,4,5)P_3$ 3-kinase preparations from various tissues (Biden *et al.*, 1987, 1988; Balla *et al.*, 1988; Daniel *et al.*, 1988; Johanson *et al.*, 1988). In the experiments presented here, addition of calmodulin to homogenates had no effect on the phosphorylation rate, probably because the endogenous calmodulin content of the homogenized tissue was sufficient to supply all binding sites of the 3-kinase with calmodulin. Addition of W-7, a potent calmodulin inhibitor in other Ca^{2+} /calmodulin-dependent enzyme systems, failed to inhibit $Ins(1,4,5)P_3$ phosphorylation at concentrations up to 100 μM , a result similar to that previously reported for the 3-kinase from RINm5F cells (Biden *et al.*, 1987). This W-7 concentration, however, is 10–20 times higher than that necessary for the half-maximal inhibition of cyclic AMP phosphodiesterase or myosin light-chain kinase respectively (Hidaka & Tanaka, 1983). Purified $Ins(1,4,5)P_3$ 3-kinase from bovine brain reportedly has a high affinity for calmodulin, and yet is similarly insensitive to W-7 and other calmodulin antagonists (Li *et al.*, 1989). The reason for the relative insensitivity of the enzyme to calmodulin antagonists, as well as the demonstration that a significant portion of total 3-kinase activity is entirely Ca^{2+} -independent as reported in the present study and elsewhere (Johanson *et al.*, 1988; Biden *et al.*, 1988; Heacock *et al.*, 1990), is unclear.

Clearly, whether the 3-kinase is activated or not, a principal effect of receptor activation in these cells is a rapid increase in $Ins(1,4,5)P_3$, and the possibility arises that such large changes in substrate concentration may themselves significantly influence the partitioning between phosphorylation and dephosphorylation pathways. The results show that the preference for the phosphorylation reaction at very low substrate concentrations disappears with increasing $Ins(1,4,5)P_3$ concentrations and that more than 90% of the substrate turnover is via dephosphorylation at 10 μM (Fig. 3). This can be explained by the $Ins(1,4,5)P_3$ 3-kinase possessing a higher affinity for $Ins(1,4,5)P_3$ and a lower maximal velocity than the 5-phosphatase, as reported in a variety of tissues (for review see Shears, 1989a). The physiological relevance of such findings could be that a significant elevation of the intracellular $Ins(1,3,4,5)P_4$ concentration can be achieved rapidly after the onset of the receptor-mediated polyphospho-

inositide hydrolysis. This effect, it should be noted, is entirely independent of any activation of the 3-kinase. At higher substrate concentrations, however, the phosphorylation rate in homogenates becomes virtually independent of the intracellular Ins(1,4,5)P₃ concentrations (Fig. 3, inset). An important implication of this is that the Ins(1,3,4,5)P₄ concentration in intact cells under stimulated conditions may depend much more on the breakdown of Ins(1,3,4,5)P₄ than on its production rate. In homogenates, the dephosphorylation of Ins(1,3,4,5)P₄ occurs very rapidly (Fig. 4). Ins(1,3,4)P₃, the dephosphorylation product of Ins(1,3,4,5)P₄, accumulated in the absence of ATP, but was phosphorylated to an InsP₄ [mostly Ins(1,3,4,6)P₄] in the presence of ATP and an ATP-generating system, a result confirmed in incubations with exogenous Ins(1,3,4)P₃ (Figs. 5a and 5b). These experiments further suggested that the metabolism of Ins(1,3,4)P₃ may be a rate-limiting step in the InsP₃/InsP₄ pathway, as there was relatively little dephosphorylation even in the absence of competition for Ins(1,3,4)P₃ by phosphorylating enzymes (Figs. 4b, 5b, 5c). Examination of the concentration-dependence of Ins(1,3,4)P₃ metabolism revealed that the proportion of phosphorylation to dephosphorylation changed in a characteristic manner (Fig. 6). Although at low Ins(1,3,4)P₃ concentrations the metabolism of this compound was almost entirely via a phosphorylation pathway to an InsP₄, with increasing Ins(1,3,4)P₃ concentrations the dephosphorylation reaction became more significant and, at 1 µM-Ins(1,3,4)P₃, reached a rate similar to that of the phosphorylation reaction. Which pathway is preferred under different circumstances in cells will depend on the kinetic properties of the respective enzymes. With Ins(1,3,4)P₃ as a substrate, very different K_m values for the inositol polyphosphate 1-phosphatase have been reported in the literature, ranging from 0.9 µM (Hansen *et al.*, 1989) to 20 µM (Inhorn & Majerus, 1987), whereas the only K_m value reported for the Ins(1,3,4)P₃ 6-kinase was very low (0.29 µM; Hansen *et al.*, 1988). This could explain the preference for the phosphorylation reaction seen at very low substrate concentrations (Fig. 6). Since Ins(1,3,4)P₃ concentrations in stimulated parotid acinar cells have been estimated to reach values up to 60 µM (Hughes *et al.*, 1989), the dephosphorylation pathway is likely to be the most important metabolic route for the conversion of Ins(1,3,4)P₃ *in vivo*.

When comparing the phosphorylation rate of Ins(1,4,5)P₃ (Fig. 3) with the conversion rate of Ins(1,3,4)P₃ (Fig. 6), it becomes clear that Ins(1,3,4)P₃ has to reach approx. 10 times the concentration of Ins(1,4,5)P₃ before a stable balance of Ins(1,3,4,5)P₄ and Ins(1,3,4)P₃ production and breakdown can be established. These observations could provide a basis for the observed accumulation of Ins(1,3,4)P₃ seen in intact cells during the first minutes after receptor activation (Fig. 1).

A key question is whether the Ins(1,3,4,5)P₄ signal observed in intact cells can be elicited or modulated by [Ca²⁺]_i acting, for instance, on the Ins(1,4,5)P₃ 3-kinase via its Ca²⁺/calmodulin-activation. Our measurements of [³H]inositol phosphate generation in intact cells under conditions of sustained polyphosphoinositide hydrolysis, but with controlled levels of [Ca²⁺]_i, suggest that this is not so. By loading the cells with excess quin2 (Tsien *et al.*, 1982; Rink, 1988) to buffer transient changes in [Ca²⁺]_i and controlling sustained changes in [Ca²⁺]_i by modulating extracellular Ca²⁺ concentrations, inositol phosphate accumulation was determined in stimulated cells in which [Ca²⁺]_i was clamped within the physiological range at either < 100 or ~ 400 nM (Fig. 7a). The data obtained indicate that neither the generation of Ins(1,3,4,5)P₄ nor the Ins(1,4,5)P₃/Ins(1,3,4,5)P₄ ratio was changed by different intracellular Ca²⁺ concentrations within the physiological range (Fig. 7b). Furthermore, the accumulation of [³H]Ins(1,3,4)P₃, a very sensitive indicator for turnover changes in the InsP₃/InsP₄ pathway (see above), was

also unchanged, although no significant amounts of its breakdown products could be found.

Taken together, these results indicate that, at least in avian nasal-gland cells, the partitioning of Ins(1,4,5)P₃ metabolism upon receptor activation and the resulting generation of Ins(1,3,4,5)P₄ seem to be much more dependent on the cellular Ins(1,4,5)P₃ concentration than on the possible Ca²⁺/calmodulin-regulation of the 3-kinase activity. Since it is the rate of the receptor-mediated polyphosphoinositide hydrolysis which determines the primary Ins(1,4,5)P₃ production it seems to be this which determines the fate of Ins(1,4,5)P₃ in the cell as well as the production and the concentration of Ins(1,3,4,5)P₄, rather than any modulation of the 3-kinase activity by increases in [Ca²⁺]_i.

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