Evidence for Muscarinic 3 Receptor Mediated Ion Transport in HT29 Cells Studied by X-ray Microanalysis

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ABSTRACT. Changes in elemental content in response to muscarinic drugs in HT29 cells were investigated by X-ray microanalysis. Acetylcholine (ACh) and carbachol (Cch), both agonists binding to muscarinic receptors, induced a decrease of the intracellular Cl and K content. This agrees with the notion that these agonists induce electrolyte and water secretion. Atropine, a non-selective antagonist of muscarinic receptors, inhibited the decrease in K and Cl caused by ACh and Cch, and instead caused an increase of the Cl and K concentrations. A similar inhibition was found in the case of the selective muscarinic 3 receptor antagonist P-F-HHSiD. In contrast, the selective muscarinic 2 receptor antagonist AF-DX 116 did not inhibit Cch-activated secretion of K and Cl. A slight inhibition of ACh induced ion secretion was seen, but this inhibition was weak compared to that caused by P-F-HHSiD. Treatment with U-73122, an inhibitor of phospholipase C, blocked ACh or Cch induced ion secretion. These results suggest that ACh and Cch stimulated secretion of Cl and K is mediated by muscarinic 3 receptors via the inositol-1,4,5-trisphosphate (IP3) dependent pathway.

Cholinergic receptors play a central role in mediating the complicated process of secretion and absorption of water and electrolytes in intestinal epithelia (2, 8, 18, 26). It is known that there are two structurally and functionally different types of cholinergic receptors, namely, nicotinic acetylcholine receptors and muscarinic acetylcholine receptors. The latter have been divided into 5 subtypes (M1-M5) based on differences in gene structure, in activated pathways and in affinity to muscarinic ligands (4, 11, 20). Recent studies have provided evidence for the expression of M3 receptors in submucosal neurons and intestinal epithelial cells, including rat colonic epithelia (17), T84 cells (7) and HT29 cells (14). In contrast to epithelial cells, submucosal neurons and autonomic ganglia have, in addition, nicotinic receptors. Therefore, epithelial cell cultures such as HT29 cells are a good model system to determine the role of muscarinic receptor subtypes in the regulation of ion secretion in intestinal epithelia. The presence of M3 receptors has been demonstrated in these cells, but little is known about their role in ion secretion.

In the present study, X-ray microanalysis in the electron microscope was used as a method to measure the concentration of the intracellular elements. This method, based on generation of element-specific X-rays by the electron beam, can measure the concentrations of several elements in cultured cells simultaneously (24, 25). This is an advantage especially if several ions are secreted or taken up concurrently. Moreover, X-ray microanalysis can help elucidate the dynamic process of ion transport if different time points after stimulation are taken (29). The purpose of this study was to investigate the ion response to muscarinic drugs, to examine which muscarinic receptor subtype is involved in activating this ion transport, and to investigate which signal pathway is involved in secretion.

MATERIALS AND METHODS

Chemicals. AF-DX116 (11-((2-(diethylamino)methyl)-1-piperidinyl)-acetyl)-5,11-dihydro-6H-pyrido(2,3-b)-(1,4)-benzodiazepin-6-ontelen-zepine) and P-F-HHSiD (p-fluoro-hexa-hydrosiladifenidol) were a gift from Dr. A. Rinken, Dept. of Medical and Physiological Chemistry, Uppsala University. The phospholipase C inhibitor U-73122 was from Research Biochemicals International, Natick, MA, USA. Cellulose nitrate filters were from Schleicher and Schuell, Dassel, Germany. Atropine, acetylcholine and carbachol were from Sigma, St. Louis, MO, USA. Reagents of analytical grade were

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Abbreviations: ACh; acetylcholine, Cch; carbachol, DMEM; Dulbecco's Modified Eagle's Medium, IP3; inositol-1,4,5-trisphosphate, AF-DX116; 11-((2-(diethylamino)methyl)-1-piperidinyl)-acetyl)-5,11-dihydro-6H-pyrido(2,3-b)-(1,4)-benzodiazepin-6-ontelen-zepine, P-F-HHSiD; p-fluoro-hexahydrosiladifenidol.

used.

Cell culture. HT29 human colon adenocarcinoma cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% inactivated fetal calf serum and 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. A confluent cell culture was subcultured after seeding for 5–7 days. The cells were detached by 1 mM EDTA in Hank's balanced salt solution without Ca and Mg, and were transferred to a small piece of cellulose nitrate filter (about 0.8 cm²). After waiting for one hour to allow attachment of the cells to the filters, 3–4 ml of the complete medium was added and the cells were allowed to grow for 2–3 days before the experiment.

Experimental procedure. Samples were prepared for microanalysis as described previously (24, 25, 29). Before the incubation, the cell culture was rinsed with Ringer's solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.5 mM CaCl₂, 5 mM Hepes, and 5 mM glucose, pH 7.4) to remove the culture medium because this could interfere with the agonists or antagonists. Then the cells were incubated with the agonists and/or antagonists (dissolved in Ringer's solution) at room temperature. In the blocking experiments, the cells were first incubated with atropine, AF-DX116 or P-F-HHSiD for 30 min to block a specific subtype of muscarinic receptor. This was followed by incubation with ACh and Cch for different periods. The control cells were not exposed to agonists or antagonists. At the end of the incubation, the filters with the cells were removed from the incubation medium and rinsed immediately in stirred distilled water for 5 sec to remove the salt-rich Ringer's solution. The filters were blotted on paper to remove excess water. Then, the filters were frozen in liquid propane cooled by liquid nitrogen (at about -190°C). The filters were dried overnight at -30°C. The dried filters were coated with a conductive carbon layer to avoid charging during microanalysis.

X-ray microanalysis. The cells frozen on filters were examined in a Philips 525 scanning electron microscope (Philips Electron Optics, Eindhoven, The Netherlands) and analyzed with a Link AN 10000 energy-dispersive X-ray microanalysis system (Oxford Instruments, Oxford, U.K.) at 20 kV. Each spectrum was acquired for 100 sec. Methods for quantitative analysis have been reviewed in (21). In brief, quantitative analysis was carried out by comparing the spectra from the specimen with those from a standard, consisting of known concentrations of mineral salts in a 20% gelatin/5% glycerol matrix which was frozen and freeze-dried in the same way as the specimen (22). The ratio of the characteristic X-ray signal to the background signal (peak to background ratio) was compared for specimen and standard (21). Only one spectrum was obtained from each cell. Because of the thickness of the freeze-dried cell, the analytical volumn comprises a large part of a cell.

Statistical analysis. The values determined are given as mean ± standard deviation (SD). The data were analysed by an unpaired Student's *t* test.

RESULTS

Treatment of the HT29 cells with 10 μM ACh induced a significant decrease of the intracellular content of Cl and K from 1 min to 30 min after stimulation (Fig. 1a), but only a slight decrease of Na. Treatment with 10 μM Cch caused a response similar to that observed after ACh; the decrease in Cl and K was significant at 5 min and 15 min (Fig. 1b).

In order to study the effect of blocking muscarinic re-
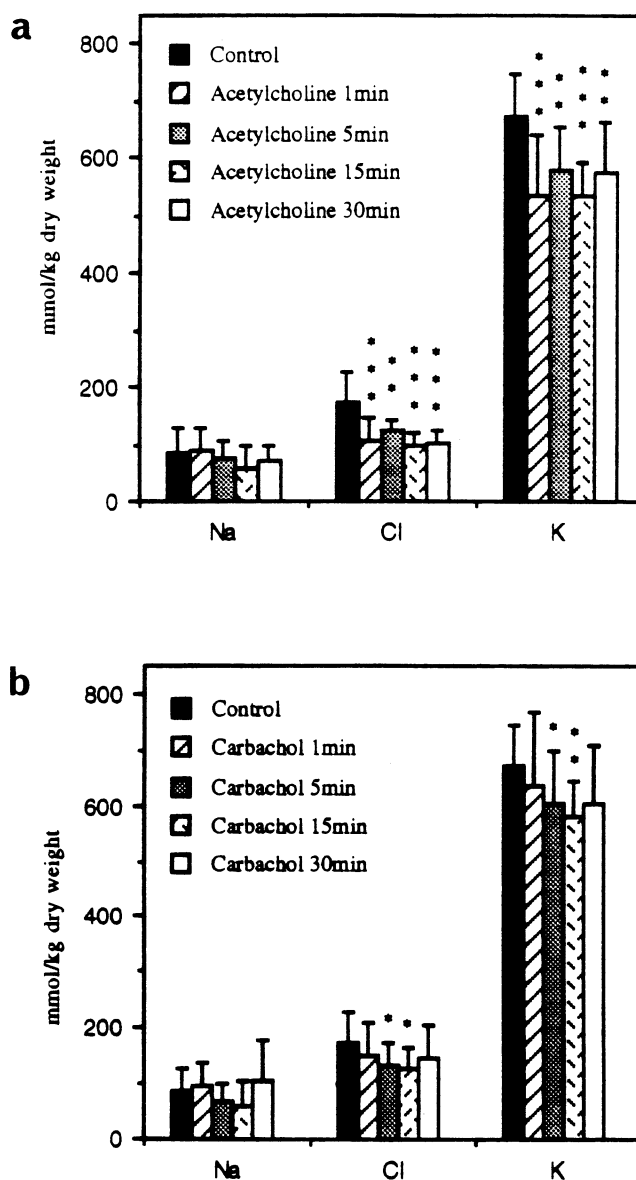
Fig. 1. The effect of stimulation of HT29 cells by (a) ACh (10 μM) and (b) Cch (10 μM). Data show the elemental concentrations of Na, Cl and K in mmol/kg dry weight. Values are mean ± SD (n = 26 for each time point). Significant differences from the control are indicated by asterisks (*** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$).

ceptors, the cells were pretreated with atropine (10 μM) for 30 min, and then exposed to ACh (10 μM) or Cch (10 μM). Pretreatment with atropine for 30 min did not significantly affect the Cl and K content of the cells, but caused a minor increase in the Na content. An increase in intracellular Na, Cl, and K was observed at 5 and 15 min in cells treated with atropine+ACh (Fig. 2a),

whereas a slight increase of Na and Cl at 1 min, and no change of K was observed after treatment with atropine + Cch (Fig. 2b). These results indicate that atropine can block ACh and Cch induced ion secretion.

To further investigate muscarinic receptor subtypes, selective muscarinic receptor antagonists were used. The cells were pretreated with AF-DX116 (10 μM) for
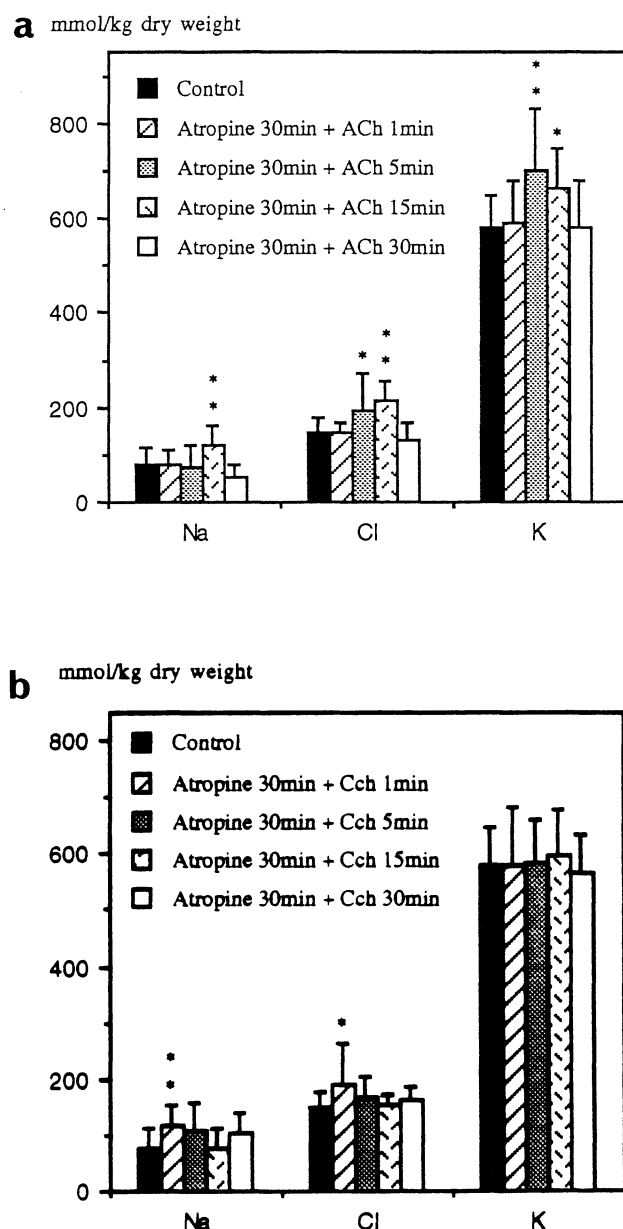
Fig. 2. The effect of atropine (10 μM) with ACh (10 μM) (a) or Cch (10 μM) (b) on the concentration of Na, Cl and K in HT29 cells. Preincubation with atropine for 30 min was followed by incubation with ACh or Cch for 1, 5, 15 or 30 min (n=26 for each time point). Significant differences from the control are indicated by asterisks (*** p <0.001, ** p<0.01, and * p<0.05).
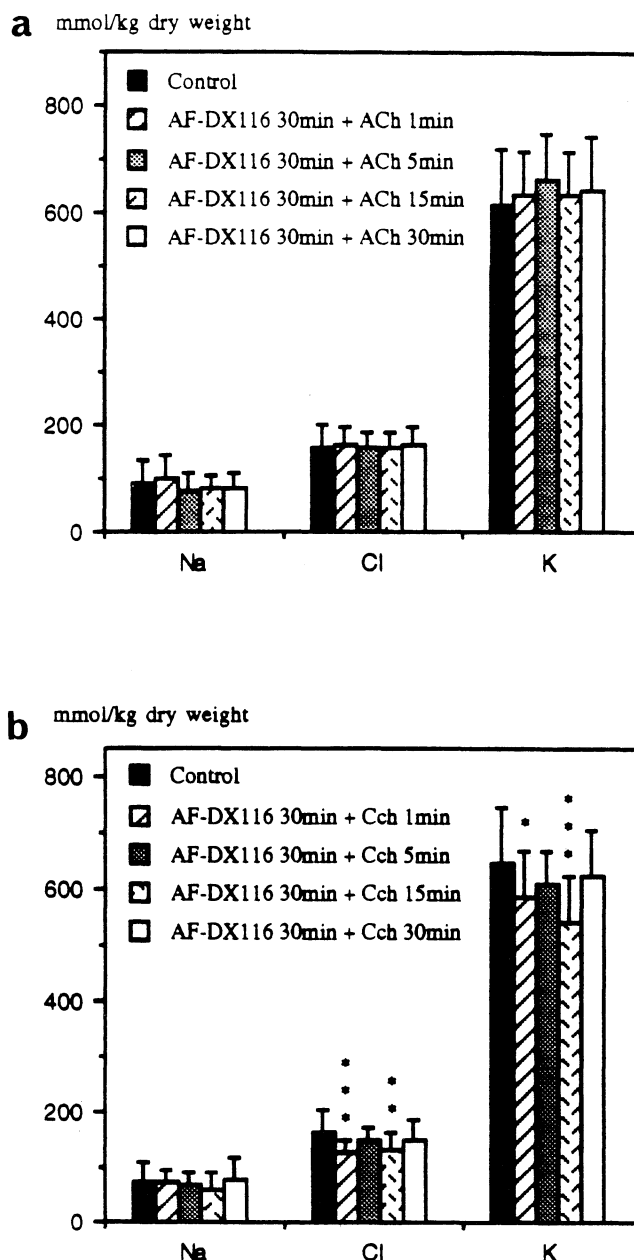
Fig. 3. Changes in ion concentration induced by 10 μM ACh (a) or 10 μM Cch (b) after pretreatment with 10 μM AF-DX116 (n=26 for each time point). In fig 3a no significant effect of ACh was found. In Fig. 3b significant differences from the control are indicated by asterisks (*** p<0.001, ** p<0.01, and * p<0.05).

30 min, and subsequently exposed to ACh (10 μM). No significant changes in elemental concentrations were seen, indicating that the action of ACh was blocked by AF-DX116 (Fig. 3a). However, addition of Cch in the presence of AF-DX116 induced a response similar to that following Cch alone (Fig. 3b), suggesting that AF-

DX116 does not inhibit the effect of Cch at dose of 10 μM. On the other hand, preincubation of the cells with P-F-HHSiD (5 μM) for 30 min, followed by addition of ACh (10 μM) caused a response which was the opposite of that of ACh, namely an increase in Cl and K (Fig. 4a). Addition of Cch after pretreatment with P-F-HHSiD induced a similar response, with a significant
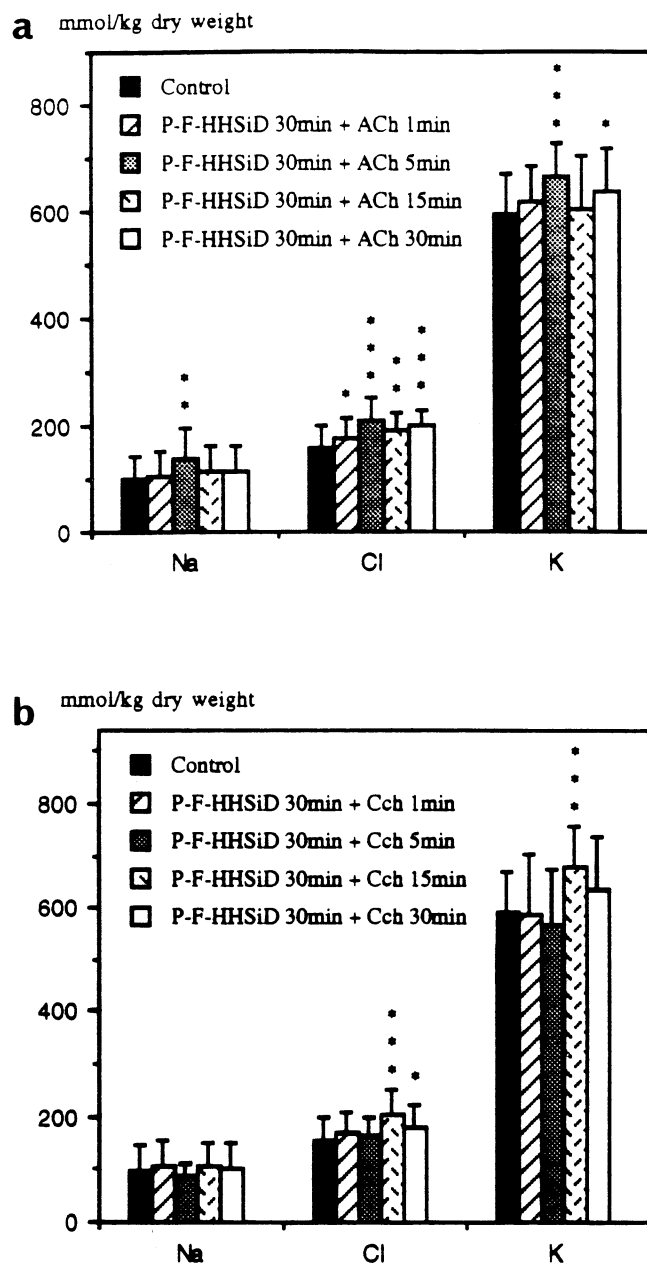

Fig. 4. The effect of PF-HHSiD (5 μM) with ACh (10 μM) (a) or Cch (10 μM) (b) on the concentration of Na, Cl and K in HT29 cells. Preincubation with PF-HHSiD for 30 min was followed by incubation with ACh or Cch for different times (n=26 for each time point). Significant differences from the control are indicated by asterisks (*** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$).
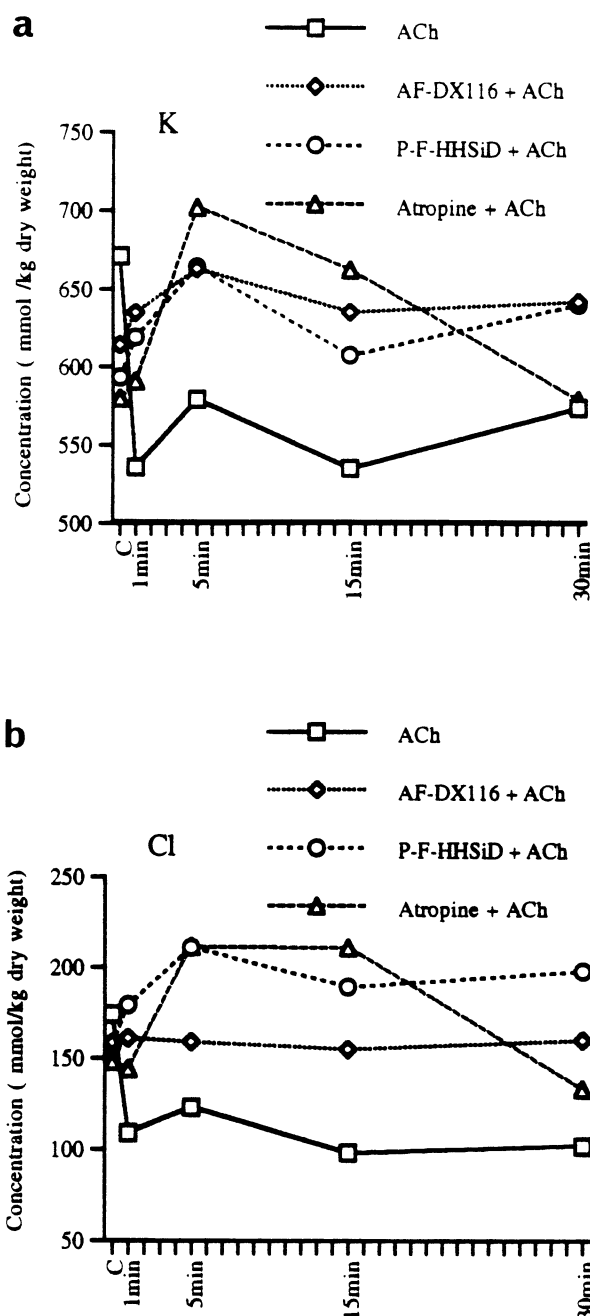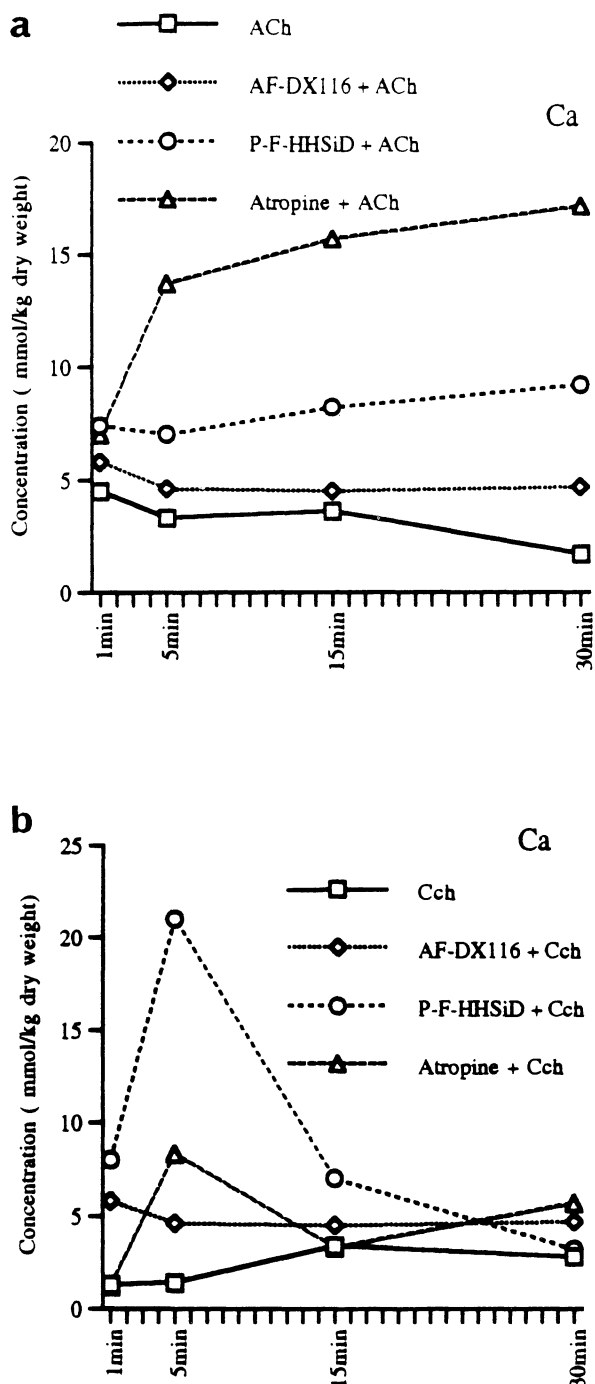
Fig. 5. Concentrations of (a) K and (b) Cl in HT29 cells in response to ACh stimulation, showing parallel changes in K and Cl. (Data from Figs. 1–4).

increase of intracellular Cl and K at 15 min (Fig. 4b). Compared with AF-DX116, P-F-HHSiD showed a strong inhibitory effect on both ACh and Cch induced

ion response. Pretreatment with AF-DX116 alone or P-F-HHSiD alone for 30 min did not affect the Cl and K content of the cells significantly, and caused only a minor increase of the Na concentration.

To explore the interaction between K and Cl in response to agonist alone or antagonist plus agonist, the time course of changes in K and Cl was studied. After stimulation with ACh or Cch, the decrease in K appeared to be approximately parallel to that in Cl (Figs.

Fig. 6. The concentration of Ca in HT29 cells in response to drugs affecting muscarinic receptors, showing an increase in Ca after pretreatment of the cells with different antagonists followed by stimulation with Ach (a) or Cch (b). Data from experiments presented in Figs. 1–4.
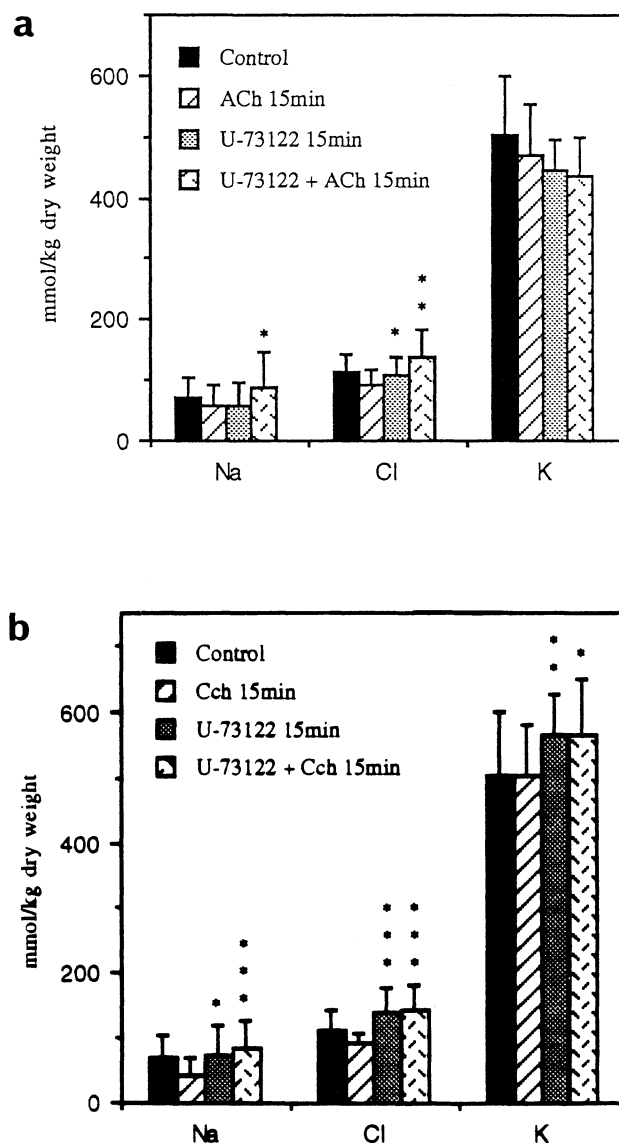
Fig. 7. Effect of U-73122 (10 μM) with or without ACh (a) or Cch (b) on the Na, Cl and K concentrations in HT29 cells. Na, Cl and K were increased in the presence of U-73122. A significant increase of Na and Cl was observed in the presence of U-73122 combined with ACh (a) or Cch (b), compared to ACh or Cch, respectively, alone (n = 22 for each experiment). Significant differences from the control are indicated by asterisks (*** p<0.001, ** p<0.01, and * p<0.05).

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5a and b). Similarly, after exposure to atropine followed by Ach or Cch, a simultaneous increase in K and Cl occurred.

The total Ca levels in the cell did not change significantly in response to ACh and Cch stimulation. However, pretreatment with P-F-HHSiD or atropine followed by stimulation with ACh or Cch induced a significant increase in Ca (Figs. 6a and b).

To determine whether the IP3 pathway was important for ACh or Cch-induced ion transport, the cells were preincubated with U-73122 (10 μM), an inhibitor of phospholipase C, for 5 min and then incubated with a solution containing U-73122 and ACh or Cch for 15 min. A significant increase in Cl and Na was observed in the presence of U-73122, compared to ACh or Cch alone (Fig. 7a, b). This result indicates that U-73122 can block ion secretion induced by agonists of muscarinic receptors.

DISCUSSION

The results of the present study show that ACh and Cch induce a decrease in intracellular Cl and K, indicating that these agonists of muscarinic receptors induce ion secretion. This is in good agreement with previous studies (10, 12). It is thought that cholinergic agonists activate K channels in the basolateral membrane of the cells, where functional cholinoceptors are present (15, 16). This initial activation of K channels causes subsequent hyperpolarization which gives rise to Cl efflux through channels in the apical membrane (9, 23, 27). Consistently, we observed parallel changes in K and Cl in response to binding of agonists to muscarinic receptors. The effects of both ACh and Cch can be blocked by preincubating the cells with atropine, which supports the notion that these effects are mediated by muscarinic receptors. Previous radioligand binding studies have shown that the M3 receptor is a major subtype of muscarinic receptors on intestinal epithelia (7, 14, 17). In the present study, we found that P-F-HHSiD, a selective antagonist of the M3 receptor, inhibits the decrease in K and Cl induced by ACh and Cch in a similar way as atropine, although atropine appears to have a higher affinity to muscarinic receptors than P-F-HHSiD. Moreover, the M3 receptor has been reported to be present in the HT29 cells. Interestingly, both atropine and P-F-HHSiD give rise to a characteristic ion response, which was not only to block the effect of ACh or Cch, but also to induce an increase in Cl and K, concomitant with an increase in Ca. In contrast to P-F-HHSiD, AF-DX 116 only showed a weak inhibitory effect but it did not increase Cl and K. This may be due to the fact that AF-DX116 has a low affinity to the M3 receptor, but a relatively high affinity to the M2 receptor. It should be noted that a different response to Cch and ACh was ob-

served after pretreatment with AF-DX116. Usually, Cch acts longer than ACh, as ACh is rapidly hydrolysed by acetylcholine esterase. Our results, in conclusion, suggest that K and Cl secretion induced by muscarinic agonists is probably mediated via the M3 receptor subtype.

We did not observe a marked increase in Ca after treatment with agonists of muscarinic receptors. X-ray microanalysis only measures total elemental Ca, and changes in free Ca^{2+} ions are relatively small in relation to the total amount of Ca in the cell. Interestingly, we observed an increase in Ca especially after exposure of the cells to atropine plus agonists, indicating an apparent uptake of calcium into the cells. The reason for this is unclear. The regulation of intracellular free Ca^{2+} concentration is associated with the inositol 1,4,5-triphosphate (IP3) dependent pathway. The activation of this pathway promotes the formation of IP3 which causes a rapid increase of cytoplasmic Ca^{2+} by releasing Ca^{2+} from intracellular Ca^{2+} pools and stimulates Ca^{2+} entry from the extracellular compartment (1, 6, 19).

The stimulation of muscarinic receptors by their ligands results in the activation of second-messenger pathways that may mediate the ion response. It has been reported that cAMP can induce efflux of K and Cl from HT29 cells (5, 13, 16). This was also observed by X-ray microanalysis (24). However, previous studies indicated that Cch increased the level of IP_3 but not that of cAMP in intestinal epithelial cells (7, 14, 17). In the present study we demonstrated that U-73122, a inhibitor of phospholipase C (3, 28), can block the ACh or Cch-induced secretion, suggesting that the action of these agonists on ion transport is indeed mediated via an IP3 dependent pathway.

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