Biochimica et Biophysica Acta, 551 (1979) 95—108

BBA 78264

TRANSEPITHELIAL PERMEABILITY IN THE RABBIT PANCREAS *

J.W.C.M. JANSEN, J.J.H.H.M. DE PONT and S.L. BONTING

Department of Biochemistry, University of Nijmegen, Nijmegen (The Netherlands)

(Received May 24th, 1978)
(Revised manuscript received September 13th, 1978)

Key words: Permeability; Paracellular route; (Pancreatic epithelium)

Summary

1. The transepithelial permeability in the isolated rabbit pancreas has been studied with the aid of radioactive markers added to the bathing medium.

2. After addition of these compounds in 2 mM concentration to the medium, they equilibrate within 30 min to a steady-state concentration in the secreted fluid. The latter concentrations, expressed as percent of those in the bathing medium, are: urea 100%, glycerol 90%, erythritol 95%, mannitol 60%, lactose 5%, sucrose 4% and inulin 3%.

3. Addition of 10^{-5} M carbachol to the bathing medium after 60 or 90 min of incubation results in an increase of the concentrations of mannitol, lactose sucrose and inulin in the secreted fluid. Maximal concentrations, reached about 35 min after addition of the stimulant, are: mannitol 65%, lactose 31%, sucrose 23%, inulin 8%.

4. No change in the concentration of urea is observed, while the concentrations of glycerol and erythritol increase always to 100% after addition of 10^{-5} M carbachol.

5. For sucrose and lactose the increase in permeability appears to be dependent on the concentration of carbachol.

6. There is no increase in the extracellular space for lactose, sucrose and inulin after incubating fragments of the rabbit pancreas with 10^{-5} M carbachol.

7. Addition of atropine 5 min or more after carbachol stimulation has no effect on enzyme secretion, but markedly inhibits the increase in sucrose permeability.

8. These results indicate that: (a) the permeability of the transcellular transport route in the isolated rabbit pancreas is determined by the size of the permeating molecules, (b) this route is probably extracellular, (c) its perme-

* A preliminary report has been presented at the Symposium on Gastric Ion Transport, July 24—28, 1977, Uppsala, Sweden (Ref. 30).

ability is increased by a cholinergic agent in dose-dependent fashion, (d) the increase in permeability is not caused by the enzyme secretion as such.

Introduction

In the pancreas the transtubular potential difference is near zero [2—5] and the secreted fluid is isosmotic with plasma or bathing medium [6—9]. These two properties are characteristic for a leaky epithelium [1]. This implies that inorganic ions and small non-electrolytes should easily pass from plasma or bathing medium to the lumen of the pancreas.

In previous studies [10,11] we have observed that Ca^{2+} and Mg^{2+} are partially secreted via a paracellular transport way. Mannitol behaves in the same way. K^+ may also pass through a paracellular pathway, since the concentration of this ion in the secreted fluid follows that in the bathing medium when the latter concentration is enhanced [12]. In our studies on calcium, we have observed that the permeability of the paracellular transport route is increased by addition of carbachol, which acetylcholine derivative stimulates the enzyme secretion.

The purpose of this study is to investigate the properties of the paracellular transport route and in particular its increased permeability upon stimulation by carbachol. The experiments indicate that the permeability of the transcellular transport route for various substances in the isolated rabbit pancreas is determined by their molecular size, and that the increase in permeability induced by carbachol is not a consequence of the enzyme secretion evoked by this stimulus.

Materials and Methods

Chemicals. Carbachol, the carbamyl analogue of acetylcholine, is purchased from ACF Chemiefarma, Naarden (The Netherlands). Cerulitide (caerulein) is donated by Dr. R. de Castiglione (Farmitalia, Milan, Italy). Secretin and pancreozymin-C-octapeptide are gifts from Prof. Dr. H. Beyerman (Department of Organic Chemistry, Technical University, Delft, The Netherlands) and Dr. M. Ondetti (The Squibb Institute for Medical Research, Princeton, NJ, U.S.A.), respectively. Atropine is obtained from Merck, Darmstadt.

[^{14}C]Urea (60 Ci/mol), [^{14}C]glycerol (38 Ci/mol), [^{14}C]erythritol (2.3 Ci/mol), [^{14}C]mannitol (60 Ci/mol), [^{14}C]lactose (60 Ci/mol), [^{14}C]sucrose (381 Ci/mol) and [^{3}H]inulin (900 Ci/mol) are purchased from The Radiochemical Centre, Amersham, U.K.

Hyamine hydroxide 10-X is obtained from Packard Instrument International S.A., Zürich (Switserland) and ChromAR-Sheet 500 from Mallinckrodt, St. Louis, U.S.A. Aquasol is obtained from New England Nuclear (Boston, MA, U.S.A.).

The enzymes hexokinase, glucose-6-phosphate dehydrogenase and β-fructosidase as well as adenosine 5′-triphosphate and nicotinamide-adenine dinucleotide phosphate (oxidized form) are purchased from Boehringer, Mannheim, Germany.

Preparation of the isolated rabbit pancreas. Male and female New Zealand white rabbits, weighing 2—3 kg, are used. The animals are killed by a blow on

the neck, immediately followed by carotic exsanguination. The rabbit pancreas is further prepared and mounted essentially according to Rothman [13].

Preparation of pancreas fragments. Fragments of about 60 mg wet weight are cut from the pancreatic tissue stretched between the spleen and the rectum.

Incubation medium. Both preparations of the rabbit pancreas are incubated in a balanced Krebs-Ringer bicarbonate medium, containing (in mmol/l): Na^+ 143.5, K^+ 4.9, Ca^{2+} 2.5, Mg^{2+} 1.2, HCO_3^- 25.0, $H_2PO_4^-$ 1.2, Cl^- 130.7 and glucose 5.5. Before incubation the pH of the solution is adjusted to 7.4 by addition of HCl. During incubation the medium is continuously gassed with 95% O_2 + 5% CO_2 and maintained at 37°C.

Incubation and fraction collection. The isolated rabbit pancreas is incubated for 1 h after mounting in a bath containing 350 ml incubation medium, in order to reach a steady-state condition. After this period, the incubation medium is replaced by a medium containing the radioactive substance in 2 mM concentration, except that [^{3}H]inulin is added in a trace amount of 10 μCi to the bathing medium.

The pancreatic fluid is collected in 5-min fractions in pre-weighed plastic counting vials. The volume of secreted fluid is determined by weighing. Thereafter 5 μl is taken for protein determination, while the remaining volume is mixed with 10 ml Bray's solution [14] and subjected to radioactive counting.

During incubation carbachol and atropine are applied as indicated in the tables and figures.

Determination of the extracellular space. The rabbit pancreas fragments are preincubated in the Krebs-Ringer bicarbonate medium for 30 min and continuously gassed with carbogene, a mixture of 95% O_2 and 5% CO_2, at 37°C. They are then transferred to another vessel, containing the same medium but to which 0.4 μCi [^{14}C]substance/ml or 0.8 μCi [^{3}H]inulin/ml has been added. The total incubation time at 37°C with continuous gassing by carbogene is 90 min. Carbachol is added after 60 min.

After the incubation period two 50-μl supernatant medium samples are taken for counting. From the remaining medium the tissue fragments are then quickly removed, blotted and weighed in pre-weighed glass counting vials. Thereupon, 2 ml hyamine hydroxide 10-X is added to the fragments as well as to the 50-μl medium samples. After 16 h at 50°C the vial contents are thoroughly mixed. After cooling to room temperature 100 μl 30% H_2O_2 (Merck Darmstadt) is added to the vials, which are kept at 50°C for 3 h. After cooling again to room temperature 10 ml Aquasol is added and after mixing radioactivity is measured by liquid scintillation spectrometry. Quenching is corrected for by means of the external standard method.

The extracellular space for the radioactive substance (V_e), expressed as percent of total tissue water, is calculated from the cpm of ^{14}C-labeled substance or [^{3}H]inulin in 50 μl incubation medium (C_m), cpm of ^{14}C-labeled substance or [^{3}H]inulin in the pancreatic fragments (C_s), the wet weight of the fragments (W, in mg) and the average dry weight percentage (D) by means of the formula:

$$V_e = 50 \cdot 10^4 \cdot \frac{C_s}{C_m} \cdot \frac{1}{W} \cdot \frac{1}{100 - D}$$

Thin-layer chromatography. Thin-layer chromatography of the secreted fluid is carried out on ChromAR Sheet 500 using chloroform/methanol/acetic acid (70 : 30 : 15, v/v) as developing solvent. After development the sheet is dried and cut in 1-cm^2 pieces. Each piece is placed in a counting vial with 1 ml water and 10 ml Aquasol. After mixing, the radioactivity of each piece is measured by liquid scintillation spectrometry.

Analytical methods. Protein is determined according to Lowry et al. [15] on a microscale, bovine serum albumin (Behringwerke) serving as a standard.

In the experiments with non-radioactive sucrose, the latter compound is analysed by measuring the amount of NADPH formed after hydrolysis of the sucrose with β-fructosidase, followed by phosphorylation of the resulting glucose with hexokinase and ATP, and oxidation of the glucose 6-phosphate with glucose-6-phosphate dehydrogenase and NADPH [16].

The radioactivity present in the collected fractions, fragments and thin-layer pieces is measured in a liquid scintillation analyzer (Philips).

Results

Permeability for the radioactive substances before and after addition of carbachol

In general, when the radioactive subtance is added to the bathing medium of the isolated rabbit pancreas (Figs. 1—4), the compound begins immediately to be secreted and reaches a steady-state concentration after about 30 min. Addition of carbachol (10^{-5} M) to the bathing medium after 60 or 90 min of incubation always results in a marked increase in enzyme secretion, which reaches a peak 15—20 min after addition of carbachol (Figs. 1—4). This 15—20 min delay mainly reflects the time elapsing for transport of the enzymes from the point of origin to the end of the pancreatic duct. The flow is always constant for at least 4 h with the exception of a transient decrease in the 5—

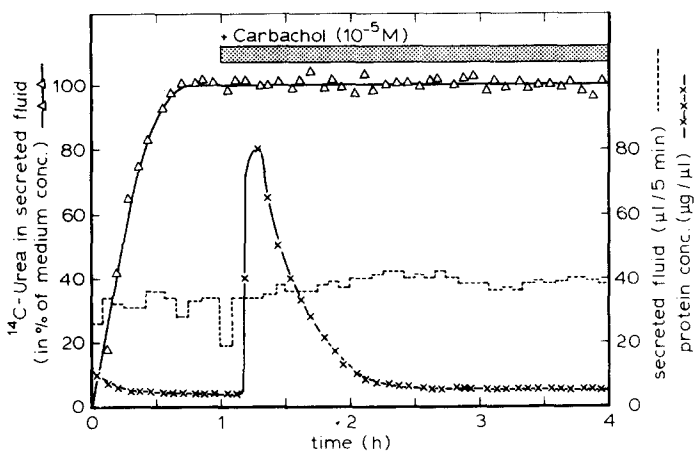

Fig. 1. Permeability of the isolated rabbit pancreas to [^{14}C]urea. At t = 0 2 mM [^{14}C]urea and at t = 1 h 10^{-5} M carbachol are added to the bathing medium. Rate of fluid secretion (— — —), and the concentrations of protein (X———X) and [^{14}C]urea (△———△) in the secreted fluid are measured. Representative for three experiments.

TABLE I

CONCENTRATIONS OF THE RADIOACTIVE NON-ELECTROLYTES IN THE SECRETED FLUID OF THE ISOLATED RABBIT PANCREAS BEFORE AND AFTER STIMULATION WITH 10^{-5} M CARBACHOL

Values are given with S.E., while *n* indicates the number of experiments.

Compound	Average concentration in secreted fluid (in percent of that in the bathing medium)			*n*
	30 min period before stimulation	25—40 min after stimulation *	60—90 min after stimulation **	
Urea	99 ± 0.8	103 ± 6.7	99 ± 7.0	3
Glycerol	88 ± 3.5	94 ± 2.7	98 ± 2.4	5
Erythritol	94 ± 1.7	101 ± 2.5	103 ± 3.7	4
Mannitol	59 ± 4.6	65 ± 3.0	56 ± 3.8	5
Lactose	5.1 ± 0.6	31 ± 1.0	27 ± 3.3	3
Sucrose	3.5 ± 0.2	23 ± 3.1	19 ± 3.0	4
Inulin	2.6 ± 0.4	7.8 ± 1.8	5.5 ± 0.8	3

* Period during which peak permeability is reached for the larger molecules.
** Period during which permeability reaches a steady-state value.

10 min period immediately after stimulation with 10^{-5} M carbachol. The cause of the latter effect is unknown.

Table I summarizes the average concentrations of the non-electrolytes used in the secreted fluid, expressed as percentage of the respective concentrations in the bathing medium. It appears that after equilibration (30—40 min after addition of the compounds to the bathing medium at $t = 0$, Fig. 1) the concentration of urea in the secreted fluid is the same as in the bathing medium. It is not influenced by addition of 10^{-5} M carbachol. [^{14}C]Erythritol (Fig. 2) and [^{14}C]glycerol reach after 30—40 min concentrations of 94 and 88% of those in the bathing medium, which rise after stimulation to about 100%.

Previous experiments in our laboratory have shown that mannitol reaches a

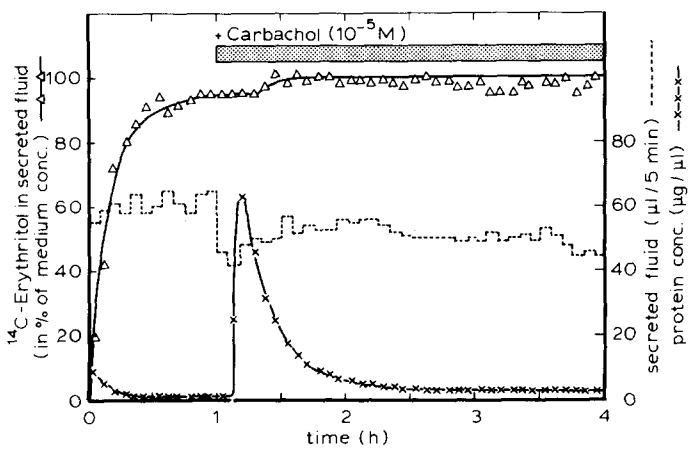

Fig. 2. Permeability of the isolated rabbit pancreas to [^{14}C]erythritol. At $t = 0$ 2 mM [^{14}C]erythritol and at $t = 1$ h 10^{-5} M carbachol are added to the bathing medium. Rate of fluid secretion (— — —), and concentrations of protein (X———X) and [^{14}C]erythritol (△———△) in the secreted fluid are measured. Representative for four experiments.

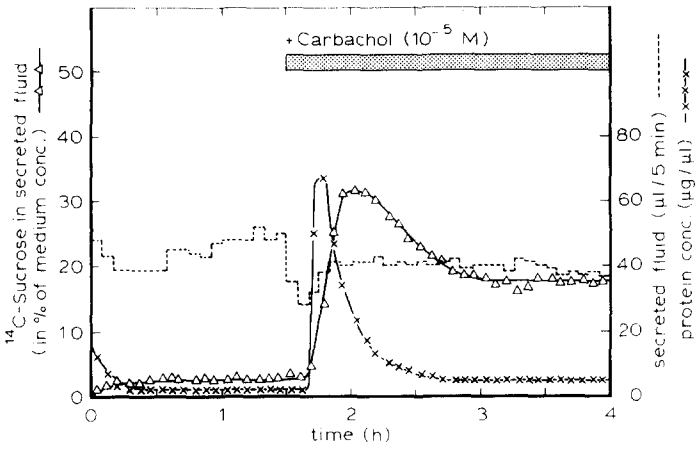

Fig. 3. Permeability of the isolated rabbit pancreas to [^{14}C]sucrose. At t = 0 2 mM [^{14}C]sucrose and at t = 1.5 h 10^{-5} M carbachol are added to the bathing medium. Rate of fluid secretion (— — —), and concentrations of protein (X———X) and [^{14}C]sucrose (△———△) in the secreted fluid are measured. Representative for four experiments.

concentration in the secreted fluid of approx. 60% of that in the bathing medium, which is increased after stimulation with 10^{-5} M carbachol to maximally 70% [10]. This has been confirmed in the present series of experiments (Table I). When [^{14}C]sucrose (Fig. 3) and [^{14}C]lactose are added to the bathing medium, these compounds appear in the secreted fluid with a steady-state concentration of about 4 and 5%, respectively, of that in the bathing medium (Table I). Stimulation with 10^{-5} M carbachol results in a marked increase in the concentrations of these compounds in the secreted fluid. A peak is reached about 35 min after addition of carbachol (i.e. 20 min after the appearance of the enzyme peak). Thereafter the concentration of the radioactive compounds

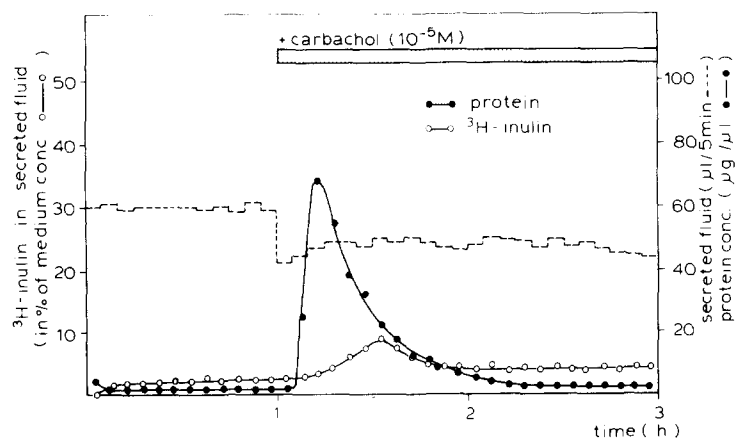

Fig. 4. Permeability of the isolated rabbit pancreas to [^{3}H]inulin. At t = 0 10 µCi [^{3}H]inulin and at t = 1 h 10^{-5} M carbachol are added to the bathing medium. Rate of fluid secretion (— — —), and concentrations of protein (●———●) and [^{3}H]inulin (○———○) are measured in the secreted fluid. Representative for three experiments.

in the secreted fluid decreases again, but remains elevated (Table I). Even inulin (Fig. 4) appears in the secreted fluid (3% relative to the bathing medium), and stimulation with 10^{-5} M carbachol results in a slight increase (up to 8%) of this concentration (Table I).

Dose dependence of the carbachol effects

The observed permeability increase after addition of carbachol is dose dependent. This has been studied for sucrose and lactose. While with 10^{-5} M carbachol a peak in the sugar secretion is observed at 25—40 min after stimulation (cf. Fig. 3), we find with 10^{-6} M carbachol a gradual increase of the sugar secretion to a steady-state level (after approx. 30 min). With 10^{-7} M carbachol no significant increase of either the sugar or the enzyme secretion is observed. This is illustrated in Table II, where the ratios of the sugar concentration in the secreted fluid before and after stimulation with these three carbachol concentrations are shown. The ratios are given for the period of peak permeability (25—40 min after stimulation) as well as for the period of steady-state secretion (60—90 min after stimulation). There appears to be a parallelism between the increase in sugar permeability and the increase in protein secretion.

Effects of other stimulants

When, instead of carbachol, $2 \cdot 10^{-8}$ M secretin is added to the bathing medium, a slight increase in the flow rate is observed, but there is no increase in the sucrose concentration in the secreted fluid. Caerulein ($6 \cdot 10^{-9}$ M) and

TABLE II

EFFECT OF CARBACHOL ON THE CONCENTRATIONS OF SUCROSE, LACTOSE AND PROTEIN IN THE FLUID SECRETED BY THE ISOLATED RABBIT PANCREAS

Period I, 30 min before stimulation; period II, 25—40 min after stimulation; period III, 60—90 min after stimulation. Protein ratio, amount of protein secreted in the 30 min period after to the amount of protein secreted in the 30 min period before stimulation. Values are given with S.E. and the number of determinations (n).

Carbachol concentration (M)	Average sugar concentration ratio		Protein ratio	n
	Period II / Period I	Period III / Period I		
Sucrose				
10^{-5}	6.7 ± 1.4	5.4 ± 0.9	12.6 ± 3.7	4
10^{-6}	1.6 ± 0.1	2.1 ± 0.2	5.9 ± 1.6	4
10^{-7}	1.2 ± 0.1	1.5 ± 0.2	1.2 ± 0.1	2
0	1.3 ± 0.1 *	1.5 ± 0.1 **	1.0 ± 0.1 ***	4
Lactose				
10^{-5}	6.2 ± 0.9	5.5 ± 1.1	10.3 ± 3.5	3
10^{-6}	1.8 ± 0.2	2.3 ± 0.2	7.2 ± 0.1	2
0	1.2 ± 0.1 *	1.7 ± 0.3 **	1.3 ± 0.1 ***	3

* Ratio of the average sucrose concentration 115—130 min to that 60—90 min after starting the experiment.

** Ratio of average sugar concentration 180—210 min to that 60—90 min after starting the experiment.

*** Ratio of the amount of protein secreted in the 90—120 min period of that of the 60—90 min period after starting the experiment.

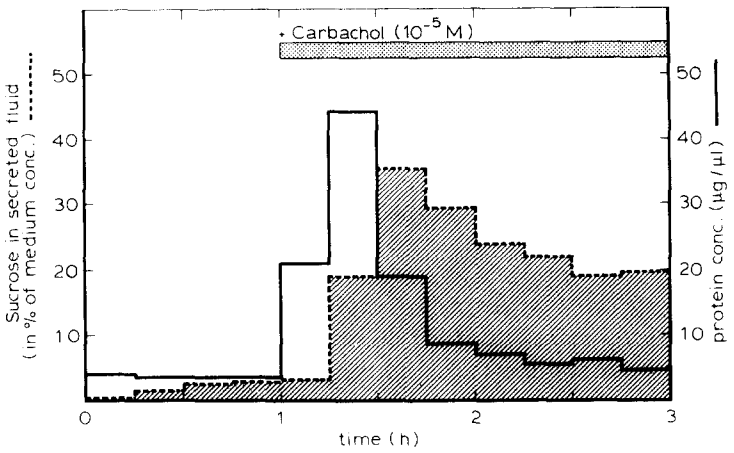

Fig. 5. Permeability of the isolated rabbit pancreas to sucrose. At $t = 0$ 5 mM sucrose and at $t = 1$ h 10^{-5} M carbachol are added to the bathing medium. Concentrations of protein (———) and sucrose (dark area, — — —) in the secreted fluid are measured. Representative for two experiments.

pancreozymin-C-octapeptide ($2 \cdot 10^{-8}$ M) give essentially the same results as 10^{-5} M carbachol.

Check on metabolism of sucrose

In the previous experiments we have added a radioactive compound to the bathing medium and analysed the secreted fluid for radioactivity. We have also checked in one case whether the compounds are metabolized by the tissue and thus that the radioactivity would be due to degradation products of the compound. Two control experiments have been carried out with sucrose, which seemed to be the most likely candidate for metabolism apart from glycerol. First, the secreted fluid has been analysed by thin-layer chromatography. All radioactivity is found in the sucrose spot (R_F 0.38), and no radioactivity is detected in the glucose spot (R_F 0.58). Secondly, non-radioactive sucrose in a 5 mM concentration has been added to the bathing medium and the sucrose content in the secreted fluid has been analysed enzymatically. Essentially the same secretion curve (Fig. 5) is found as when radioactivity is followed in the case of ^{14}C-labelled sucrose (cf. Fig. 3).

Extracellular space

The extracellular space occupied by the tested substances gives an indication how these molecules find their way to the secreted fluid: via an intracellular or an extracellular pathway. The extracellular spaces have been determined in rabbit pancreatic fragments with and without addition of carbachol, and are expressed as percent of total tissue water reached by the radioactive substances (Table III). The spaces occupied by lactose, sucrose and mannitol are hardly or slightly larger than for inulin, a molecule which is considered to be an extracellular marker. The high values for urea, glycerol and erythritol seem to indicate that these substances can penetrate into the cells. The value for glycerol, which is well above 100%, suggests that this compound is metabolized by the cells.

TABLE III

PERCENT OF TOTAL TISSUE WATER REACHED BY RADIOACTIVE COMPOUNDS IN RABBIT PANCREATIC FRAGMENTS

Values are given with S.E. and between parentheses the number of determinations. Carbachol (10^{-5} M) has been used as stimulant and is present during the last 30 min of the 90 min incubation period.

Compound	Unstimulated	Stimulated
Urea	90 ± 3.2 (3)	—
Glycerol	157 ± 7.6 (4)	—
Erythritol	77 ± 4.3 (4)	—
Mannitol	50 ± 4.6 (4)	—
Lactose	40 ± 1.1 (7)	45 + 1.0 (2)
Sucrose	42 ± 2.4 (9)	42 ± 1.7 (6)
Inulin	36 ± 1.0 (11)	37 ± 1.4 (6)

After addition of carbachol there is hardly any increase in the extracellular spaces of the three substances tested (lactose, sucrose and inulin), which suggests that this stimulant does not make cells more permeable. This means that the increased permeability for the marker substances is not due to their uptake in the cells.

Effect of atropine added at various times after stimulation with carbachol

The increase in the permeability of sucrose and other compounds reaches a maximum about 20 min after the appearance of the enzyme peak. We have investigated the relation between the increased sucrose permeability and the enzyme secretion by adding atropine (10^{-4} M) simultaneously with or somewhat later than carbachol (10^{-5} M). Fig. 6 and Table IV show that addition of atropine simultaneously with or 2.5 min after addition of carbachol markedly inhibits the enzyme secretion and abolishes the effect of carbachol on the

TABLE IV

EFFECTS OF ATROPINE (10^{-4} M), ADDED AT VARIOUS TIME INTERVALS AFTER CARBACHOL (10^{-5} M) STIMULATION, ON PROTEIN AND SUCROSE SECRETION BY THE ISOLATED RABBIT PANCREAS

Values are given with S.E. and the number of determinations (n).

Time interval (min)	Amount of protein (mg) *	Sucrose concentration ratio **	n
—	7.9 ± 1.2	6.7 ± 1.4 ***	4
0	1.0 ± 0.4	1.3 ± 0.1	3
2.5	2.4 ± 0.7	1.3 ± 0.1	2
5	6.8 ± 1.7	2.2 ± 0.4	4
10	9.5 ± 1.9	3.2 ± 0.6	3
15	8.6 ± 2.5	3.6 ± 0.5	3

* Amount of protein secreted in the period 0—30 min after stimulation with carbachol.

** Ratio of average sucrose concentration in the period 5—15 after atropine addition to average sucrose concentration in the 30 min period before stimulation with carbachol.

*** Ratio of average sucrose concentration in the period 30—40 min after stimulation with carbachol to that before stimulation.

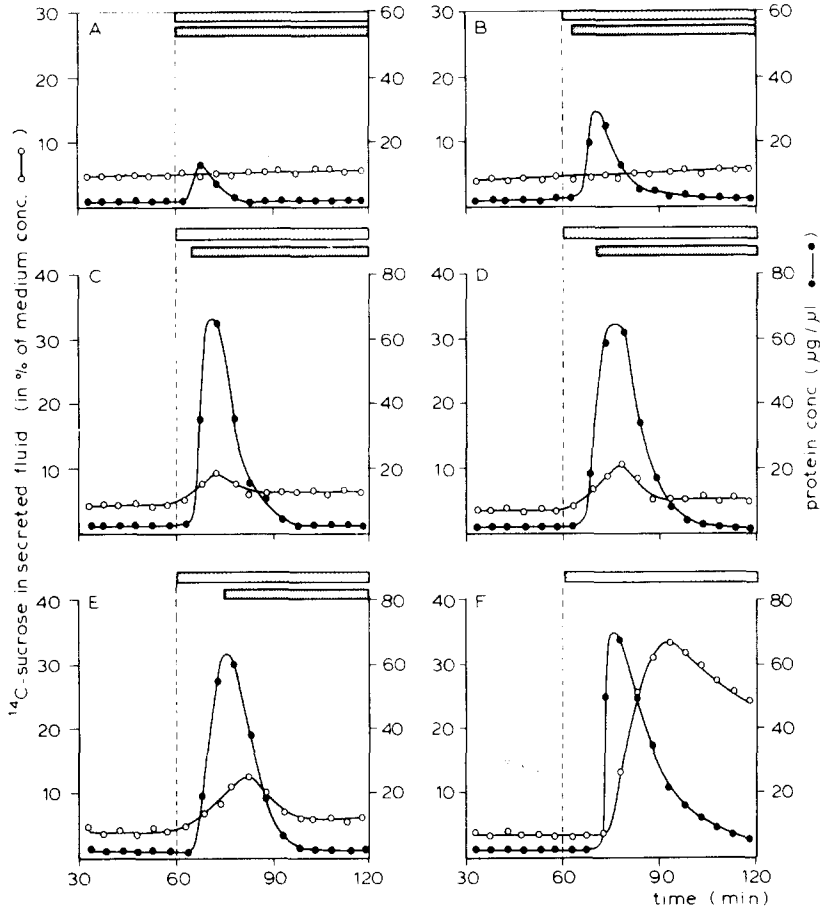

Fig. 6. Effects of 10^{-4} M atropine on protein secretion and sucrose permeability of the isolated rabbit pancreas. Atropine (▣) is given at 0 (A), 2.5 (B), 5 (C), 10 (D) and 15 min (E) after addition of 10^{-5} M carbachol (▣). F represents a control experiment, where no atropine is added to the bathing medium. In the secreted fluid the concentrations of protein (●——●) and [^{14}C]sucrose (○——○) are measured. Representative for 3—4 experiments in each case.

sucrose secretion. However, when atropine is added 5—15 min after addition of carbachol, the enzyme secretion is not much affected any more, while the increase in sucrose permeability is still very much decreased. This suggests that the enzyme secretion process and the increase in the paracellular permeability are affected independently by the interaction of carbachol with its receptor(s).

Discussion

The conductance ratio of the cellular and the extracellular routes is the most reliable criterion for the leakiness of an epithelium [27]. For the pancreas no data are available on this criterion, but the low transtubular potential difference and the isosmotic character of the secreted fluid relative to plasma or bathing medium [2—9] strongly favour the assumption of a leaky epithelium [1].

Considering that the junctional complex between the epithelial cells is probably the most important barrier in the paracellular route, electron microscopic studies also indicate that the pancreas belongs to the class of the relatively leaky epithelia [18,19] on the basis of the classification of junctional complexes of Claude and Goodenough [20]. The latter classification has, however, been disputed [21].

Our functional experiments confirm the results of these morphological studies and give an indication of the molecular size of organic molecules which can pass the barrier between the bathing medium and the lumen in the isolated rabbit pancreas. From our results it appears that urea, and to a lesser extent erythritol and glycerol, can pass this epithelium without difficulty. Since these compounds also penetrate the intracellular space, their main transport route cannot be determined. Mannitol shows an intermediate behaviour, but lactose, sucrose and inulin do not enter the intracellular space. Hence we may conclude that the latter three substances reach the lumen only by way of the paracellular route.

Dewhurst et al. [22] have recently investigated the non-electrolyte permeability of the isolated perfused cat pancreas. They added a number of non-electrolytes to the perfusate in a 0.1 M concentration and compared the reduction in fluid secretion with that obtained by an equal concentration of sucrose. Assuming that sucrose is not permeable at all, they calculated the apparent reflexion coefficient for a number of compounds. They found that molecules with a molecular volume equal to or larger than arabinose are not permeable, whereas smaller molecules are more permeable the smaller their molecular volume. Quantitatively there are a number of important differences between their observations and ours. Whereas in our study urea, glycerol and erythritol are 88—99% permeable, in their study there is a large difference between the apparent reflexion coefficients of these three compounds. Whereas in our study mannitol is permeable for 59% and sucrose for 3.5%, in their study both compounds are not permeable. Whether the observed difference is due to the difference in species or preparation or to a methodical difference cannot yet be established.

In addition to these uncharged substances, ions can also pass through the pancreatic epithelium via the paracellular pathway. In view of the equality of the potassium concentration in the perfusate and the secreted fluid of the cat pancreas, Case and Scratcherd [12] suggest that potassium diffuses passively from the extracellular fluid to the pancreatic fluid via a paracellular pathway. Previous experiments in our laboratory indicate that Ca^{2+} and Mg^{2+} also pass to the lumen via a paracellular transport route [10,11].

In two recent publications [10,11] we have reported that after stimulation of the enzyme secretion of the isolated rabbit pancreas with 10^{-5} M carbachol the concentrations of ^{28}Mg, ^{45}Ca and [^{3}H]mannitol in the secreted fluid are increased, reaching peak values about 40 min after stimulation. In the present paper we confirm the results for mannitol [10], and in addition we now observe the same phenomenon for lactose, sucrose and inulin. Since the latter three substances do not significantly enter the cells after stimulation, we conclude that during stimulation of the enzyme secretion with carbachol the permeability of the paracellular route is increased.

The molecular size of the molecules, which can pass the barrier between plasma or bathing medium and lumen is limited. Inulin (mol. wt. approx. 5000) can pass this barrier, and its permeability is slightly increased after stimulation with 10^{-5} M carbachol. By means of histochemical techniques it has been shown that horseradish peroxidase (M_r = 40 000) can pass the paracellular route between the acinar cells of the submondibular gland after stimulation with adrenalin [23], while ferritin (M_r = 250 000) is unable to pass the junctional complexes both in unstimulated and in pilocarpine-stimulated rat pancreas [24].

In this respect, it is interesting to consider the recent finding of Isenman and Rothman [25] that amylase (M_r = 45 000) appears in the bathing medium of the isolated rabbit pancreas. They attribute this to leakage of the enzyme across the basolateral membrane of the acinar cells. Their findings can, however, equally well be interpreted as due to leakage of the enzyme from lumen to bath through the extracellular pathway. Its molecular size would seem to be small enough to permit such leakage. In their experiments there is always a 60—400-fold concentration gradient for the enzyme between lumen and bath. Their observation that lactate dehydrogenase, a normal cytoplasmic enzyme, does not appear in the bathing medium is also consistent with this, since this enzyme is not secreted into the lumen. The increased amylase concentration in the bath after stimulation with acetyl-β-methylcholine chloride is also in accord with the increased permeability of the paracellular pathway after carbachol addition in our experiments. The absence of an increased amylase concentration in the bath after stimulation with pancreozymin may be due to their use of a low pancreozymine concentration ($5 \cdot 10^{-9}$ M), since they also find only a small effect on the ductal amylase concentration in this case.

The question has been considered whether the two phenomena, enzyme secretion and paracellular permeability, are coupled processes. At first sight, the dose dependence of both phenomena for carbachol and the stimulation of both effects by caerulein and pancreozymin-C-octapeptide as well as by carbachol might suggest that they are coupled. However, we have found multiple evidence against this. The permeability effect occurs 15—20 min later than the enzyme secretion peak. When atropine, an antagonist of carbachol, is added 5 min or more after addition of carbachol, a normal enzyme peak is obtained, while the sucrose permeability is still inhibited. Moreover, we have recently observed that upon addition of 10 mM 2,4,6-triaminopyrimidine to the bathing medium of the isolated rabbit pancreas normal stimulation of the enzyme secretion is obtained with 10^{-5} M carbachol after 1 h, while the relative sucrose concentration in the secreted fluid hardly increases (Jansen et al., to be published). Another indication that these two processes are uncoupled is shown by our previous observation [10] that repeated stimulation of the isolated rabbit pancreas with acetylcholine hardly results in a second enzyme peak but does lead to an increased $^{45}Ca^{2+}$ concentration in the selected fluid. These observations lead us to the conclusion that stimulation of the enzyme secretion is not responsible for the increase in paracellular permeability, but that stimulation of both processes can proceed through activation of the acetylcholine receptor(s).

The above findings suggest that under certain conditions the epithelial paracellular pathway increases in permeability. This raises the question of the

physiological significance of this effect. If the increased permeability represents a normal secretion route, this could provide a mechanism whereby, besides small proteins, the role of which is not clear, substances such as Ca^{2+} can reach the lumen. The extra amount of calcium, thus secreted upon stimulation of the enzyme secretion, could be important for the biological activity of the digestive enzymes in the intestine.

There remain, however, some unsolved problems. One problem is why the radioactive markers reach a maximum concentration in the secreted fluid only 35 min after addition of 10^{-5} M carbachol. Subtracting from this value the 15—20 min time required for transport, then there still remains a gap of 15—20 min. The longer pathway and the lower concentration gradient compared to those for the secreted protein might be responsible to some extent, but no data in support of this suggestion are available. The continuing elevated secretion of the radioactive substances into the pancreatic fluid after the transient peaks may well be an artifact of the isolated pancreas preparation in the sense that the permeability increase of the junctional complexes is not reversed in this preparation.

Another unsolved problem is the location of the paracellular pathway. After duct ligation in the rat pancreas the strands of the junctional complexes between the acinar cells are seen to be disarranged [26]. Permeability of both ductal and acinar junctional complexes of the submandibular gland to horse-radish peroxidase, injected either into the ductal lumen [27,28] or arterially [23], has been reported. In rat parotid gland ductal injection of horse-radish peroxidase at low pressure leads to penetration of the acinar junctional complex only after stimulation with isoproterenol [29], but no information is given about the ductal junctional complex. Hence, neither from our experiments, nor from other published data is it possible to conclude whether the pathway is located between the acinar or the ductal cells or both.

Acknowledgements

The excellent technical assistance of Miss A.M.M. Jakobs is gratefully acknowledged. This investigation was supported in part by the Netherlands Organization for the Advancement of Basic Research (Z.W.O.), through the Netherlands Biophysics Foundation.

References

1 Frömter, E. and Diamond, J. (1972) Nat. New Biol. 235, 9—13
2 Schulz, I., Yamagata, A. and Weske, M. (1969) Pflügers Arch. 308, 277—290
3 Swanson, C.H. and Solomon, A.K. (1973) J. Gen. Physiol. 62, 407—429
4 Way, L.W. and Diamond, J.M. (1970) Biochim. Biophys. Acta 203, 298—307
5 Greenwell, J.R. (1977) Pflügers Arch. 367, 265—270
6 De Zilwa, L.A.E. (1904) J. Physiol. (London) 31, 230—233
7 Gilman, A. and Cowgill, G.R. (1933) Am. J. Physiol. 104, 476—479
8 Case, R.M., Harper, A.A. and Scratcherd, T. (1968) J. Physiol. (London) 196, 133—149
9 Rothman, S.S. and Brooks, F.P. (1965) Am. J. Physiol. 208, 1171—1176
10 Schreurs, V.V.A.M., Swarts, H.G.P., De Pont, J.J.H.H.M. and Bonting, S.L. (1975) Biochim. Biophys. Acta 404, 257—267
11 Schreurs, V.V.A.M., Swarts, H.G.P., De Pont, J.J.H.H.M. and Bonting, S.L. (1976) Biochim. Biophys. Acta 436, 664—674

12 Case, R.M. and Scratcherd, T. (1974) J. Physiol. (London) 242, 415—428
13 Rothman, S.S. (1964) Nature 204, 84—85
14 Bray, A. (1960) Anal. Biochem. 1, 279—285
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
16 Bergmeyer, H.U. and Bernt, E. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), Vol. 3, pp. 1176—1179, Verlag Chemie, Weinheim/Academic Press Inc., New York
17 Augustus, J., Bijman, J., van Os, C.H. and Slegers, J.F.G. (1977) Nature 268, 657—658
18 Galli, P., Brenna, A., De Camilli, P. and Meldolesi, J. (1976) Exp. Cell Res. 99, 178—183
19 Metz, J., Forssman, W.G. and Ito, S. (1977) Cell Tissue Res. 177, 459—474
20 Claude, P. and Goodenough, D.A. (1973) J. Cell Biol. 58, 390—400
21 Mollgard, K., Malinowska, D.H. and Saunders, N.R. (1976) Nature 264, 293—294
22 Dewhurst, D.G., Hadi, N.A., Hutson, D. and Scratcherd, T. (1978) J. Physiol. (London) 277, 103—114
23 Parsons, P.A., Klinger, A.H. and Garrett, J.R. (1977) Histochem. J. 9, 419—433
24 Geuze, J.J. and Poort, C. (1973) J. Cell Biol. 57, 159—174
25 Isenman, L.D. and Rothman, S.S. (1977) Proc. Natl. Acad. Sci. U.S. 74, 4068—4072
26 Metz, J., Merlo, M., Billich, H. and Forssman, W.G. (1978) Cell Tissue Res. 186, 227—240
27 Garrett, J.R. and Parsons, P.A. (1976) Histochem. J. 8, 177—189
28 Parsons, P.A. and Garrett, J.R. (1977) Med. Biol. 55, 249—260
29 Oliver, C. and Hand, A.R. (1978) J. Cell Biol. 76, 207—220
30 De Pont, J.J.H.H.M., Jansen, J.W.C.M. and Bonting, S.L. (1978) Acta Physiol. Scand. Special Suppl. 373—380