Fluoroaluminate activation of different components of the calcium signal in an exocrine cell

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In isolated cells from the avian supra-orbital nasal gland, used as a model for exocrine ion secretion, addition of NaF (2–15 mM) produced a slow Al^{3+} -enhanced increase in intracellular Ca^{2+} concn. ($[\text{Ca}^{2+}]_i$), resulting in a more than 2-fold sustained elevation in $[\text{Ca}^{2+}]_i$. Simultaneously, cellular $\text{Ins}(1,4,5)\text{P}_3$ contents became markedly elevated, suggesting an AlF_4^- activation of a phospholipase C-specific G-protein. Subsequent addition of the muscarinic agonist carbachol failed to produce any further sustained increase in $[\text{Ca}^{2+}]_i$, indicating that the AlF_4^- -induced increase in $[\text{Ca}^{2+}]_i$ involves a Ca^{2+} -entry pathway identical with that activated by carbachol. In low- Ca^{2+} media (extracellular $[\text{Ca}^{2+}] = 0.04 \text{ mM}$) no such increase in $[\text{Ca}^{2+}]_i$, either sustained or transient, is seen, although cellular $\text{Ins}(1,4,5)\text{P}_3$ levels were markedly elevated. Despite the failure to observe any change in $[\text{Ca}^{2+}]_i$ in the low- Ca^{2+} medium, estimation of the size of the agonist-sensitive Ca^{2+} stores (determined as the magnitude of the transient change in $[\text{Ca}^{2+}]_i$ induced by carbachol) revealed that these are progressively emptied by the action of AlF_4^- . However, the onset of this emptying showed an initial lag period of at least 2 min (with 5 mM-NaF plus 10 μM - AlCl_3). In marked contrast, determinations of the magnitude of the Ca^{2+} -entry pathway under identical conditions showed that this was significantly activated after as little as 1 min of AlF_4^- treatment. This suggests that, under these conditions, activation of Ca^{2+} entry in these cells preceded the release of Ca^{2+} from agonist-sensitive stores, contradicting current models in which the receptor-enhanced entry of extracellular Ca^{2+} is entirely dependent on, and subsequent to, the prior release of Ca^{2+} from the intracellular stores.

INTRODUCTION

Increases in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in response to appropriate receptor activation are known to constitute a major signalling pathway for the stimulation of ion and fluid secretion in a variety of exocrine glands. Such increases have been shown to be associated with the generation of $\text{Ins}(1,4,5)\text{P}_3$, resulting from the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ under the influence of the enzyme phospholipase C (PLC). This increase in $[\text{Ca}^{2+}]_i$ has been shown to comprise two distinct components: a release of Ca^{2+} from intracellular stores, probably mediated via $\text{Ins}(1,4,5)\text{P}_3$, and an enhanced entry of Ca^{2+} from the extracellular medium, the nature of which is far from clear. By analogy with the adenylate cyclase signalling system, it has been suggested that receptor occupation by an appropriate ligand may be coupled to the activation of PLC via a guanine-nucleotide-binding protein (G-protein) (for references see Taylor & Merritt, 1986; Litosch, 1987; Fain *et al.*, 1988). Several lines of evidence indicating the involvement of a G-protein (or proteins) in such responses exist for a variety of different cell types, including the fact that guanine nucleotides modulate high-affinity agonist binding to Ca^{2+} -mobilizing receptors, that receptor-mediated activation of PLC in cell-free preparations is dependent on the presence of guanine nucleotides, and that Ca^{2+} -mobilizing agonists stimulate guanine nucleotide hydrolysis and/or exchange.

A common feature of the G-proteins examined so far is that they can be activated directly by NaF in the presence of Al^{3+} ions (Sternweis & Gilman, 1982). The suggestion is that the active moiety is the fluoroaluminate complex (AlF_4^-), which interacts at the guanine-nucleotide-binding site, mimicking the terminal γ -phosphate of GTP and inducing activation (Bigay *et al.*, 1985), although alternative explanations do exist (Stadel & Crooke,

1989). Among exocrine cell types there are several reports demonstrating such actions of AlF_4^- on the Ca^{2+} -signalling pathway. For example, Blackmore *et al.* (1985), using hepatocytes, showed that NaF produced a concentration-dependent, AlCl_3 -enhanced, rise in $[\text{Ca}^{2+}]_i$, an increase in Ca^{2+} efflux, a depletion of PtdInsP_2 , and an increase in $\text{Ins}(1,4,5)\text{P}_3$ levels. It was concluded that AlF_4^- mimics the effects of Ca^{2+} -mobilizing hormones by activating a G-protein involved in the coupling of the receptors for such hormones to the PLC-mediated hydrolysis of PtdInsP_2 . A similar NaF-induced formation of inositol phosphates was shown for parotid acinar cells by Taylor *et al.* (1986), and for pancreatic acini by Matozaki *et al.* (1988).

In the study reported here, I have employed the supra-orbital nasal salt gland of birds as a model for exocrine ion secretion and its control (Shuttleworth & Thompson, 1989). Using dispersed isolated cells from this tissue, I first demonstrate that NaF induces increases in intracellular $\text{Ins}(1,4,5)\text{P}_3$ contents and $[\text{Ca}^{2+}]_i$ to levels similar to, or even greater than, those seen after muscarinic-receptor activation, and the increases observed are shown to be specifically augmented by the addition of AlCl_3 . However, these experiments further revealed that, whereas the initiation of the muscarinic response occurs within seconds, the AlF_4^- -generated response is much slower, taking several minutes. Such a slow or gradual development of the normal $[\text{Ca}^{2+}]_i$ response after addition of fluoride has previously been reported in a variety of other cell types, such as neutrophils (Strnad & Wong, 1985), hepatocytes (Hughes & Barritt, 1987), parotid cells (Merritt & Rink, 1987), pancreatic acini (Matozaki *et al.*, 1988), adrenal glomerulosa cells (Baukal *et al.*, 1988) and myometrium (Marc *et al.*, 1988), and raised the possibility of allowing a more precise dissection of the time course for the activation of the separate components of the overall Ca^{2+} signal.

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; $[\text{Ca}^{2+}]_o$, extracellular Ca^{2+} concentration; PLC, phospholipase C; G-protein, guanine-nucleotide-binding protein.

METHODS

Cells

Cells were isolated from the supra-orbital nasal salt glands of ducklings (*Anas platyrhynchos*), 4–10 days old, after 48 h of salt loading administered *ad lib.* as 1% NaCl in drinking water as previously described (Shuttleworth & Thompson, 1989). Briefly, the glands were removed, finely minced, and the tissue was incubated for 90 min at 38 °C in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's nutrient F12 medium (Sigma) containing 0.25% trypsin (Boehringer) and 23 mM- NaHCO_3 , gassed with O_2/CO_2 (19:1), pH 7.4. Remaining tissue fragments were dispersed by flushing through pipettes of decreasing diameter, and the resulting suspension was filtered through nylon mesh to remove any undissociated tissue, and the filtrate spun at 60 *g* for 10 min at room temperature. Trypsin action was terminated by resuspension of the cells in Hepes-buffered saline containing 0.2 mg of trypsin inhibitor (Sigma)/ml. The saline contained (in mM) 118.7 NaCl, 4.8 KCl, 1.3 CaCl_2 , 23.0 NaHCO_3 , 1.2 MgSO_4 , 1.2 KH_2PO_4 , 6.0 glucose, 15.0 Hepes, pH 7.4 when gassed with 5% CO_2 in air. After 10 min of incubation, the suspension was centrifuged and the cells were resuspended in Hepes-buffered saline. The suspension was filtered again through nylon mesh and then stored in the above Hepes-buffered saline at 38 °C and gassed with 5% CO_2 in air until used.

Determination of $[\text{Ca}^{2+}]_i$

$[\text{Ca}^{2+}]_i$ was determined in suspensions of dispersed cells by using the fluorescent probe indo-1 (Grynkiewicz *et al.*, 1985), as previously described (Shuttleworth & Thompson, 1989). Cells were loaded at 38 °C by incubation in saline containing 5 μM of the acetoxymethyl ester form, indo-1/AM (Calbiochem), for 20 min. The cells were then washed twice by centrifugation at 60 *g* for 10 min and resuspension in air-equilibrated Hepes-buffered saline (NaHCO_3 replaced with the sodium salt of the Hepes buffer, plus 3.9 mM- Na_2SO_4). Loaded cell suspensions were maintained in the dark at 38 °C until use (max. 2 h). Immediately before measurement, each portion of cells was centrifuged at 100 *g* for 60 s and resuspended in air-equilibrated Hepes-buffered saline in which the Ca^{2+} concentration had been lowered to 0.5 mM in order to avoid possible solubility problems after addition of fluoride. Measurements were made at 38 °C in a spectrofluorimeter (Perkin-Elmer LS-5B) with a magnetically stirred thermostatically controlled cuvette holder, by using excitation and emission wavelengths of 332 nm and 400 nm respectively. Output from the spectrofluorimeter was directed to a pen recorder and simultaneously digitized and stored in a micro-computer. Calibration of the resulting signal and calculation of $[\text{Ca}^{2+}]_i$ were essentially by the methods of Tsien *et al.* (1982) and Lückhoff (1986), as previously described (Shuttleworth & Thompson, 1989).

Binding assay for $\text{Ins}(1,4,5)\text{P}_3$

In these experiments, a specific binding-protein assay (Amersham) was used to determine the cellular content of $\text{Ins}(1,4,5)\text{P}_3$. Isolated cells, prepared as described above, were washed by centrifugation at 60 *g* for 10 min and resuspension in fresh air-equilibrated Hepes-buffered saline. After a second centrifugation, cells were resuspended in the appropriate medium (see the Results section) and divided into 250 μl portions. For each medium, one portion of cells was taken for analysis of protein content by a Coomassie Blue assay (Pierce). Remaining portions of cells were incubated under the appropriate conditions in a shaking water bath at 38 °C in large centrifuge tubes (50 ml), and incubations were terminated at the times indicated by the

addition of an equal volume of ice-cold 10% (v/v) HClO_4 . The tubes were left on ice for 15 min, then centrifuged at 15000 *g* for 2 min. Water-soluble components were extracted from samples of the supernatant after addition of EDTA (pH 7.0; final concn. 2 mM), by adding an excess of a 1:1 (v/v) mixture of Freon (1,1,2-trichlorotrifluoroethane) and tri-*n*-octylamine, followed by vortex-mixing and centrifugation at 2000 *g* for 4 min at 4 °C. Samples of the uppermost phase (of three) were assayed as described in the Amersham kit. Basically this involves incubation of the samples on ice in buffer (pH 9.0) containing 1.0 mM-EDTA in the presence of the specific binding protein and tracer amounts of ^3H -labelled $\text{Ins}(1,4,5)\text{P}_3$. The protein-bound fractions are then separated by centrifugation at 1000 *g* for 4 min at 4 °C and the pellets counted for radioactivity in a liquid-scintillation counter after addition of scintillant (Ecoscint A, National Diagnostics), and a calibration curve is constructed. The $\text{Ins}(1,4,5)\text{P}_3$ content of the samples is then determined, after appropriate correction for non-specific binding, from a calibration curve constructed from known $\text{Ins}(1,4,5)\text{P}_3$ standards assayed in an identical manner.

RESULTS

The effect of addition of NaF (10 mM) plus AlCl_3 (10 μM) on $[\text{Ca}^{2+}]_i$ in isolated avian nasal-gland cells is shown in Fig. 1, and compared with the effect of muscarinic-receptor activation by using carbachol (500 μM). As discussed above, these experiments were performed at an extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) of 0.5 mM. As previously reported (Shuttleworth & Thompson, 1989; Shuttleworth, 1990), carbachol produced an extremely rapid increase in $[\text{Ca}^{2+}]_i$ from resting values of around 100 nM to reach a peak value of 450 nM within 5 s before declining to a sustained elevated concentration of approx. 230 nM, or some 2.3 times the resting value. This sustained value is somewhat lower than those previously reported, presumably reflecting the decreased $[\text{Ca}^{2+}]_o$ used in these experiments. It has previously been noted that the sustained value of $[\text{Ca}^{2+}]_i$ in carbachol-stimulated nasal-gland cells is critically dependent on $[\text{Ca}^{2+}]_o$ (Shuttleworth & Thompson, 1989). Addition of NaF plus AlCl_3

Fig. 1. Effect of carbachol and NaF plus AlCl_3 on $[\text{Ca}^{2+}]_i$

Measurement of fluorescence and calculation of the corresponding $[\text{Ca}^{2+}]_i$ were performed on isolated cells loaded with indo-1 as described. Cells were suspended in saline with $[\text{Ca}^{2+}]_o = 0.5$ mM, and either carbachol (500 μM) or NaF (10 mM) plus AlCl_3 (10 μM) as indicated (A). Subsequently, atropine (100 μM) was added to the carbachol-stimulated cells (B), or carbachol (500 μM) was added to the NaF + AlCl_3 -stimulated cells (C). Representative traces are shown.

Fig. 2. Enhancement by Al³⁺ and concentration-dependence of the NaF effect on $[Ca^{2+}]_i$.

Isolated cells were loaded with indo-1, suspended in saline with $[Ca^{2+}]_o = 0.5$ mM and $[Ca^{2+}]_i$ was determined as described. (a) NaF (10 mM), AlCl₃ (10 μM) or NaF plus AlCl₃ was added at the point indicated. (b) AlCl₃ (10 μM) plus NaF at the various concentrations indicated was added where shown. Traces are displayed superimposed for comparison and are representative of at least five separate experiments.

to the extracellular medium also resulted in an increase in $[Ca^{2+}]_i$, but, in contrast with the effect of carbachol, the rise was gradual, taking approx. 3–4 min before reaching a new stable elevated level. Despite the marked difference in the time course of the changes in $[Ca^{2+}]_i$, it should be noted that the final value of the sustained elevated intracellular concentration is similar in cells after muscarinic-receptor activation with carbachol and in cells stimulated by addition of NaF and AlCl₃ (Fig. 1). The elevated $[Ca^{2+}]_i$ resulting from addition of carbachol was entirely sensitive to atropine, as addition of the antagonist (100 μM) to carbachol-treated cells rapidly restored $[Ca^{2+}]_i$ to values indistinguishable from resting levels. However, similar addition of atropine to NaF + AlCl₃-treated cells failed to affect the elevated levels of $[Ca^{2+}]_i$ seen in such cells (results not shown). Furthermore, the subsequent addition of carbachol to cells treated with NaF plus AlCl₃ and whose $[Ca^{2+}]_i$ had stabilized at the new elevated level resulted in a decreased transient increase in $[Ca^{2+}]_i$ which then returned to a stable level similar to that seen before carbachol stimulation (see Fig. 1).

Addition of NaF (10 mM) alone also produced a slow increase in $[Ca^{2+}]_i$, but the rate of its action was greatly enhanced by the simultaneous presence of 10 μM-AlCl₃ (Fig. 2a). This concentration of AlCl₃ alone, however, does not produce any increase

Table 1. Effect of NaF and AlCl₃, either alone or in combination, on cellular Ins(1,4,5)P₃ levels

The cellular Ins(1,4,5)P₃ content in batches of cells was determined by binding assay as described. Cells were exposed to NaF (10 mM) and AlCl₃ (10 μM) either alone or in combination as indicated for 3 min. Data are means ± S.E.M. for the numbers of experiments in parentheses.

NaF concn. (mM)	AlCl ₃ concn. (μM)	Cellular Ins(1,4,5)P ₃ content (pmol/mg of protein)
0	0	26.5 ± 1.6 (5)
10	10	126.3 ± 13.6 (4)
10	0	81.3 ± 8.3 (4)
0	10	38.9 ± 4.5 (4)

in $[Ca^{2+}]_i$, which actually declines somewhat. Simple inspection of the curves generated reveals that the effect of AlCl₃ on the response to NaF is to increase the speed of the response approx. 6-fold. The effect of different concentrations of NaF, all in the presence of 10 μM-AlCl₃, on $[Ca^{2+}]_i$ is illustrated in Fig. 2(b). It is clear that the principal effect of the concentrations of NaF on the $[Ca^{2+}]_i$ response is on the rate of the increase in $[Ca^{2+}]_i$ induced, rather than on the final level attained, which was remarkably similar for all the concentrations tested (ranging from 208 ± 10.6 at 2 mM-NaF to 228 ± 15.8 nM at 15 mM-NaF; $n = 5$ in each case). Furthermore, as illustrated in the example shown in Fig. 1, subsequent addition of carbachol (500 μM) to cells pretreated with NaF (5–15 mM) plus AlCl₃ did not result in any further sustained elevation of $[Ca^{2+}]_i$ (increase in $[Ca^{2+}]_i$ after carbachol = $2.5 \pm 1.9\%$; $n = 15$). Only at the lowest concentration of NaF used (2 mM) did the subsequent addition of carbachol significantly increase the stable $[Ca^{2+}]_i$ (by $16.2 \pm 2.0\%$; $n = 5$).

The effect of NaF (10 mM) plus AlCl₃ (10 μM) on cellular Ins(1,4,5)P₃ levels was also studied. Table 1 shows that, after 3 min of exposure, cellular Ins(1,4,5)P₃ levels had increased almost 5-fold over control levels. Under the same conditions, NaF (10 mM) alone increased cellular Ins(1,4,5)P₃ levels approx. 3-fold, whereas AlCl₃ (10 μM) alone produced only a 47% increase in Ins(1,4,5)P₃. Clearly, the presence of AlCl₃, which alone has only a relatively small effect, greatly enhances the increase in Ins(1,4,5)P₃ induced by NaF.

Effects of $[Ca^{2+}]_o$

Although other possibilities exist (see below), the observed effects of NaF on $[Ca^{2+}]_i$ in these cells could clearly result from the release of Ca²⁺ from sites of sequestration within the cell (intracellular Ca²⁺ stores), or by an enhanced entry of Ca²⁺ from the extracellular medium, or from a combination of both of these processes. A protocol that has been frequently used as a means of distinguishing the contributions from these two sources in receptor-activated cells is to compare the responses in the absence of extracellular Ca²⁺ (or at a low $[Ca^{2+}]_o$) with those in presence of normal Ca²⁺ concentrations. In our previous studies on the avian nasal-gland cells we have shown that complete removal of extracellular Ca²⁺ appears to produce irreversible effects on the ability to maintain normal intracellular Ca²⁺ balance, and cannot therefore be used in such protocols in these cells (Shuttleworth & Thompson, 1989). In addition, the common use of excess EGTA to obtain Ca²⁺-free media must be avoided, as EGTA is known to be a very effective chelator of Al³⁺ ions. Consequently we compared the effect of the addition of NaF (5 mM) and AlCl₃ (10 μM) on $[Ca^{2+}]_i$ in cells suspended in saline containing either

Fig. 3. Effect of NaF plus AlCl_3 on $[\text{Ca}^{2+}]_i$ at different extracellular Ca^{2+} concentrations

Indo-1-loaded cells were suspended in saline with $[\text{Ca}^{2+}]_o = 0.5$ mM or 0.04 mM as indicated. At the point indicated NaF (5 mM) plus AlCl_3 (10 μM) was added, and the changes in $[\text{Ca}^{2+}]_i$ were monitored as described. Representative traces (of at least five separate experiments) are shown.

0.5 mM- Ca^{2+} (as above) or 0.04 mM- Ca^{2+} . It has previously been shown in these cells that, in media with $[\text{Ca}^{2+}]_o$ of 0.04 mM, resting values of $[\text{Ca}^{2+}]_i$ and contents of intracellular Ca^{2+} stores are largely unaffected, yet stimulation with carbachol results in a transient increase in $[\text{Ca}^{2+}]_i$ to reach peak levels essentially the same as seen in normal media ($[\text{Ca}^{2+}]_o = 1.3$ mM), but which spontaneously return to values close to those typical of unstimulated cells. This has been interpreted as indicating the presence of a normal receptor-activated release of Ca^{2+} from intracellular stores, coupled with a very much decreased receptor-enhanced entry of extracellular Ca^{2+} , which therefore makes a negligible contribution to sustained $[\text{Ca}^{2+}]_i$ levels in the cells stimulated at this low $[\text{Ca}^{2+}]_o$. The effects of NaF (5 mM) plus AlCl_3 (10 μM) on $[\text{Ca}^{2+}]_i$ at these two values of $[\text{Ca}^{2+}]_o$ are illustrated in Fig. 3, which shows that, in contrast with the slow but marked increase in $[\text{Ca}^{2+}]_i$ in media containing 0.5 mM- Ca^{2+} ,

Fig. 4. Effect of NaF plus AlCl_3 on cellular $\text{Ins}(1,4,5)\text{P}_3$ content at different extracellular Ca^{2+} concentrations

Batches of cells were suspended in saline with $[\text{Ca}^{2+}]_o = 0.5$ mM (■) or 0.04 mM (●), and cellular $\text{Ins}(1,4,5)\text{P}_3$ content was determined by binding assay as described. At zero time, NaF (5 mM) plus AlCl_3 (10 μM) was added. Values are means $\pm$ S.E.M. for four independent experiments.

no significant effect of NaF plus AlCl_3 can be discerned in cells suspended in the low- Ca^{2+} medium.

The marked difference in the $[\text{Ca}^{2+}]_i$ response in the two media noted above could be due to a failure to elevate inositol phosphate levels adequately in the low- Ca^{2+} medium; therefore the effects of NaF (5 mM) plus AlCl_3 (10 μM) on the cellular contents of $\text{Ins}(1,4,5)\text{P}_3$ were compared in cells suspended in media with $[\text{Ca}^{2+}]_o$ of 0.5 mM or 0.04 mM. Although absolute levels were decreased by some 40–50 % in the low- Ca^{2+} medium (Fig. 4), the addition of NaF plus AlCl_3 still results in marked increases in the cellular $\text{Ins}(1,4,5)\text{P}_3$ content in both media (approx. 5-fold stimulation in both cases). Determinations of the time course for the change in $\text{Ins}(1,4,5)\text{P}_3$ levels in the low- Ca^{2+} medium indicate that they show an essentially linear increase over the entire 5 min of measurement (Fig. 4). Clearly then, suspension of the cells in the low- Ca^{2+} medium does not appear to affect the mechanism of AlF_4^- activation, but does seem to inhibit partially some component of basal inositol phosphate metabolism.

Effects on intracellular Ca^{2+} stores

The above demonstration of pronounced increases in the generation of $\text{Ins}(1,4,5)\text{P}_3$ in the low- Ca^{2+} medium suggested that, in this medium, NaF plus AlCl_3 could still be releasing Ca^{2+} from intracellular stores, despite the lack of any significant observed effect on $[\text{Ca}^{2+}]_i$. In order to investigate this possibility, the size of the agonist-sensitive intracellular Ca^{2+} pools at different times after the addition of NaF and AlCl_3 was estimated by comparing the size of the transient peak in $[\text{Ca}^{2+}]_i$ induced by 500 μM -carbachol (i.e. the maximum change in $[\text{Ca}^{2+}]_i$ induced by carbachol) in cells suspended in a low- Ca^{2+} medium ($[\text{Ca}^{2+}]_o = 0.04$ mM) in the absence and presence of NaF plus AlCl_3 . Fig. 5 shows that the magnitude of the change in $[\text{Ca}^{2+}]_i$ induced by carbachol is progressively decreased by exposure to 5 mM-NaF plus 10 μM - AlCl_3 , indicating that the agonist-sensitive pool of intracellular Ca^{2+} is being depleted under these conditions.

Fig. 5. Effect of NaF plus AlCl_3 on agonist-sensitive intracellular Ca^{2+} stores

Batches of cells loaded with indo-1 were suspended in low- Ca^{2+} ($[\text{Ca}^{2+}]_o = 0.4$ mM) saline, and NaF (5 mM) plus AlCl_3 (10 μM) was added at zero time. Subsequently, at the times indicated, the size of the agonist-sensitive Ca^{2+} stores was estimated by addition of carbachol (500 μM) and the maximum change in $[\text{Ca}^{2+}]_i$ determined (i.e. peak $[\text{Ca}^{2+}]_i$ after carbachol minus resting $[\text{Ca}^{2+}]_i$ immediately before addition of carbachol). To compensate for any variation between different cell preparations and possible progressive changes in agonist-sensitive pool size with time in the low- Ca^{2+} medium, the values obtained for the cells exposed to NaF plus AlCl_3 were expressed as a percentage of the value obtained from corresponding samples of cells isolated simultaneously and suspended for the same length of time in the low- Ca^{2+} medium in the absence of NaF and AlCl_3 .

Presumably, in view of the absence of any significant effect on $[Ca^{2+}]_i$, such emptying of the agonist-sensitive intracellular Ca²⁺ stores occurs sufficiently slowly for other processes of cellular Ca²⁺ balance to compensate. A similar slow release of Ca²⁺ from intracellular stores without resulting in a measurable increase in $[Ca^{2+}]_i$ has been reported by Pandol *et al.* (1989) in pancreatic acini after the addition of low concentrations of 4-bromo-A23187. However, an important point to note from Fig. 5 is that no significant depletion of the agonist-sensitive pools is apparent until at least 2 min after the addition of NaF and AlCl₃. This clearly differs markedly from the observed increase in cellular Ins(1,4,5)P₃ levels under identical circumstances, which, as described above, increased essentially linearly without any apparent delay or lag period (Fig. 4).

Effects on extracellular Ca²⁺ entry

The above data, although demonstrating that NaF plus AlCl₃ does induce the release of Ca²⁺ from agonist-sensitive intracellular stores, suggest that the onset of this process is delayed by at least

2 min under the conditions described. This is in marked contrast with the effect of the same concentrations of NaF and AlCl₃ on $[Ca^{2+}]_i$, which, at least at $[Ca^{2+}]_o$ of 0.5 mM, appears to increase without any obvious delay (see Fig. 3). However, such an apparent discrepancy could result from the different values of $[Ca^{2+}]_o$ in the two types of experiment and, for example, the consequent differences in cellular Ins(1,4,5)P₃ levels (see Fig. 4). To overcome this, the effect of NaF and AlCl₃ on the initiation of an enhanced entry of Ca²⁺ from the extracellular medium was determined under conditions essentially identical with those used above to evaluate the depletion of agonist-sensitive Ca²⁺ stores. Thus cells were suspended in low-Ca²⁺ media ($[Ca^{2+}]_o = 0.04$ mM) and 5 mM-NaF plus 10 μ M-AlCl₃ were added. At different times after the addition of NaF plus AlCl₃, Ca²⁺ was added to the medium to raise $[Ca^{2+}]_o$ to 0.5 mM, and the change in $[Ca^{2+}]_i$ was recorded. Previous studies have established that, although the changes in $[Ca^{2+}]_i$ seen after a sudden increase in $[Ca^{2+}]_o$ theoretically comprise several components, the initial increases predominantly reflect the rate of extracellular Ca²⁺ entry across the plasma membrane. The results obtained are illustrated in Fig. 6(a) and show that addition of NaF plus AlCl₃ does induce an enhanced rate of extracellular Ca²⁺ entry with time, and that this effect increases with time of exposure, as indicated by the progressively rapid increase in $[Ca^{2+}]_i$ seen on raising $[Ca^{2+}]_o$. Most significantly, the data show that a markedly enhanced rate of Ca²⁺ entry is observed after as little as only 1 min of exposure to NaF plus AlCl₃ (Fig. 6b), and is even more apparent 2 min after the addition of NaF plus AlCl₃. This is in marked contrast with the depletion of intracellular agonist-sensitive Ca²⁺ stores as determined above, which appears to begin only after some 2–3 min after the addition of NaF plus AlCl₃.

DISCUSSION

In this study we have demonstrated a concentration-dependent, NaF-induced, AlCl₃-enhanced elevation of cellular Ins(1,4,5)P₃ levels and $[Ca^{2+}]_i$ in the chloride-secreting exocrine cells of the avian nasal salt gland. The sustained increase in $[Ca^{2+}]_i$, together with the subsequent smaller size of the transient change in $[Ca^{2+}]_i$ induced by carbachol, suggest that the addition of NaF plus AlCl₃ results in the release of Ca²⁺ from intracellular stores and the activation of a plasma-membrane $[Ca^{2+}]_i$ -entry pathway, in a manner paralleling that produced by muscarinic-receptor activation. Separate analysis of the Ca²⁺ content of agonist-sensitive intracellular stores and the rate of entry of extracellular Ca²⁺ into the cytosol supports this suggestion. It was further shown that the subsequent addition of carbachol failed to produce a sustained level of $[Ca^{2+}]_i$ above that already produced by NaF plus AlCl₃. There is substantial evidence from a variety of cell types to indicate that the sustained elevation of $[Ca^{2+}]_i$ induced by carbachol involves an enhanced entry of Ca²⁺ into the cytosol from the extracellular medium. At a $[Ca^{2+}]_o$ of 0.5 mM, this carbachol-induced Ca²⁺-entry mechanism is far from maximal (Shuttleworth & Thompson, 1989), so it is therefore reasonable to assume that both muscarinic-receptor activation and addition of NaF plus AlCl₃ result in the activation of a common $[Ca^{2+}]_i$ -entry pathway.

Although such data are consistent with the hypothesis that the observed responses result from AlF₄⁻ activation of a PLC-specific G protein (G_p), it is always important to consider possible alternative actions of fluoride in the cell. Inhibition of phosphatases by fluoride (Lange *et al.*, 1986) could result in an increase in Ins(1,4,5)P₃ levels simply by inhibiting inositol phosphate breakdown. However, in preliminary experiments, the inhibition of inositol phosphate breakdown by phosphatases by

Fig. 6. Effect of NaF plus AlCl₃ on Ca²⁺ entry

Batches of indo-1-loaded cells were suspended in the low-Ca²⁺ medium ($[Ca^{2+}]_o = 0.04$ mM), and $[Ca^{2+}]_i$ was determined as described. (a) NaF (5 mM) plus AlCl₃ (10 μ M) was added at the point indicated and subsequently, at the times marked (Δ), Ca²⁺ was added to the medium to raise $[Ca^{2+}]_o$ to 0.5 mM. The traces represent individual representative experiments that have been superimposed to permit comparison. (b) shows the change in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) after an increase in $[Ca^{2+}]_o$ from 0.04 mM to 0.5 mM (at zero time) in cells pretreated for 1 min with NaF (5 mM) plus AlCl₃ (10 μ M) compared with control (untreated) cells. In each case the traces represent the means (—) $\pm$ S.E.M. (----) of four separate experiments.

using 10 mM-Li⁺ did not result in any significant increase in the generation of either total inositol phosphates or InsP₃, as measured by anion-exchange chromatography, in control cells (results not shown). This indicates that the turnover of inositol phosphates and the generation of InsP₃ are negligible in unstimulated cells and would therefore be unlikely to be affected by any fluoride-induced phosphatase inhibition. Furthermore, in the experiments reported here, the specific moiety measured was Ins(1,4,5)P₃ (rather than total InsP₃ or total inositol phosphates), and any inhibition of phosphatases would still leave the alternative route of Ins(1,4,5)P₃ metabolism [to Ins(1,3,4,5)P₄ via a 3-kinase] intact. Similarly, the observed elevation in [Ca²⁺]_i could have resulted from the inhibition of transport processes involved in Ca²⁺ sequestration or efflux, such as the membrane Ca²⁺-ATPase (Missiaen *et al.*, 1988). However, this would not explain the observed increases seen in cellular Ins(1,4,5)P₃ content, and is contrary to the evidence presented above indicating that fluoride and carbachol activate a common pathway for the sustained increase in [Ca²⁺]_i involving an enhanced entry of extracellular Ca²⁺. Of course fluoride activation could operate for other G-proteins besides those coupled to PLC, e.g. for adenylate cyclase. However, elevations of intracellular cyclic AMP via forskolin or membrane-permeant cyclic AMP analogues do not elevate [Ca²⁺]_i or increase inositol phosphate generation in avian nasal-gland cells (T. J. Shuttleworth, unpublished work).

It is concluded that, in these cells, a GTP-binding regulatory protein is involved in the mechanism whereby muscarinic-receptor activation induces the mobilization of Ca²⁺ from intracellular stores and increases the entry of Ca²⁺ across the plasma membrane. The simplest explanation for such a response is that the putative G-protein is a member of the so-called G_p group involved in the receptor activation of PLC, resulting in an increased generation of Ins(1,4,5)P₃ and its metabolites. On the basis of current models, the increased generation of Ins(1,4,5)P₃ resulting from activation of such a G-protein would induce the release of Ca²⁺ from specific agonist-sensitive intracellular Ca²⁺ stores, and depletion of these intracellular stores would then, by a process as yet not clearly defined, subsequently activate an enhanced entry of extracellular Ca²⁺ across the plasma membrane (Putney, 1986; Merritt & Rink, 1987; Gallacher, 1988; Irvine, 1989). The result is a sustained elevation of [Ca²⁺]_i that is critically dependent on the presence of extracellular Ca²⁺. However, in the studies reported here, detailed analysis of the time courses for activation of these two components of the overall [Ca²⁺]_i signal by NaF plus AlCl₃ revealed aspects that are inconsistent with such models. Thus, under essentially identical experimental conditions, it was possible to demonstrate a clearly enhanced entry of extracellular Ca²⁺ significantly before any detectable depletion of agonist-sensitive intracellular Ca²⁺ stores had occurred. The reason for the observed delay in the release of Ca²⁺ from the agonist-sensitive intracellular pools is not clear, but the data suggest that it is not the result of any delay in the onset of the activation of PLC, as, under identical conditions, cellular Ins(1,4,5)P₃ levels are significantly increased (more than doubled) after only 1 min and are elevated more than 3-fold after treatment with NaF plus AlCl₃ for 2 min (Fig. 4). One possibility is that Ins(1,4,5)P₃ levels have to reach a critical value before Ca²⁺ release begins and, in the low-Ca²⁺ (0.04 mM) medium, the observed decrease in the cellular content of this inositol phosphate, together with the relatively slow rise after NaF + AlCl₃ treatment, may mean that this critical level is not reached until after 2 min. If this is correct, then the data in Fig. 4 suggest that cellular levels of Ins(1,4,5)P₃ in excess of 40–45 pmol/mg of

protein are required to initiate the release of Ca²⁺ from the agonist-sensitive intracellular stores. In this context it is interesting that such values are typical of those seen in these cells after stimulation with carbachol (Shuttleworth, 1990), although, in carbachol-stimulated cells, such levels are reached within approx. 5 s after receptor activation, and the initiation of the emptying of intracellular Ca²⁺ stores is equally rapid.

Although the precise basis for the observed discrepancy between the onset of the Ca²⁺-entry process and the release of Ca²⁺ from the agonist-sensitive stores is not yet clear, it is nevertheless difficult to rationalize the data presented with current models in which such Ca²⁺ entry is dependent on, and subsequent to, the prior release of Ca²⁺ from the intracellular stores. It would seem that to be consistent with such models would require either that the Ca²⁺-entry mechanism be activated by depletion of a component of the intracellular stores that is too small to be detected by our techniques, or that AlF₄⁻ treatment somehow increased the overall number of the agonist-sensitive stores while simultaneously emptying them so that no net decrease in agonist-induced release was initially observed. Although neither of these alternative explanations can be completely eliminated at this stage, the possibility clearly remains that, contrary to most current models of Ca²⁺ signalling in exocrine cells, receptor-enhanced Ca²⁺ entry in these cells may not depend on the prior release of Ca²⁺ from agonist-sensitive stores.

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