Bicarbonate and fluid secretion evoked by cholecystokinin, bombesin and acetylcholine in isolated guinea-pig pancreatic ducts

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1. HCO_3^- secretion was investigated in interlobular duct segments isolated from guinea-pig pancreas using a semi-quantitative fluorometric method. Secretagogue-induced decreases in intracellular pH, following blockade of basolateral HCO_3^- uptake with a combination of amiloride and DIDS, were measured using the pH-sensitive fluoroprobe BCECF. Apparent secretory HCO_3^- fluxes were calculated from the initial rate of intracellular acidification.
2. In the presence of HCO_3^-, stimulation with secretin (10 nM) or forskolin (5 μM) more than doubled the rate of intracellular acidification. This effect was abolished in the absence of HCO_3^-. It was also abolished in the presence of HCO_3^- when DIDS and NPPB were applied to the luminal membrane by microperfusion. We therefore conclude that the increase in acidification rate is a useful index of secretagogue-induced HCO_3^- secretion across the luminal membrane.
3. Secretin, cholecystokinin (CCK) and bombesin each stimulated HCO_3^- secretion in a dose-dependent fashion. They evoked comparable maximal responses at about 10 nM and the EC_{50} values were 0.5 nM for secretin, 0.2 nM for CCK and 30 pM for bombesin. Acetylcholine (ACh) was also effective, with a maximum effect at 10 μM.
4. The stimulatory effect of CCK was blocked completely by the CCK_1 receptor antagonist devazepide but not by the CCK_2 receptor antagonist L365,260. The CCK analogue JMV-180 (Boc-Tyr(SO_3H)-Nle-Gly-Trp-Nle-Asp-phenylethyl ester), which is an agonist of the high-affinity CCK_1 receptor but an antagonist of the low-affinity receptor, also stimulated HCO_3^- secretion but with a smaller maximal effect than CCK. JMV-180 partially inhibited the response to a high concentration of CCK but not to a lower concentration, suggesting that both high- and low-affinity states of the CCK_1 receptor evoke HCO_3^- secretion.
5. The stimulatory effect of bombesin was blocked completely by the gastrin-releasing peptide (GRP) receptor antagonist D-Phe^6-bombesin(6–13)-methyl ester (BME) but not by the neuromedin B (NMB) receptor antagonist D-Nal-cyclo[Cys-Tyr-D-Trp-Orn-Val-Cys]-Nal-NH_2 (BIM-23127).
6. Secretagogue-evoked fluid secretion was also examined using video microscopy to measure the rate of swelling of ducts whose ends had sealed during overnight culture. Secretin, CCK, bombesin and ACh all evoked fluid secretion with maximal rates of approximately 0.6 nl min^{-1} mm^{-2}, and with concentration dependences similar to those obtained for HCO_3^- secretion.
7. We conclude that CCK, bombesin and ACh stimulate the secretion of a HCO_3^--rich fluid by direct actions on the interlobular ducts of the guinea-pig pancreas and that these responses are mediated by CCK_1 receptors, GRP receptors and muscarinic cholinoceptors, respectively.

Our knowledge of the site of production of HCO_3^--rich pancreatic juice and the mechanisms responsible for regulating its secretion are derived largely from *in vivo* studies in a variety of species, supplemented by observations in perfused glands and, more recently, in isolated pancreatic ducts. These studies, which have been summarised elsewhere (Case & Argent, 1993), present a confusing picture, partly because of genuine species variations and partly because of differences between *in vivo* and *in vitro* observations. Thus, while secretin undoubtedly evokes a HCO_3^--rich fluid secretion from the ducts of all species studied, the effects of other stimuli on fluid secretion vary greatly and their sites of action, whether ducts or acini, are usually unclear. For example, in an *in vivo* study of pancreatic secretion in anaesthetised guinea-pigs, cholecystokinin (CCK) was observed to evoke a copious HCO_3^--rich fluid secretion but it was impossible to determine whether CCK was evoking fluid secretion from the acini or from the ducts (Padfield *et al.* 1989).

In order to assess the direct actions of known and potential agonists and antagonists on ductal secretion, it is clearly necessary to study their actions on ductal tissue uncontaminated by acinar tissue. Previous studies on isolated pancreatic ducts have involved the use of micropuncture techniques, either to aspirate the secreted fluid from the duct lumen for subsequent analysis (Ashton *et al.* 1990) or to inject a pH-sensitive fluoroprobe into the duct lumen for fluorescence imaging (Ishiguro *et al.* 1998). Although informative, these experiments are technically difficult and time consuming. Therefore, in the present study, we have adopted an alternative approach in which ductal HCO_3^- secretion and fluid secretion are measured separately using simpler methods.

Our measurements of ductal HCO_3^- secretion make use of the fact that the intracellular pH (pH_i) depends upon the balance between HCO_3^- uptake across the basolateral membrane and HCO_3^- exit across the luminal membrane. Therefore, when basolateral HCO_3^- uptake is prevented, by blocking Na^+–HCO_3^- cotransport with DIDS and Na^+–H^+ exchange with amiloride, pH_i declines as HCO_3^- continues to leave the cell across the luminal membrane (Ishiguro *et al.* 1996). We have therefore estimated instantaneous HCO_3^- secretion from the initial rate of fall in pH_i as measured by microfluorometry.

Our measurements of ductal fluid secretion take advantage of the fact that during overnight culture the ends of the isolated ducts seal. Therefore, when a duct is stimulated, fluid secretion into the closed luminal space causes the duct segment to swell. Fluid secretory rate can therefore be estimated from the rate of change in duct volume. As in our previous study (Ishiguro *et al.* 1998), duct swelling was assessed by microscopy, but in this case the increase in luminal volume was measured directly by bright-field video microscopy thus avoiding the need for microinjection of fluoroprobes.

These two methods have enabled us to study the secretory responses of isolated guinea-pig pancreatic ducts following stimulation with secretin, CCK, bombesin and acetylcholine (ACh). CCK and bombesin-like peptides are widely distributed throughout the gastrointestinal tract where they exhibit a variety of actions. In general, CCK acts through two receptor subtypes, CCK_1 and CCK_2, but in addition, two different states of the CCK_1 receptor are recognised and referred to as high- and low-affinity, respectively (Wank, 1998; Noble *et al.* 1999). Bombesin-like peptides also act through two receptor subtypes, namely neuromedin B (NMB) and gastrin-releasing peptide (GRP) receptors. The actions of these two peptides are well characterised in a variety of cells and tissues including pancreatic acinar cells (Jensen, 1994). Our observations on isolated interlobular ducts described here demonstrate unequivocally that, in the guinea-pig, not only secretin but also CCK, bombesin and ACh evoke HCO_3^- and fluid secretion directly from the ducts.

A preliminary report of these findings has already been published (Szalmay *et al.* 2000).

METHODS

Animals and materials

Male tricolour guinea-pigs were obtained from Leeds University for use in Manchester, and male MD guinea-pigs from Charles River Hungary Ltd for use in Budapest. Animals weighed 300–400 g and were maintained on a normal pellet diet. All animal experiments were performed in accordance with national guidelines.

Culture media, soybean trypsin inhibitor, bovine serum albumin (BSA), porcine secretin, ACh, forskolin, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and amiloride were obtained from Sigma; 2′,7′-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein acetoxymethyl ester (BCECF AM) was from Molecular Probes Europe (Leiden, The Netherlands); collagenase (Type CLSPA) was from Worthington (Freehold, NJ, USA); CCK-8 (Asp-Tyr(SO_3)-Met-Gly-Trp-Met-Asp-Phe-NH_2) was from Research Plus Inc. (Bayonne, NJ, USA); JMV-180 was from Neosystem Laboratory (Strasbourg, France); L365,260 and devazepide (L364,718) were from Merck Sharp & Dohme Research Laboratories (Rahway, NJ, USA); Cell-Tak was from Collaborative Biomedical Products (Bedford, MA, USA); glutamine was from Research Plus Laboratories (Denville, NJ, USA); human insulin (Humulin R) was from Lilly (Gödöllö, Hungary); and dexamethasone (Oradexon) was from N.V. Organon (Oss, The Netherlands). Bombesin, D-Phe^6-bombesin(6–13)-methyl ester (BME) and D-Nal-cyclo[Cys-Tyr-D-Trp-Orn-Val-Cys]-Nal-NH_2 (BIM-23127; referred to as BIM) were a gift from Dr D. H. Coy (Department of Medicine, Tulane University Medical Center, New Orleans, LA, USA).

Solutions

The standard Hepes-buffered solution contained (mM): 130 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose and 10 Na-Hepes, and was equilibrated with 100% O_2. The Na^+-free, Hepes-buffered solution contained equimolar *N*-methyl-D-glucamine (NMDG) in place of Na^+. The standard HCO_3^--buffered solution contained (mM): 115 NaCl, 25 $NaHCO_3$, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 10 D-glucose, and was equilibrated with 95% O_2 and 5% CO_2. All solutions were adjusted to pH 7.4 at 37 °C.

Isolation and culture of pancreatic ducts

Ducts were prepared as previously described in detail (Ishiguro *et al.* 1996). Briefly, animals were killed by cervical dislocation and the whole pancreas was removed and injected with 4 ml of a digestion buffer consisting of Dulbecco's modified Eagle's medium (DMEM) containing 40–80 U ml^{-1} collagenase, 400 U ml^{-1} hyaluronidase, 0.2 mg ml^{-1} soybean trypsin inhibitor (SBTI) and 2 mg ml^{-1} BSA. The tissue was chopped with scissors, top-gassed with 5% CO_2–95% O_2 and incubated in the digestion buffer for 30–60 min at 37 °C. The digested tissue was then washed and resuspended in DMEM containing 0.2 mg ml^{-1} SBTI and 30 mg ml^{-1} BSA. Interlobular ducts were microdissected from samples of tissue suspension under a dissecting microscope using 25-gauge hypodermic needles, and transferred to McCoy's 5A tissue culture medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 0.15 mg ml^{-1} SBTI, 0.1 i.u. ml^{-1} human insulin and 4 μg ml^{-1} dexamethasone. They were cultured at 37 °C in 5% CO_2 in air overnight, during which time the ends of the ducts sealed spontaneously.

Measurement of intracellular pH

Intracellular pH was measured by microfluorometry as described in detail elsewhere (Ishiguro *et al.* 1996). In outline, isolated ducts were loaded with the pH-sensitive fluoroprobe BCECF by incubation in Hepes-buffered solution containing the acetoxymethyl ester BCECF AM (1–2 μM) for 10–30 min at room temperature. Individual ducts were then transferred to a temperature-controlled Perspex perfusion chamber (volume, 200 μl) mounted on the stage of a Nikon Diaphot inverted microscope and allowed to attach to a coverslip pre-coated with Cell-Tak in the base of the chamber. The chamber was perfused at 2 ml min^{-1} with either the Hepes- or HCO_3^--buffered solution at 37 °C. A small part of the ductal epithelium was illuminated alternately at 440 and 490 nm and the fluorescence intensities (F_{440} and F_{490}, respectively) were measured at 530 nm. Intracellular pH was calculated from the F_{490}/F_{440} ratio using calibration data that were obtained with the nigericin–K^+ method (Thomas *et al.* 1979).

In order to estimate secretory HCO_3^- flux, the ducts were first superfused for 5 or 10 min with the HCO_3^--buffered solution. An agonist (or vehicle alone in control experiments) was then added to the superfusate and, after 5 or 10 min of stimulation, DIDS (0.1 mM) and amiloride (0.3 mM) were added simultaneously (in the continued presence of the agonist) to block the basolateral uptake of HCO_3^-. The initial rate of decrease in pH_i (acidification rate) following the addition of the inhibitors was measured by linear regression (see Fig. 1*A*).

Measurement of intracellular buffering capacity

Intrinsic intracellular buffering capacity (β_i) was measured as a function of pH_i according to the method of Weintraub & Machen (1989). Ducts were first superfused with the Na^+-free, Hepes-buffered solution for 30 min and then exposed to stepwise increases and decreases in NH_4Cl concentration (5, 10, 20, 10 and 5 mM). The changes in pH_i caused by the stepped changes in $[NH_4^+]_o$, and the corresponding changes in $[NH_4^+]_i$ (calculated from pH_i assuming that the pK_a of NH_4^+ was 8.9), were used to estimate the value of β_i at the mid-point of each pH interval:

$$\beta_i = \Delta[NH_4^+]_i/\Delta pH_i.$$

Measurements of β_i at higher pH_i values were obtained in the normal Hepes-buffered solution using trimethylamine (TMA^+, pK_a = 9.4) in place of NH_4^+ (Szatkowski & Thomas, 1989). The pooled NH_4^+ and TMA^+ data for β_i were averaged over 0.2 pH unit bins, plotted as a function of pH_i and fitted with a polynomial curve. The predicted total buffering capacity (β_t) in the presence of HCO_3^-/CO_2 was calculated using:

$$\beta_t = \beta_i + 2.3[HCO_3^-]_i$$

(Weintraub & Machen, 1989). Apparent HCO_3^- fluxes could then be calculated from the pH_i measurements obtained in the experiments described above by multiplying the rate of change of pH_i by the appropriate value of β_t.

Microperfusion of isolated interlobular ducts

Luminal microperfusion of isolated interlobular ducts was performed using a modification (Ishiguro *et al.* 1999) of the method originally developed for electrophysiological studies of rat pancreatic ducts (Novak & Greger, 1988*a*, *b*). After overnight culture, the ends of the duct were cut open using 25-gauge hypodermic needles and the ductal epithelial cells were loaded with BCECF as described above. The duct was then transferred to a temperature-controlled Perspex perfusion chamber, mounted on the stage of a Nikon Diaphot inverted microscope, for simultaneous luminal perfusion and measurement of pH_i. One end of the duct was gently aspirated into a holding pipette with a tip diameter of 120 μm and a recessed constriction. A concentric perfusion pipette (tip diameter, 10–20 μm) was then advanced into the duct lumen. The other end of the duct was anchored to the glass coverslip at the base of the chamber using Cell-Tak adhesive.

The duct lumen was perfused at a flow rate of 10–20 μl min^{-1} using an exchange pipette (fused silica capillary; o.d., 440 μm; i.d., 320 μm; Polymicro Technologies Inc., Phoenix, AZ, USA) which was inserted into the shank of the perfusion pipette. Pressurised nitrogen gas was applied to the reservoirs supplying the luminal perfusion solutions, and a steady flow of perfusate, via a waste line from the back of the perfusion pipette to a reservoir located approximately 1 m above the chamber, allowed rapid changes of luminal fluid composition. The elongated perfusion chamber was perfused at 3 ml min^{-1} in the same direction as the flow of luminal perfusate. This was sufficient to sweep away the luminal perfusate emerging from the open end of the duct, thus preventing access of luminally applied transport inhibitors to the outer surface of the duct. Intracellular pH was measured by microfluorometry as described above.

Measurement of fluid secretion

Fluid secretion into the closed luminal space of ducts cultured overnight was analysed by video microscopy (Steward *et al.* 1998). Briefly, ducts were mounted in a temperature-controlled Perspex perfusion chamber on the stage of a Nikon TMS inverted microscope. Bright-field images of a group of ducts were acquired at 1- or 2-min intervals using a CCD camera (model 902A, Watec Corp., Las Vegas, NV, USA or model 4913, Cohu Inc., San Diego, CA, USA) connected to the frame-grabber board (LG-3, Scion Corp., Frederick, MD, USA) of a personal computer.

For the first 10 min of each experiment the ducts were superfused with the Hepes-buffered solution and then, from 10 to 30 min, with the HCO_3^--buffered solution. An agonist (or vehicle alone in control experiments) was then added to the superfusate from 20 to 30 min. At the end of each experiment the ducts were exposed to a hypotonic solution (the standard Hepes-buffered solution diluted 50% with distilled water) in order to evoke osmotic swelling of the luminal space and thus confirm that the ends of the duct were completely sealed. Data from the few ducts that did not show a normal swelling response to the hypotonic challenge were discarded.

Digital images of the ducts were analysed by using Scion Image software (Scion Corp.) to measure the area corresponding to the luminal space in each image. Luminal area measurements from individual images were normalised to the mean of the first few in the series (A_0) thus giving values for the relative area ($A_R = A/A_0$). These were then converted to relative luminal volumes ($V_R = V/V_0$), assuming that the lumen was cylindrical and taking into account the relative increases in width and length in each series. The secretory

rate, calculated from the rate of change of luminal volume, was divided by the luminal surface area of the epithelium and expressed in nl min^{-1} mm^{-2}.

Statistical analysis

Data are presented as means ± S.E.M., where the value of n quoted is the total number of ducts. These were generally obtained from at least three different animals. Student's unpaired t tests were used for the statistical comparison of data; $P < 0.05$ was chosen as the limit for statistical significance. Concentration–response data were fitted with a polynomial curve using Prism software (GraphPad Software Inc, San Diego, CA, USA), which provided estimates of the EC_{50} and maximal response values.

RESULTS

HCO_3^- entry across the basolateral membrane of the guinea-pig pancreatic duct is closely coupled to HCO_3^- secretion across the luminal membrane. Consequently, duct cell pH_i remains remarkably constant during the transition from the unstimulated state to maximal HCO_3^- secretion (Ishiguro *et al.* 1996). However, when HCO_3^- entry is blocked by the application of transport inhibitors, the continuing efflux of HCO_3^- across the luminal membrane leads to a fall in pH_i as a result of the net loss of base from the cell. The initial rate of fall in pH_i is therefore an index of instantaneous HCO_3^- efflux and we have used this measurement to investigate the physiological regulation of HCO_3^- secretion in isolated guinea-pig ducts.

In these experiments, the rate of HCO_3^- efflux was calculated from the rate of intracellular acidification following blockade of HCO_3^- uptake across the basolateral membrane, taking into account the intracellular buffering capacity. The underlying assumption is that, during steady-state secretion, HCO_3^- efflux is balanced by HCO_3^- uptake through Na^+–H^+ exchange and Na^+–HCO_3^- cotransport and that these are totally inhibited by 0.3 mM amiloride and 0.1 mM DIDS, respectively (Ishiguro *et al.* 1996).

Figure 1*A* shows averaged data from four experiments in which pH_i was measured in isolated guinea-pig ducts loaded with BCECF. As mentioned above, application of 10 nM secretin at 5 min had no effect on pH_i but, when amiloride and DIDS were applied simultaneously 5 min later, the rate of intracellular acidification was markedly faster in the secretin-stimulated ducts (-0.065 ± 0.012 min^{-1}, $n = 4$) than in the unstimulated control ducts (-0.017 ± 0.003 min^{-1}, $n = 4$; $P < 0.05$).

When the experiment was repeated in the absence of HCO_3^- (Fig. 1*B*), the initial value of pH_i was slightly greater than that in the presence of HCO_3^-, as previously observed (Ishiguro *et al.* 1996). Under these conditions, the intracellular acidification rate in unstimulated ducts following the application of amiloride and DIDS (-0.029 ± 0.003 min^{-1}, $n = 5$) was no longer increased by secretin stimulation (-0.035 ± 0.009 min^{-1}, $n = 5$; $P > 0.05$). This supports our hypothesis that the acidification rate measured in this way is an index of HCO_3^- efflux from the cell.

Intracellular buffering capacity

The acidification rate measured in these experiments depends not only on the acid and base fluxes across the plasma membrane but also on the intracellular buffering capacity, which is itself a function of pH_i. In order to calculate the net flux of HCO_3^-, we determined the

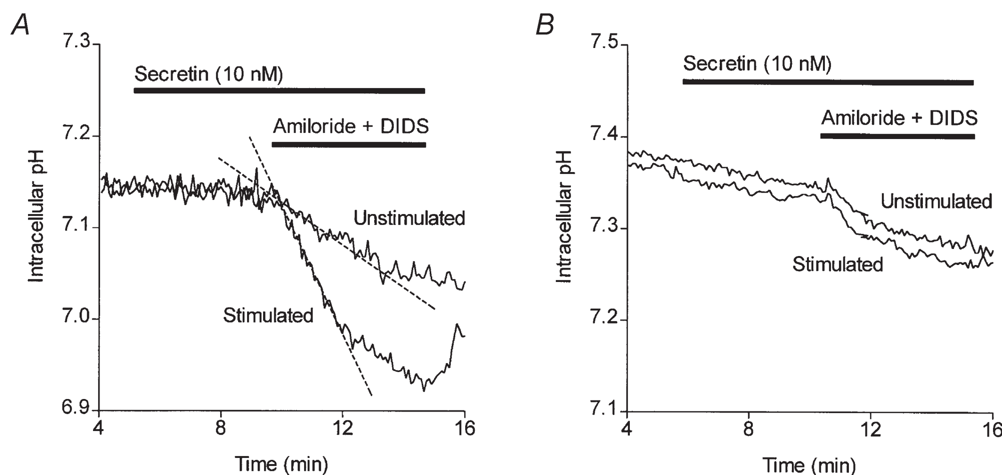

Figure 1. Secretin-evoked HCO_3^- efflux in isolated guinea-pig pancreatic ducts

A, averaged changes in intracellular pH in isolated guinea-pig pancreatic ducts following simultaneous inhibition of basolateral Na^+–H^+ exchange and Na^+–HCO_3^- cotransport with 0.3 mM amiloride and 0.1 mM DIDS, respectively. Ducts were either unstimulated throughout ($n = 4$) or stimulated with 10 nM secretin from 5 min ($n = 4$). The dashed lines show the initial slopes of the changes in pH_i used to calculate the intracellular acidification rate, which was used as an index of HCO_3^- secretion. *B*, averaged data from similar experiments performed in nominally HCO_3^--free, Hepes-buffered solutions ($n = 5$).

buffering capacity by measuring the changes in pH_i resulting from stepped changes in the extracellular concentration of the weak bases NH_3 and TMA. Figure 2*A* shows a typical experiment in which an isolated duct loaded with BCECF was exposed first to a Hepes-buffered solution in which Na^+ was totally replaced by $NMDG^+$. Replacement of Na^+ had the effect of both lowering the initial pH_i value into an appropriate range and inhibiting any Na^+-dependent pH-regulatory mechanisms. NH_4Cl was then added to the superfusate, initially at a concentration of 5 mM, which was then increased in two further steps to 20 mM and then reduced stepwise to zero. From the changes in measured pH_i and the changes in the calculated intracellular concentration of NH_4^+ for each step, values of the intrinsic buffering capacity β_i were determined over a range of pH_i values (see Methods). To measure β_i at higher values of pH_i, TMA was added to the normal Hepes-buffered solution at concentrations of 5, 10 and 20 mM.

Mean results from all 26 experiments are shown in Fig. 2*B* where the data have been fitted with a polynomial function (continuous line). The dashed line in Fig. 2*B* includes the predicted additional contribution of the HCO_3^-/CO_2 buffering system, and these are the values of β_t that have been used throughout the rest of this paper to convert acidification rates to acid/base fluxes.

Effects of luminal inhibitors

To test further the assumption that the reduction in pH_i upon inhibition of the basolateral transporters is due to efflux of HCO_3^- to the lumen of the duct, a microperfusion technique was used to apply anion transport inhibitors to the luminal membrane. To simulate physiological conditions, the lumen was perfused with a solution containing 139 mM HCO_3^- and 10 mM Cl^-, while the bath was perfused with the normal HCO_3^--buffered solution. In the experiments shown in Fig. 3*A*, unstimulated ducts, and ducts stimulated from 5 min with 5 μM forskolin, were exposed at 15 min to basolateral application of 0.3 mM amiloride and 0.1 mM DIDS. As with secretin (Fig. 1*A*), forskolin markedly accelerated the intracellular acidification resulting from inhibition of the basolateral transporters (Fig. 3*A*).

Inclusion of 0.1 mM NPPB and 0.1 mM DIDS in the luminal perfusate had no significant effect on the intracellular acidification evoked by basolateral amiloride and DIDS in the unstimulated ducts (data not shown). However, the stimulatory effect of forskolin was completely abolished (Fig. 3*A* and *B*). Since both NPPB and DIDS inhibit a variety of anion channels and transporters, it is impossible to identify the luminal channels and/or transporters involved in the acidification response. Whatever the mechanism, however, these results confirm that the increase in the acidification rate elicited by forskolin is the result of increased acid/base fluxes, most probably HCO_3^- secretion across the luminal membrane.

Secretin-evoked HCO_3^- secretion

Secretin is a major physiological stimulus for pancreatic HCO_3^- secretion. Therefore our first objective was to test the validity of this new technique by characterising the secretory response of the isolated ducts to this hormone.

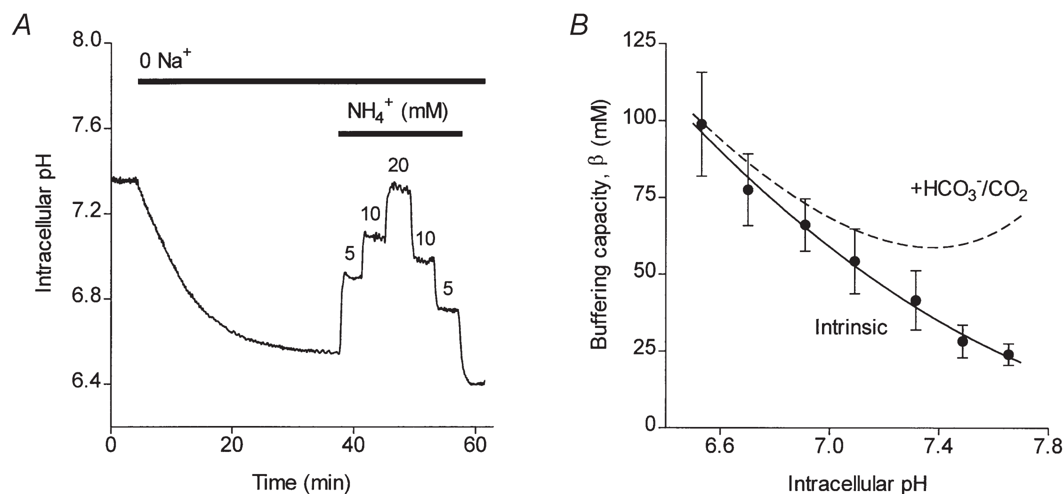

Figure 2. Intracellular buffering capacity in isolated interlobular ducts

A, changes in intracellular pH in an isolated guinea-pig duct exposed first to a Na^+-free Hepes-buffered solution, and then to increasing and decreasing concentrations of NH_4Cl. *B*, intracellular buffering capacity plotted as a function of intracellular pH using data obtained from experiments of the type shown in *A*, and similar experiments using trimethylamine to induce step changes in pH_i. Data from 26 ducts (from 6 preparations) were averaged within 0.2 pH unit bins and the error bars represent S.E.M. The continuous line, representing the intrinsic buffering capacity, was obtained by non-linear regression using a polynomial function. The dashed line includes the additional, theoretical contribution of the HCO_3^-/CO_2 buffering system.

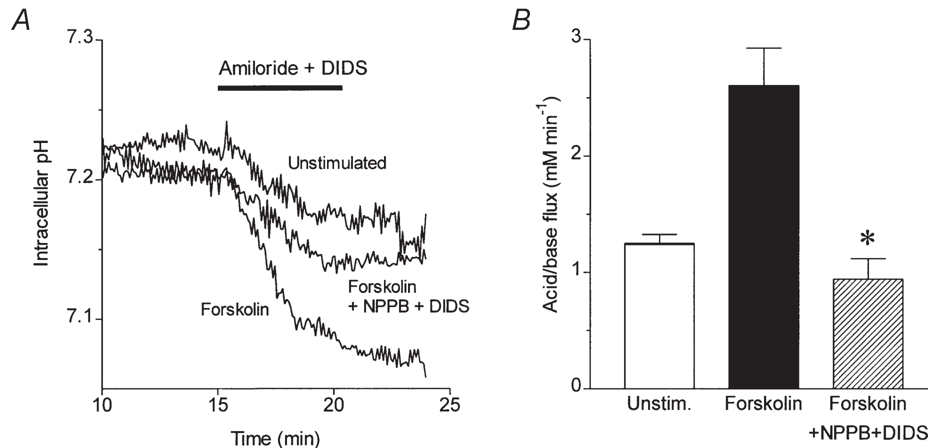

Figure 3. Inhibition of luminal membrane anion transporters by microperfusion

A, changes in intracellular pH in luminally microperfused guinea-pig duct exposed at 15 min to 0.3 mM amiloride and 0.1 mM DIDS. Three representative experiments show the pH_i changes in an unstimulated duct, in a duct stimulated from 5 min with 5 μM forskolin, and in a duct stimulated with forskolin during luminal microperfusion with 0.1 mM NPPB and 0.1 mM DIDS to block the luminal membrane anion transporters. *B*, mean acid/base fluxes calculated from the rate of intracellular acidification induced by basolateral application of amiloride and DIDS in the experiments shown in *A* ($n = 12$ in each series). The asterisk indicates a significant difference from the response to forskolin alone ($P < 0.05$, Student's t test). The error bars represent S.E.M.

As mentioned earlier, stimulation with secretin had no significant effect on pH_i but the subsequent acidification, evoked by application of amiloride and DIDS, was accelerated by secretin in a dose-dependent fashion (Fig. 4*A*). The apparent HCO_3^- fluxes, calculated from the acidification rate, taking into account the intracellular buffering capacity, are shown as a function of secretin concentration in Fig. 4*B*. Although a component of the calculated flux may be due to other sources of intracellular acidification, such as proton influx and metabolism, the secretin-stimulated component shows a sigmoid concentration–response curve with an EC_{50} of 0.5 nM ($\log EC_{50} = -9.3 \pm 0.5$) and a maximal acid/base flux of 4.5 ± 0.6 mM min^{-1}. The EC_{50} value is comparable

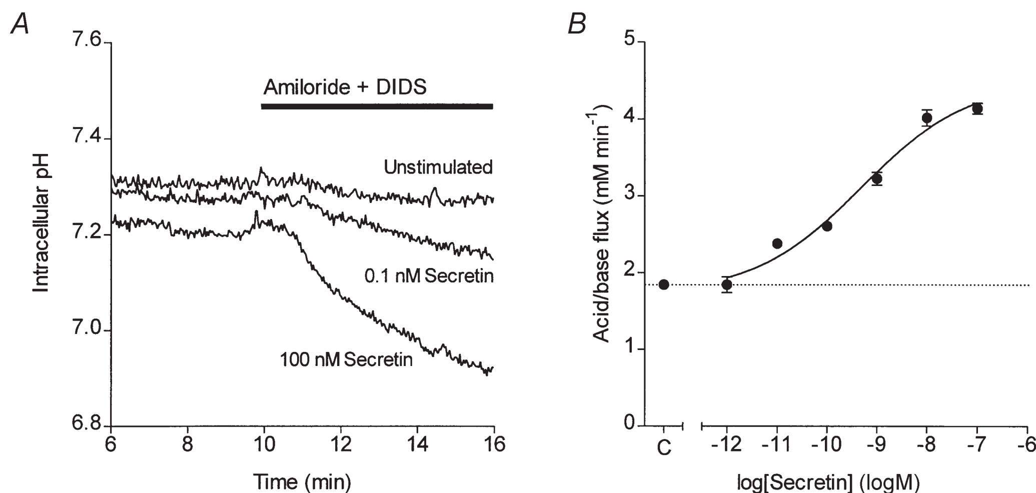

Figure 4. Secretin-evoked HCO_3^- secretion in isolated guinea-pig pancreatic ducts

A, changes in intracellular pH in guinea-pig pancreatic ducts exposed at 10 min to 0.3 mM amiloride and 0.1 mM DIDS. Three representative experiments show the pH_i changes in an unstimulated duct and in ducts stimulated from 5 min with either 0.1 or 100 nM secretin. *B*, concentration–response curve for secretin-stimulated HCO_3^- secretion. Data show mean values of acid/base flux in response to different concentrations of secretin ($n = 6$) and the error bars represent S.E.M. The continuous line was obtained by fitting a sigmoid curve. The dotted line represents the unstimulated control value (C) obtained from ducts exposed to 0.3 mM amiloride and 0.1 mM DIDS but not secretin ($n = 15$).

with that obtained previously by fluorescence imaging measurements of fluid secretion in isolated guinea-pig ducts (0.3 nM, $\log EC_{50} = -9.5 \pm 0.5$; Ishiguro *et al.* 1998). On the other hand, the EC_{50} is more than an order of magnitude greater than that obtained by micropuncture measurements of fluid secretion in rat ducts (20 pM; Argent *et al.* 1986), perhaps indicating a significant species difference in the secretin receptor characteristics.

CCK-evoked HCO_3^- secretion

In the anaesthetised guinea-pig, CCK stimulates a copious secretion of HCO_3^--rich pancreatic juice (Padfield *et al.* 1989). To determine whether this secretion represents a direct effect of CCK on ductal tissue, CCK was tested on isolated ducts using the same protocol as that described for secretin. Figure 5*A* shows that, like secretin, CCK evoked a dose-dependent increase in acidification rate upon application of amiloride and DIDS at 10 min. The concentration–response curve (Fig. 5*B*) has an EC_{50} of 0.2 nM ($\log EC_{50} = -9.9 \pm 0.4$) and the maximal acid/base flux was 4.1 ± 0.4 mM min^{-1}, which is comparable with that obtained with maximal secretin stimulation.

Figure 6*A* shows the effects on HCO_3^- secretion of pretreatment with selective CCK receptor antagonists. The stimulatory effect of CCK was completely inhibited by the CCK_1 receptor antagonist devazepide (L364,718, 1 μM) but was unaffected by the CCK_2 receptor antagonist L365,260 (1 μM). This indicates the presence of CCK_1 rather than CCK_2 receptors on guinea-pig pancreatic ducts.

The CCK_1 receptor is known to exist in two alternative affinity states on pancreatic acini (Jensen, 1994). These two states can be distinguished using the CCK derivative JMV-180, which is an agonist at the high-affinity sites but an antagonist at the low-affinity sites (Matozaki *et al.* 1989). When applied alone to the isolated pancreatic ducts, 3 μM JMV-180 evoked a modest HCO_3^- secretion (Fig. 6*B*). However, in combination with a maximal dose of CCK (10 nM), JMV-180 caused a partial inhibition of HCO_3^- secretion, while at a mid-range CCK concentration (1 nM) it had neither stimulatory nor inhibitory effects (Fig. 6*B*). These results suggest that the lower end of the CCK dose–response curve is the result of occupation of high-affinity receptors while the upper end also involves occupation of low-affinity sites.

Bombesin-evoked HCO_3^- secretion

Like secretin and CCK, bombesin stimulation alone had no effect on the pH_i of interlobular ducts isolated from guinea-pig pancreas. However, application of amiloride and DIDS to block basolateral HCO_3^- uptake again revealed a marked dose–dependent stimulation of HCO_3^- secretion, as reflected by a steep increase in the rate of intracellular acidification (Fig. 7*A*). The concentration–response curve for the effect of bombesin on HCO_3^- secretion (Fig. 7*B*) was again sigmoid, with an EC_{50} of 30 pM ($\log EC_{50} = -10.6 \pm 0.4$) and a maximal acid/base flux of 3.6 ± 0.1 mM min^{-1}, comparable with the maximal values obtained with secretin and CCK.

In mammals, bombesin-related peptides are thought to act either on GRP receptors or on NMB receptors. To distinguish between these, we examined the effects of selective bombesin receptor antagonists on the HCO_3^- fluxes evoked by bombesin stimulation. As shown in

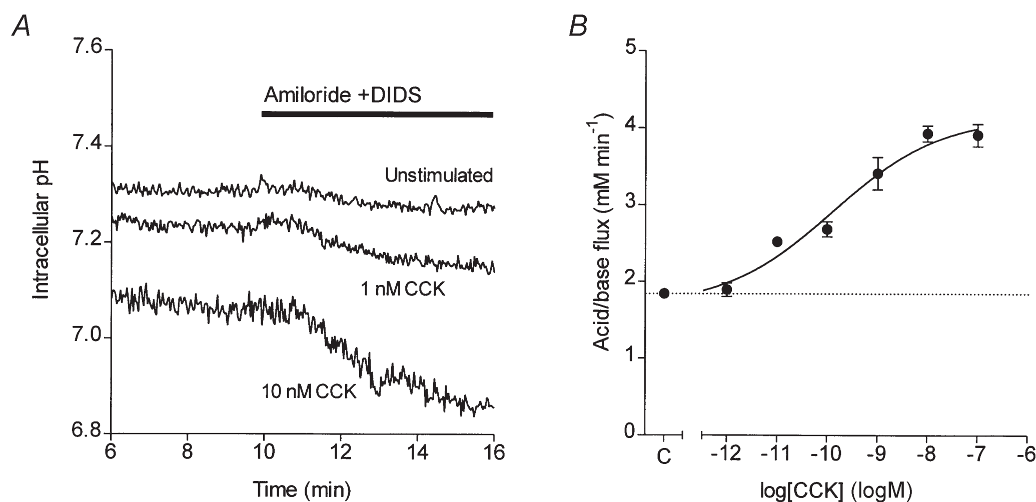

Figure 5. CCK-evoked HCO_3^- secretion in isolated guinea-pig pancreatic ducts

A, changes in intracellular pH in guinea-pig pancreatic ducts exposed at 10 min to 0.3 mM amiloride and 0.1 mM DIDS. Three representative experiments show the pH_i changes in an unstimulated duct and in ducts stimulated from 5 min with either 1 or 10 nM CCK. *B*, concentration–response curve for CCK-stimulated HCO_3^- secretion. Data show mean values of acid/base flux in response to different concentrations of CCK ($n = 9$) and the error bars represent S.E.M. The continuous line was obtained by fitting a sigmoid curve. The dotted line represents the unstimulated control value (C) obtained from ducts exposed to 0.3 mM amiloride and 0.1 mM DIDS but not CCK ($n = 15$).

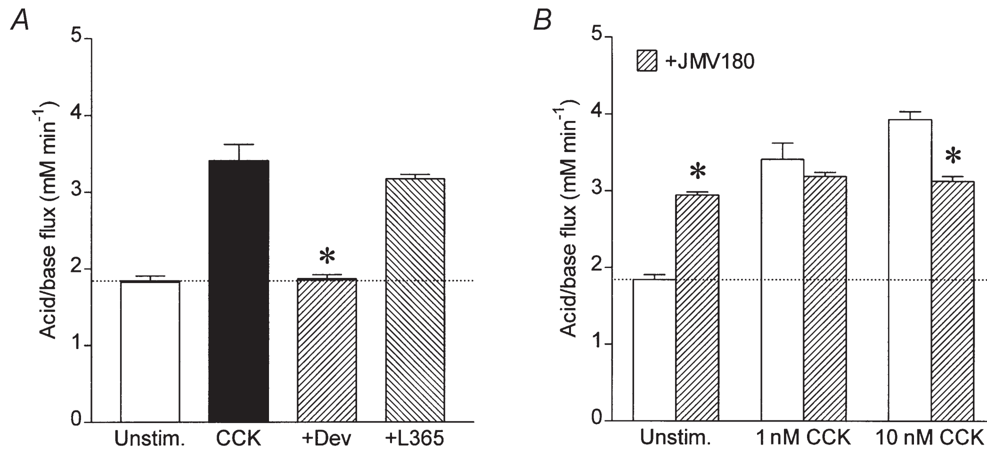

Figure 6. Effects of CCK receptor antagonists on CCK-stimulated HCO_3^- secretion in isolated guinea-pig pancreatic ducts

Mean acid/base fluxes calculated from the rate of intracellular acidification induced by basolateral application of 0.3 mM amiloride and 0.1 mM DIDS. *A*, ducts were either unstimulated (Unstim., $n = 15$) or stimulated with 1 nM CCK (CCK, $n = 9$), 1 nM CCK + 1 μM devazepide (+Dev, $n = 9$) or 1 nM CCK + 1 μM L365,260 (+L365, $n = 9$). The horizontal line represents the unstimulated control value. The asterisk indicates a significant difference from the response to CCK alone ($P < 0.05$, Student's *t* test). The error bars represent S.E.M. *B*, ducts were either unstimulated (Unstim., $n = 15$) or stimulated with 1 nM ($n = 9$) or 10 nM CCK ($n = 9$) in the presence (▨) or absence (□) of the partial CCK_1 receptor agonist JMV-180 (3 μM). The horizontal line represents the unstimulated control value. The asterisks indicate significant differences from the corresponding responses in the absence of JMV-180 ($P < 0.05$, Student's *t* test). The error bars represent S.E.M.

Fig. 8, the GRP receptor antagonist BME (1 μM) completely abolished the stimulatory effect of 10 nM bombesin while the NMB-type receptor antagonist BIM (1 μM) had no inhibitory effect, suggesting that the effect of bombesin is mediated through the GRP receptor.

ACh-evoked HCO_3^- secretion

Similar experiments were also performed with ACh, which has previously been shown to evoke fluid secretion in rat pancreatic ducts (Ashton *et al.* 1993). Using a

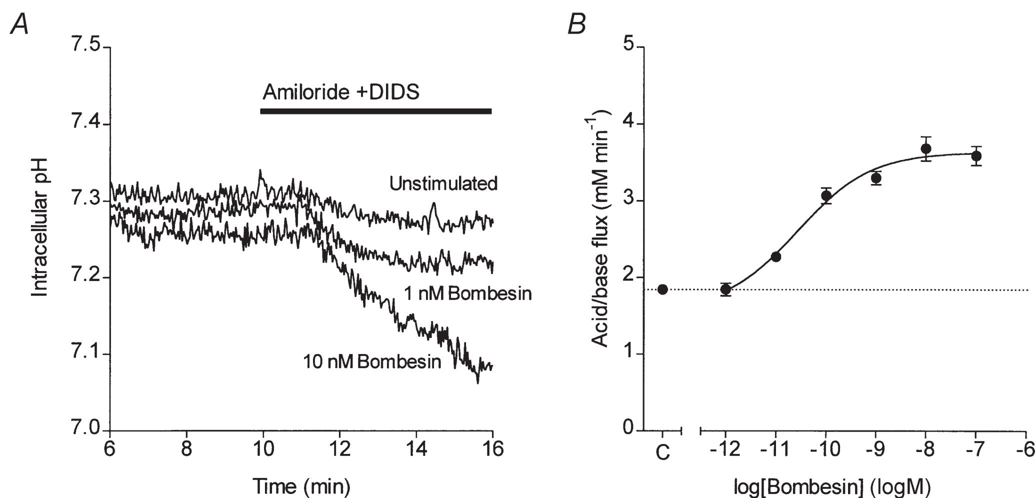

Figure 7. Bombesin-evoked HCO_3^- secretion in isolated guinea-pig pancreatic ducts

A, changes in intracellular pH in guinea-pig pancreatic ducts exposed at 10 min to 0.3 mM amiloride and 0.1 mM DIDS. Three representative experiments show the pH_i changes in an unstimulated duct and in ducts stimulated from 5 min with either 1 or 10 nM bombesin. *B*, concentration–response curve of bombesin-stimulated HCO_3^- secretion. Data show mean values of acid/base flux in response to different concentrations of bombesin ($n = 9$) and the error bars represent S.E.M. The continuous line was obtained by fitting a sigmoid curve. The dotted line represents the unstimulated control value (C) obtained from ducts exposed to 0.3 mM amiloride and 0.1 mM DIDS but not bombesin ($n = 15$).

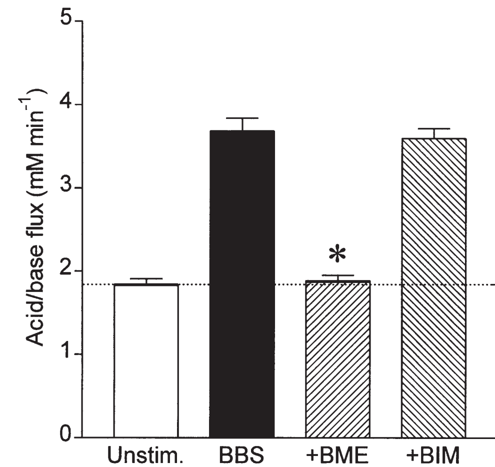

Figure 8. Effects of bombesin receptor antagonists on bombesin-stimulated HCO_3^- secretion in isolated guinea-pig pancreatic ducts

Mean acid/base fluxes calculated from the rate of intracellular acidification induced by basolateral application of 0.3 mM amiloride and 0.1 mM DIDS. Ducts were either unstimulated (Unstim., $n = 15$) or stimulated with 10 nM bombesin (BBS, $n = 9$), 10 nM bombesin + 1 μM BME (+BME, $n = 9$) or 10 nM bombesin + 1 μM BIM (+BIM, $n = 9$). The horizontal line represents the unstimulated control value. The asterisk indicates a significant difference from the response to bombesin alone ($P < 0.05$, Student's t test). The error bars represent S.E.M.

relatively high concentration of ACh (10 μM), the calculated HCO_3^- flux in the guinea-pig ducts was 3.8 ± 0.2 mM min^{-1}, comparable with the maximal values obtained with secretin, CCK and bombesin. Smaller responses were obtained with 1 and 3 μM ACh. As in the rat ducts, the response to ACh was blocked by pretreatment with 10 μM atropine (data not shown), indicating the probable involvement of muscarinic receptors.

Fluid secretion evoked by CCK, bombesin and ACh

Because the ends of isolated guinea-pig pancreatic ducts seal during overnight culture, fluid secretion to the lumen causes the ducts to swell. The change in volume of the ducts can therefore be measured by video microscopy as a simple index of fluid secretory rate (Steward *et al.* 1998). Figure 9*A* shows mean data for the changes in relative duct volume occurring in the presence and absence of 1 nM CCK. Initially, in the HCO_3^--free Hepes solution, the duct volume remained relatively constant. When the perfusion fluid was switched to the HCO_3^--buffered solution at 10 min the ducts began to swell steadily, as a result of spontaneous fluid secretion. Application of 1 nM CCK at 20 min markedly accelerated ductal swelling, indicating that the secretory rate had increased.

Using this method, fluid secretory rates were measured with several of the secretagogues over a range of concentrations. In general, the concentrations that were

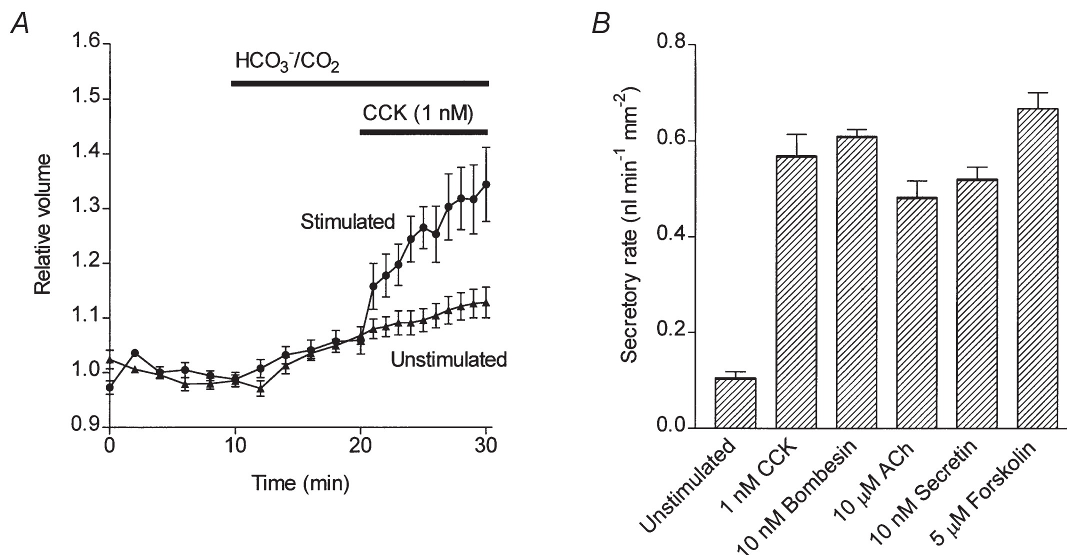

Figure 9. Measurement of fluid secretion in response to secretagogues by video microscopy

A, mean changes in relative luminal volume of isolated guinea-pig pancreatic ducts. The bath solution was switched from the Hepes-buffered solution to the HCO_3^-/CO_2 solution at 10 min. Mean data from two series of experiments show the volume changes in unstimulated ducts ($n = 10$) and in ducts stimulated with 1 nM CCK from 20 min ($n = 9$). *B*, mean secretory rates calculated from the initial rate of increase in relative luminal volume following stimulation with a range of secretagogues. Mean and S.E.M. values were calculated from 6–23 individual experiments together with equivalent data from unstimulated control ducts ($n = 13$).

effective in stimulating fluid secretion were comparable with those that stimulated HCO_3^- secretion. Fluid secretory rates obtained with maximal doses of each of the agonists are plotted in Fig. 9*B*. Taken together with the intracellular acidification data, our results indicate that, like secretin, CCK, bombesin and ACh are all capable of directly evoking a HCO_3^--rich fluid secretion in the interlobular ducts of the guinea-pig pancreas.

DISCUSSION

This paper describes a relatively simple fluorometric method for measuring the secretion of HCO_3^- in isolated pancreatic ducts. This is achieved by measuring the initial rate of intracellular acidification that occurs when the basolateral pH regulatory mechanisms of the cells are abruptly inhibited. The main transporters serving this function, and thereby responsible for the basolateral uptake of HCO_3^- during secretion, are a Na^+–H^+ exchanger which is blocked by amiloride and a Na^+–HCO_3^- cotransporter which is blocked by DIDS (Ishiguro *et al.* 1996). In our experiments, simultaneous application of the two inhibitors caused a fall in pH_i that was markedly accelerated by pretreatment with secretin (Fig. 1). This increased rate of acidification evoked by secretin pretreatment was dependent on the presence of HCO_3^-/CO_2. We attribute this to a secretin-evoked increase in the efflux of HCO_3^- from the cells. In order to determine whether the direction of HCO_3^- efflux was to the duct lumen or to the bath, we microperfused the lumen to selectively block anion transport pathways at the luminal membrane (Fig. 3). Combined application of NPPB and DIDS to the luminal membrane completely abolished the increase in HCO_3^- efflux evoked, in this case, by forskolin stimulation. We therefore conclude that the acid/base flux measured by this technique is principally due to HCO_3^- secretion across the luminal membrane.

The intracellular acidification observed in unstimulated ducts using this technique may be due partly to spontaneous HCO_3^- secretion and partly to other, background sources of intracellular acidification such as H^+ leakage into the cells, HCO_3^- efflux to the bath, and acidic products of metabolism, any of which could also change in response to secretagogue stimulation. Therefore, while our technique does not provide a pure measure of HCO_3^- secretion, it does represent a relatively simple assay for investigating the effects of putative regulators on ductal HCO_3^- secretion.

In order to convert the intracellular acidification rate to an apparent acid/base flux, it was necessary to measure the intracellular buffering capacity of the duct cells (Fig. 2). Our data indicate that, within the pH_i range at which these measurements were made (7.1–7.4), the intracellular buffering capacity is approximately 60 mM. Thus a typical secretin-evoked increase in the intracellular acidification rate of 0.04 min^{-1} would be due to a net efflux of HCO_3^- of approximately 2 mM min^{-1}.

While many peptide receptors have been identified on guinea-pig pancreatic acini (Jensen, 1994), rather little is known about the existence and role of such receptors on guinea-pig pancreatic ducts. Measurements of HCO_3^- and fluid secretion on isolated ducts permit such an analysis. In this paper, we have characterised the responses to CCK and bombesin, and also to the parasympathetic agonist ACh. Our results clearly demonstrate the existence of CCK_1 receptors, GRP-type bombesin receptors and muscarinic ACh receptors in this tissue, and that occupation of all three receptors evokes both HCO_3^- and fluid secretion.

Physiological studies of pancreatic fluid and electrolyte secretion in the guinea-pig are very limited. However, an early study in our laboratory on anaesthetised guinea-pigs clearly showed that not only secretin but also CCK evoked a copious secretion of HCO_3^--rich pancreatic juice (Padfield *et al.* 1989). It was impossible to say from that study whether CCK was acting on the ducts or the acini to evoke fluid secretion. The data in the current paper show unequivocally that, in the guinea-pig, CCK acts directly on the ducts. Furthermore, the duct swelling measurements parallel the HCO_3^- flux data and, like those for secretin, indicate that the secreted fluid is rich in HCO_3^-. This contrasts with the rat where CCK evokes a Cl^--rich secretion (Sewell & Young, 1975), most probably derived from the acini, and where the CCK analogue caerulein has been shown to have no effect on ductal fluid secretion (Argent *et al.* 1986).

CCK receptors were originally classified as CCK_A and CCK_B receptors (now CCK_1 and CCK_2) on the basis of their relative affinities for different peptides. The subsequent availability of specific antagonists has allowed further investigations into the physiological actions of the peptide (Scarpignato *et al.* 1993; Wank, 1998; Noble *et al.* 1999). There is good evidence for the presence of functional CCK_1 receptors on pancreatic acini, on gallbladder smooth muscle, on other smooth muscle in the digestive tract, and in the enteric nervous system and brain (Wank, 1998; Noble *et al.* 1999). However, the presence and identity of functional CCK receptors on pancreatic ducts has not previously been studied. In the present study we used two benzodiazepine derivatives to selectively block CCK_1 and CCK_2 receptors. Devazepide, used at a concentration that blocks CCK_1 but not CCK_2 receptors (Huang *et al.* 1989), completely inhibited the effect of CCK on ductal HCO_3^- secretion. L365,260, which blocks CCK_2 but not CCK_1 receptors (Huang *et al.* 1989), did not alter the secretory response to CCK. Our data therefore indicate that it is the CCK_1 receptor subtype that mediates the effect of CCK on guinea-pig pancreatic ducts.

In pancreatic acinar cells, the CCK_1 receptor exists in two alternative affinity states which activate different

intracellular messenger pathways (Jensen, 1994). The low-affinity receptors activate phospholipase $C_{\beta 1}$, resulting in generation of inositol trisphosphate and hence a rise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), while the high-affinity receptors activate phospholipase A_2 causing liberation of arachidonic acid which also elevates $[Ca^{2+}]_i$. Despite the convergence of the signalling pathways, occupancy of the high-affinity sites stimulates enzyme secretion while occupation of the low-affinity sites inhibits it. Consequently, the dose–response curve for CCK-evoked enzyme secretion is bell-shaped.

The effects of CCK-like peptides on gastrointestinal smooth muscle, however, are quite different. Our observations on pyloric, fundic and antral smooth muscle in rats suggest a lack of motor activity following occupation of high-affinity receptors, and a strong contractile activity following occupation of low-affinity receptors (Kisfalvi *et al.* 2001). Similar results have been obtained with gallbladder smooth muscle in guinea-pig and rabbit (Maubach *et al.* 1991; Taniguchi *et al.* 1995), suggesting that the absence of high-affinity CCK_1 receptors on gastrointestinal smooth muscle is a general phenomenon.

JMV-180, which is an agonist of the high-affinity receptors and an antagonist of the low-affinity receptors, is a useful tool for differentiating the two receptor states and their functions (Bianchi *et al.* 1994; Rivard *et al.* 1994; Tsunoda & Owyang, 1995; Tsunoda *et al.* 1996). In rat and mouse pancreatic acini, JMV-180 acts on the high-affinity receptors to give a full secretory response similar to that of CCK. In the guinea-pig, however, it is only a partial agonist at the high-affinity receptors (Bianchi *et al.* 1994; Sjodin *et al.* 1997). At the low-affinity receptors it blocks the response to CCK in all three species including the guinea-pig. In gastrointestinal smooth muscle, where only the high-affinity receptors induce contraction, JMV-180 shows little or no agonist activity but displays a competitive antagonism against CCK stimulation (Maubach *et al.* 1991; Taniguchi *et al.* 1995; Kisfalvi *et al.* 2001).

In the present study, JMV-180 when given alone showed partial agonist activity compared with CCK. Furthermore, JMV-180 did not modify the ductal secretion evoked by 1 nM CCK, a concentration that evokes maximal enzyme secretion from acini through full occupation of high-affinity receptors. However, it significantly inhibited the secretory response to 10 nM CCK, a concentration that is generally regarded to activate not only high- but also low-affinity CCK receptors. This suppression of ductal secretion suggests that only the low-affinity component of the CCK receptor activation was inhibited by JMV-180.

Our conclusions from these studies with JMV-180 are in line with those drawn from CCK concentration–response relationships in a variety of tissues. In pancreatic acini, the curve is biphasic with an EC_{50} value for amylase secretion of around 30 pM (Jensen, 1994; Taniguchi *et al.* 1995; Kisfalvi *et al.* 2001), corresponding to an effect mediated by high-affinity receptors. On smooth muscle preparations, the contractile effect of CCK has an EC_{50} value of around 3 nM and the curve appears to be monophasic and is probably mediated by the low-affinity receptors (Jensen, 1994; Taniguchi *et al.* 1995; Kisfalvi *et al.* 2001). In the present work, the effect of CCK on HCO_3^- secretion was also monophasic but the EC_{50} value (around 0.2 nM) was half-way between the EC_{50} values obtained in acini and smooth muscle. Taken together with the JMV-180 data, this suggests that occupancy of both high- and low-affinity CCK_1 receptor sites evokes HCO_3^- secretion in guinea-pig pancreas.

Bombesin-like immunoreactivity has been demonstrated throughout the gastrointestinal tract including the pancreas (Bunnett, 1994). The peptide has a wide range of biological effects amongst which its stimulatory action on pancreatic enzyme secretion is especially well characterised (Jensen, 1994). By contrast, physiological studies on bombesin-stimulated pancreatic fluid and electrolyte secretion are very limited. An early study in our laboratory on anaesthetised guinea-pigs clearly showed that not only secretin and CCK, but also bombesin, evoked a copious secretion of HCO_3^--rich pancreatic juice (Padfield *et al.* 1989). The results of the present paper suggest that, like secretin and CCK, bombesin also directly stimulates ductal HCO_3^- and fluid secretion in this species. This observation is in line with studies in rats where bombesin also stimulates fluid secretion from isolated pancreatic ducts (Ashton *et al.* 1990, 1991).

The concentration–response curve for bombesin in our study is similar to that obtained previously with guinea-pig acini. In both cell types the EC_{50} values fall within the 10–100 pM range (Jensen, 1994), and it seems likely that the bombesin receptor subtypes are probably the same. Of the known subtypes, only one has been described in pancreatic acinar cells and this is the GRP-preferring bombesin receptor, so-called because of its preferential affinity for GRP (Jensen & Coy, 1991). A highly potent and selective antagonist for this receptor is D-Phe^6-bombesin(6–13)-methyl ester (BME) which has an affinity constant of 1.29 nM (Jensen & Coy, 1991; Varga *et al.* 1991; Coy *et al.* 1992). A second subtype, first identified in oesophageal smooth muscle, is the NMB-preferring bombesin receptor which has a preferential affinity for NMB (Von Schrenck *et al.* 1989, 1990). Antagonists for this receptor include D-Nal-cyclo[Cys-Tyr-D-Trp-Orn-Val-Cys]-Nal-NH_2 or BIM-23127 (BIM) which has a greater than 100-fold preference for the NMB receptor over the GRP receptor (Orbuch *et al.* 1993; Ladenheim *et al.* 1994; Milusheva *et al.* 1998). In the present study, we used BME and BIM to determine the receptor subtype that mediates the stimulatory effect of bombesin on ductal HCO_3^- secretion. We observed a complete inhibition of bombesin-stimulated secretion

with BME but none with BIM, suggesting that the effect of bombesin on ducts (like that on acini) is mediated by the GRP-preferring bombesin receptors.

In contrast to CCK and bombesin, the effects of ACh on pancreatic ductal secretion have been investigated in a number of studies (Ashton *et al.* 1993; Hootman *et al.* 1993; Evans *et al.* 1996). These have clearly revealed that ACh stimulates fluid secretion from isolated rat pancreatic ducts with maximal rates comparable with those evoked by secretin (Ashton *et al.* 1993; Evans *et al.* 1996). In the present study we have also observed that ACh directly evokes a HCO_3^--rich secretion in the pancreatic duct of the guinea-pig. Maximal secretory responses were similar to those obtained with secretin. Atropine abolished the stimulatory effect, suggesting the involvement of muscarinic cholinoceptors. Our observation is in line with previous results obtained in binding studies on isolated guinea-pig pancreatic ductal cells, which indicated the presence of M2 and M3 muscarinic receptors on these cells (Hootman *et al.* 1993).

As expected, the effects of CCK, bombesin and ACh on HCO_3^- secretion were paralleled by their effects on fluid secretion. This suggests that agonist-evoked fluid secretion in guinea-pig pancreatic ducts is invariably coupled to HCO_3^- secretion across the luminal membrane. The maximum rates of HCO_3^- and fluid secretion evoked by all of the agonists tested were similar in this species. In other words, CCK, bombesin (or GRP) and ACh each have the potential to significantly influence ductal HCO_3^- and fluid secretion. Whether they do so under physiological conditions remains to be determined.

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