ION TRANSPORT REGULATION IN INTESTINAL BRUSH BORDER MEMBRANES

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REGULATIE VAN IONENTRANSPORT IN BORSTELZOOMMEMBRANEN VAN DARMEPITHEEL

PROEFSCHRIFT

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Voor mijn ouders
Voor Stytia

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CONTENTS

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LIST OF ABBREVIATIONS

AC	adenylate cyclase
ADP	adenosine 5′-diphosphate
ATP	adenosine 5′-triphosphate
BB	brush border caps
BBM	brush border membrane
BBMV	brush border membrane vesicle
BLM	basolateral membrane
CaM	calmodulin
cAMP	adenosine cyclic 3′,5′-monophosphate
cGMP	guanosine cyclic 3′,5′-monosphosphate
CT	cholera toxin
CTP	cytidine 5′-triphoshate
DAG	1,2-diacylglycerol
DIDS	4,4′-diisothiocyanato stilbene-2,2′-disulfonic acid
2,3-DPG	2,3-diphosphoglycerate
ED_{50}	concentration giving half-maximal effect
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis (β-amino ethyl ether) N,N,N′,N′-tetraacetic acid
ER	endoplasmic reticulum
G_i	inhibitory GTP binding protein
G_s	stimulatory GTP binding protein
G_o	a GTP binding protein of unknown function discovered in the brain
G_p	a GTP binding protein regulating PIP_2 hydrolysis
GC	guanylate cyclase
Gpp(NH)p	guanyl-5′-yl $\beta\gamma$-imidodiphosphate
GTP	guanosine 5′-triphosphate
GTPγS	guanosine 5′-(3-O-thio)triphosphate
Hepes	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
HPTLC	high performance thinlayer chromatography
IP	inositolphosphates
IP_1	inositolmonophosphate
IP_2	inositolbisphosphate

IP_3	inositoltrisphosphate
I_{SC}	short-circuit current
IC_{50}	concentration giving half-maximal inhibition
K_D	dissociation constant
K_i	inhibitory constant
K_m	Michaelis-Menten constant
LT	E.coli heat-labile toxin
M	mucosa
P	permeability
^{32}P	$[^{32}P]H_2PO_4^-$
PA	phosphatidic acid
PDB	4β phorbol 12,13-dibutyrate
PI	phosphatidylinositol
PIP	phosphatidylinositol 4-phosphate
PIP_2	phosphatidylinositol 4,5-bisphosphate
PK-A	cAMP-dependent protein kinase
PK-C	Ca^{2+}/phospholipid-dependent protein kinase
PK-G	cGMP-dependent protein kinase
PMA	4β phorbol 12-myristate,13-acetate
S	serosa
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
SITS	4-acetamido-4'isothiocyanatostilbene-2,2'-disulfonate
ST	E.coli heat-stable toxin
TFP	trifluoperazine
Tris	tris (hydroxymethyl)aminomethane
VIP	vasoactive intestinal polypeptide

CHAPTER I

GENERAL INTRODUCTION

WATER AND SALT TRANSPORT IN THE SMALL INTESTINE

In human small intestine 80-90% of the orally ingested and internally secreted water and salt are absorbed, together with nutrients like sugar, proteins and fat and other compounds. Another 10-20% of the water is absorbed in the colon while only 1-3% leaves the body unabsorbed with the faeces [1].

The small intestine is a so-called leaky epithelium which means that it has a relatively low paracellular electrical resistance compared to the so-called tight epithelia, like distal colon and urinary bladder [2]. Other leaky epithelia are for example the proximal tubule of the kidney and the gall bladder [2]. Leaky epithelia are relatively permeable to salts but especially to water, which precludes the formation of large chemical or osmotic gradients across the epithelial barrier. This also implies that the uptake of water is osmotically coupled to the absorption or secretion of salt across the tissue [2].

In a number of leaky epithelia like ileum, gall bladder and flounder intestine the absorption of Na^+ and Cl^- is coupled by an electrically neutral mechanism [3]. In tissues with a low electrical resistance this is far more efficient than the electrical coupling between Na^+ and Cl^- as observed in tight epithelia, like distal colon. In the latter tissue the influx of Na^+ into the cell down its electrochemical gradient occurs trough a Na^+ channel and results in a large transepithelial potential difference, which promotes the absorption of Cl^-.

The neutral absorption of NaCl in mammalian ileum probably results from a combined action of a Na^+/H^+ exchanger and a Cl^-/HCO_3^- exchanger in the apical membrane which are coupled by the intracellular pH [4-6]. In rabbit and Necturus gall bladder the simultaneous presence of a single Na^+-Cl^- carrier and Na^+/H^+ and Cl^-/HCO_3^- exchangers has been described [7,8] while in the intestine of the winter flounder the neutral absorption of salt is performed by a bumetanide-sensitive Na^+-K^+-Cl^--cotransporter [9]. In all cases the electrical chemical gradient of Na^+ across the membrane of the epithelial cells, generated by the Na^+/K^+ pump in the basolateral membrane, provides the main driving force promoting NaCl absorption [3].

Conversely, the transport of Na^+ and Cl^- in jejunum does not seem to be obligatory coupled, but at least part of the Na^+ is transported presumably by a Na^+/H^+ exchanger, which through proton extrusion stimulates the absorption of HCO_3^- [10].

That the small intestine is not only able to absorb but may also actively secrete salt and water, is dramatically shown in cases of secretory diarrhoea as exemplified by patients infected with Vibrio cholerae bacteria. In this pathological state the patients may loose several litres of isotonic fluid per day into their intestine and die of dehydration if the loss of water and salt is not compensated by intraveneous infusion or oral rehydration [11]. The secretion of water is secondary to an electrogenic hyper secretion of Cl^- into the intestinal lumen provoked by cholera toxin [3]. Quite the opposite phenomenon is seen in the genetic disease cystic fibrosis in which secretion of Cl^- seems to be impaired not only in the lungs, where it results in a thick and viscous mucus, but also in the intestine [12]. The lack of secretion in the intestine however does not lead, except from some constipation, to a major problem in the functioning of the intestine, provided that an pancreatic enzyme preparation is administered to compensate for the malfunctioning of the exocrine pancreas. This implies that a hyper secretion is potentially more dangerous than a hypo secretion in the intestine.

The net flow of water and salt in the intestine is determined by the balance between the absorption and secretion across this tissue. Both processes are found to be under a strict neurohormonal control [13].

MODULATION OF SALT TRANSPORT IN SMALL INTESTINE BY NEUROHUMORAL SUBSTANCES AND BACTERIAL TOXINS. GENERATION OF SECOND MESSENGERS

A number of hormones and neurotransmitters like VIP, substance P, acetylcholine and epinephrine, but also substances involved in an inflammatory reaction like prostaglandins and bradykinin and some bacterial toxins like cholera toxin (CT) and E. coli heat-stable toxin (ST) are all capable of modulating salt and water transport across small intestinal epithelium [13]. In general, following binding to their specific receptors on the surface membrane of the enterocyte, these substances activate or inhibit a system which generates intracellular (so-

called "second") messengers. These second messengers then directly or indirectly modulate salt transporters (Na^+, Cl^-,HCO_3^- and K^+) in the epithelial membranes.

It is now generally accepted that at least the following second messengers may play a role in the regulation of salt transport in the enterocyte: adenosine cyclic 3',5'-monophosphate (cAMP) [3], guanosine cyclic 3',5'-monophosphate (cGMP) [14], ionized Ca^{2+} [15], 1,2-diacylglycerol (DAG) [16] and inositol 1,4,5-trisphosphate (IP_3) [17-19]. In Fig. 1 a number of substances affecting salt transport in the small intestine and the various mechanisms by which they influence the levels of second messengers are summarized.

- cAMP is generated from ATP by the enzyme adenylate cyclase (AC) which is located in the basolateral membrane of the enterocyte [20]. The activity of its catalytic subunit is controlled by two GTP binding proteins G_s and G_i which respectively stimulate and inhibit AC and which may also interact with protein receptors for secretagogues and anti-secretory agents, respectively [21]. The A_1 subunit of CT and E. coli heat-labile toxin (LT) can irreversibly activate the AC through ADP-ribosylation of G_s [22].
- cGMP is formed from GTP by the enzyme guanylate cyclase (GC). The major guanylate cyclase activity in the enterocyte is associated with a specific, particulate isoenzyme which is confined to the apical membrane and which is activated, probably by formation of mixed disulfide bridges, in response to ST [23-25], but not to atrial natriuretic factor (ANF) [25], a known activator of particulate GC in a number of other tissues [26].
- DAG and IP_3 are generated by the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2)in the epithelial membrane by a specific phospholipase C. Studies of signal transduction in other tissues have recently provided evidence for the existence of a GTP binding protein different from G_s or G_i (called G_p) acting as a coupling factor between hormone receptors and PIP_2-specific phospholipase C.[27,28]. This system is formally not yet demonstrated in the enterocyte but is likely to be present in the basolateral membrane in view of our observation that secretagogues which are known to activate this pathway in other tissues (e.g. acetylcholine, bradykinin) only modulate the transport of salt in small intestine

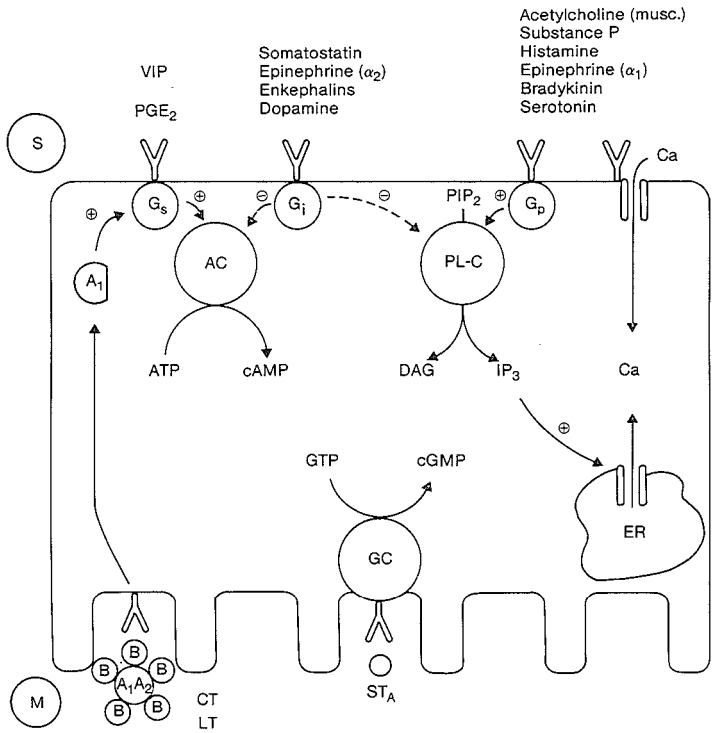

Figure 1.
Transmembrane signalling of hormones, neurotransmitters and toxins capable of modulating transepithelial transport of salt and water.

PIP_2: phosphatidylinositol-4,5-bisphosphate
PL-C: phosphatidylinositolphosphate diesterase
CT : choleratoxin (A_1, A_2, B: toxin subunits)
AC : adenylate cyclase
VIP : vasoactive intestinal peptide
G_S, G_i and G_p: GTP binding proteins
S : serosa
: receptor

ST_A : E.coli heat-stable toxin (A)
LT : E.coli heat-labile toxin
GC : guanylate cyclase
ER : endoplasmic reticulum
PGE_2: prostaglandin E_2
M : mucosa
: Ca^{2+} channel

when administered from the serosal side (De Jonge unpublished observations).

- Ca^{2+} levels in the enterocyte which are believed to be around 10^{-7} M in the basal state can be raised by opening of receptor mediated Ca^{2+} channels in the basolateral membrane [28], but also by release of Ca^{2+} from intracellular stores (presumably the ER) triggered by IP_3 [16-19,27,28]. Intracellular Ca^{2+} may therefore serve as a third messenger for hormones or neurotransmitters which stimulate the PIP_2-specific phospholipase C and generate IP_3 signals in the enterocyte.

Intact intestine or stripped mucosa mounted in Ussing chambers is a heterogeneous tissue containing a number of cell types capable of producing compounds which can modulate salt transport across the epithelial barrier, including enterochromaffin cells, which can secrete serotonin [29], neurons which may generate VIP, acetylcholine or substance P, and various other subepithelial cells, which can produce for example prostaglandins and histamine [30]. Therefore the effect of various (anti)secretagogues on salt transport in vivo or in Ussing chambers may be enhanced or totally mediated by their interaction with endocrinic, paracrinic or neuronal cell types rather than with the enterocyte itself. According to Lundgren [31] a large part of the secretory response of the small intestine to cholera toxin in vivo could be blocked with the inhibitor of nerve conduction tetrodotoxin and thus is most plausible mediated by a nervous reflex involving serotonin and VIP. In stripped proximal colon of the rat mounted in Ussing chambers, the secretion induced by Ca^{2+}-ionophore A23187 could be largely blocked by indomethacin, an inhibitor of prostaglandin synthesis (De Jonge unpublished results), and is therefore likely to be mediated by the release of prostaglandins from the subepithelium, the major source of prostaglandin synthesis in small intestine [30].

TRANSPORT SYSTEMS AFFECTED BY THE SECRETAGOGUES

Electrophysiological studies with intestinal epithelium in Ussing chambers have revealed two major effects of secretagogues on Na^+ and Cl^- transport: (i) an inhibition of the unidirectional mucosa to serosa (m+

s) fluxes of $^{22}Na^+$ and $^{36}Cl^-$ and (ii) an increase in the serosa to mucosa (s → m) unidirectional flux of $^{36}Cl^-$ accompanied by a rise in short circuit current (I_{SC}) [32]. Field et al. have interpreted this dual effect by proposing a different action of the secretagogues on villus and crypt compartments of the intestine [32]. According to this model the rise in s → m flux of Cl^- and in I_{SC} is caused by a secretagogue-induced opening of Cl^- channels in the apical membrane of the crypt cells, whereas the decrease in m → s fluxes of Na^+ and Cl^- results from an inhibition of Na^+-Cl^- cotransporters in the apical membrane of the villus cell. Naftalin et al [33] have postulated a different model to explain the dual action of the secretagogues, based on the contraction of the lateral intercellular space during a state of secretion which causes a back flux of absorbed Na^+ and Cl^- into the mucosal solution, resulting in an apparent decrease in m → s fluxes of $^{22}Na^+$ and $^{36}Cl^-$. At variance with the Field model, the increase in s → m flux of Cl^- and the rise in I_{SC} were explained by the opening of apical Cl^- channels in both villus and crypt cells. Studies to define the anatomical site of active water and salt secretion by means of an oil overlay technique and membrane potential and resistance measurements with microelectrodes [34] clearly showed that in rabbit colon only the crypt, but not the surface epithelium, is capable of active secretion of Cl^- and water. In other epithelia, however, e.g. rabbit and Necturus gall bladder, secretagogues simultaneously inhibit Na^+-Cl^--cotransport and provoke electrogenic Cl^- secretion despite the lack of a crypt compartment in these tissues [7,35]. In flounder intestine, an epithelium which also lacks crypts, the intracellular messenger cGMP inhibits the Na^+-K^+-Cl^--cotransporter [36]. Although the absence of secretagogue-activated Cl^- channels in small intestinal villus cells is not definitely proven experimentally, Field's model has become generally accepted particularly for the ileum where the presence of a coupled Na^+-Cl^- influx mechanism has been clearly demonstrated. In the jejunum, which does not seem to posses a Na^+-Cl^- cotransport system [10], the inhibition of Na^+ and Cl^- absorption by secretagogues is less firmly established. De Jonge [37] was not able to detect an inhibition of m → s flux of Cl^- or Na^+ in ileo-jejunal segments of rats treated with cholera toxin <u>in vivo</u>. Similarly Hardcastle et al. could find an inhibition of m → s fluxes of Na^+ and Cl^- in stripped rat jejunum <u>in vitro</u> after incubation with acetylcholine, but not in response to dibutyryl cAMP [38]. In cat jejunum

in vivo no effect of CT on absorption of Na^+ in the villus tip could be detected [39]. In alle these studies however a net secretion of fluid and salt in response to CT was clearly observed.

TRANSPORT SYSTEMS AFFECTED IN THE CRYPT

The postulated effects of second messengers on the transport mechanisms for salt in the crypt cells are illustrated in Fig. 2. This model is mainly derived from studies of colonic and tracheal epithelial cells, but is also thought to be applicable to small intestine [40]. In the crypt cell cAMP, cGMP and Ca^{2+} signals provoke both the activation of a Cl^- channel in the apical membrane and of a K^+ channel in the basolateral membrane. Chloride which is pumped up above its electrochemical equilibrium by a bumetanide-sensitive Na^+-K^+-Cl^-- cotransporter in the basolateral membrane, will leave the cell by this Cl^- channel. In order to maintain a more permanent secretion of Cl^-, the K^+ which is also taken up by the Na^+-K^+-Cl^--cotransporter must recycle across the basolateral membrane through the activated K^+ channel. More importantly the opening of this Ba^{2+}-sensitive K^+ channel hyperpolarizes the membrane and counteracts the depolarization caused by the apical secretion of Cl^-. The Na^+ entering the cell across the basolateral membrane together with Cl^- and K^+ will be extruded across the same membrane through the action of the Na^+/K^+-pump [40, review]. Studies on the colonic carcinoma cell line T84 [41] and on stripped colonic mucosa treated with the prostaglandin synthesis inhibitor indomethacin [42] strongly suggest that at least in this tissue Ca^{2+} does not directly activate Cl^- channels in the apical membrane but only activates K^+ channels in the basolateral membrane while cAMP has both effects. It is not yet certain whether the K^+ channels in the basolateral membrane activated by Ca^{2+} and cAMP are identical [41,42].

TRANSPORT SYSTEMS AFFECTED IN THE VILLUS

The major effect of the second messengers in the villus cell is thought to be the inhibition of an electroneutral Na^+-Cl^--cotransport system in the brush border membrane. As have been clearly demonstrated in studies of

salt transport in membrane vesicles isolated from rat and rabbit intestine, absorption of Na^+ and Cl^- is presumably carried out by the combination of a Na^+/H^+ exchanger and a Cl^-/HCO_3^- exchanger coupled by circular proton movements [5]. It can however not be excluded yet that a single Na^+-Cl^-- or Na^+-K^+-Cl^--cotransporter detected in other leaky epithelia could also play an additional role in the modulation of salt transport in the small intestine. Such carriers may be silent in isolated brush border membranes vesicles (BBMV) from small intestine due to an inappropriate osmotic environment (cf. Ref. 43). Inhibition of the Na^+/H^+ exchange, one branch of the Na^+-Cl^--cotransport system, by cAMP has been already demonstrated in rabbit gall bladder [44], rabbit renal proximal tubule [45], a renal OK cell line [46] and chicken villus enterocytes [47].

Phorbol 12,13-dibutyrate (PDB), a non-degradable analogue of DAG was shown to inhibit Na^+/H^+ exchange in rabbit proximal colon [48]. This action is opposite to its effect in many non-epithelial tissues in which phorbol esters (PhE) cause a stimulation of the Na^+/H^+ exchange, which is followed by a rise in intracellular pH and may play a role in the regulation of cell growth [49].

These findings indicate that in the small intestinal villus cell the Na^+/H^+ exchange is the most plausible site for inhibition of neutral Na^+Cl^--cotransport. In the distal tubule of the kidney however, cAMP was found to exert part of its effects through a modulation of Cl^-/Cl^- exchange [50] which presumably reflects the action of a Cl^-/HCO_3^- exchanger.

Studies of NaCl uptake in BBMV from rabbit ileum have shown an inhibition of a Na^+Cl^--cotransport system by Ca^{2+} and calmodulin but did not reveal whether a Na^+/H^+ or Cl^-/HCO_3^- exchange or a single Na^+-Cl^--cotransporter was modulated [51].

MECHANISM OF MODULATION OF TRANSPORT SYSTEMS BY SECOND MESSENGERS IN INTESTINE

The main intracellular action of the second messengers cAMP, cGMP and DAG in mammalian tissues is to activate specific protein kinases which in

turn can modulate the activity of enzymes, transport carriers or channels by covalent attachment of a phosphate group [52]. Ca^{2+} may also act at least partially by stimulation of Ca^{2+}-dependent kinases or phosphatases leading to a change in the phosphorylation state of specific target proteins; in addition, it may act more directly by binding to the transport protein itself or to a regulatory Ca^{2+} binding protein (e.g. calmodulin) associated with the transport protein, and inducing a conformational change which leads to a change in transport activity [28,52]. There are as yet a few examples of a transport system allosterically activated by cGMP not involving protein phosphorylation, e.g. the Na^+ channel in the retinal cells [53], while there are a number of Na^+ and K^+ channels which are directly regulated by Ca^{2+} [54,55]. An obligatory role of protein phosphorylation in the regulation of salt transport in intestine by cAMP and cGMP has been confirmed recently for rat proximal colon (De Jonge, unpublished results) and winter flounder [56] by showing a reversal of cyclic nucleotide induced changes in intestinal ion transport by the isoquinolinesulfonamide H8, a potent inhibitor of cAMP- and cGMP-dependent protein kinases in vitro.

Phosphorylation studies with [γ-^{32}P]ATP and immunoblotting of apical membrane and cytosol proteins, isolated from intestinal cells have demonstrated the presence of cAMP-, cGMP-, Ca^{2+}/calmodulin- and Ca^{2+}/phospholipid-dependent protein kinases [57-59]. Two isoenzymes of cAMP-dependent protein kinase (type I and II) occur in most mammalian tissues, each composed of catalytic subunits and regulatory subunits, which become dissociated upon binding of cAMP to the regulatory subunits. The type II cAMP-dependent kinase, which differs from the type I only in the regulatory subunits but not in its catalytic subunits, is located predominantly in the microvillus membrane of the enterocyte and remains associated with BBMV prepared by a freeze-thaw procedure [60]. It is however not tightly bound to the apical membrane because it can be largely removed from isolated brush border caps by repeated washings with EDTA [58]. As judged by its localization in the apical membrane the cAMP-dependent protein kinase type II is a better candidate as a regulator of intestinal salt transport than the type I enzyme, which is enriched in the cytosolic fraction of the enterocyte. It can however not be ruled out that the catalytic subunit released from the type I regulatory subunit in

response to a cAMP signal migrates to the brush border and subsequently modulates salt transport in the apical membrane.

The major isoenzyme of cGMP-dependent protein kinase in intestinal cells is located predominantly in the apical membrane and clearly differs in its structural and functional properties from the soluble and particulate forms of cGMP-dependent kinase found in other mammalian cells [57,58,61,62]. It has a molecular weight of 86 kDa, is tightly bound to both the brush border membrane and the cytoskeleton and can be autophosphorylated *in vitro* in the presence of cGMP or relatively high concentrations of cAMP [57,58,61,62].

Ca^{2+} can activate both a Ca^{2+}/calmodulin-dependent kinase in isolated brush borders and a Ca^{2+}/phospholipid-dependent kinase present in brush border and cytosolic fractions of the enterocyte [58]. In intact cells however, DAG released by phospholipase C action on membranal phospholipids, is likely to function as the main physiological activator of the enzyme (now commonly named protein kinase C).

All these kinases are capable of phosphorylating one or more substrate proteins in the apical membrane as revealed by *in vitro* phosphorylation studies with [γ-^{32}P]ATP [see Refs. 57-59]. In view of their phosphorylation by multiple endogenous protein kinases and their possible key role in a regulatory pathway shared by two or more second messengers, at least three phosphoproteins are of special interest, i.e. (i) a 25 kDa protein, which is cophosphorylated by cGMP- and cAMP-dependent protein kinase; this protein can be extracted from the brush border by acid-chloroform-methanol [25] and in this respect resembles the 23 kDa regulator of Ca^{2+}-ATPase in heart sarcoplasmic reticulum known as phospholamban [63] (ii) a 135 kDa protein which is phosphorylated by cAMP- and Ca^{2+}/calmodulin-dependent protein kinase and (iii) a 28 kDa protein which acts as a substrate for Ca^{2+}/calmodulin-dependent and C-kinase.

In vivo phosphorylation studies in rat intestinal epithelium prelabeled by intraluminal injection of inorganic ^{32}P did not reveal any difference between sham-operated and CT- or ST-treated jejunal segments (De Jonge, unpublished results), but ^{32}P-preloaded rabbit ileal mucosa displayed a stimulation of ^{32}P incorporation into a 32 kDa and a 52 kDa protein in response to both Ca^{2+}-ionophore and theophylline [64]. Sofar a causitive role in ion transport regulation across the apical membrane has not yet

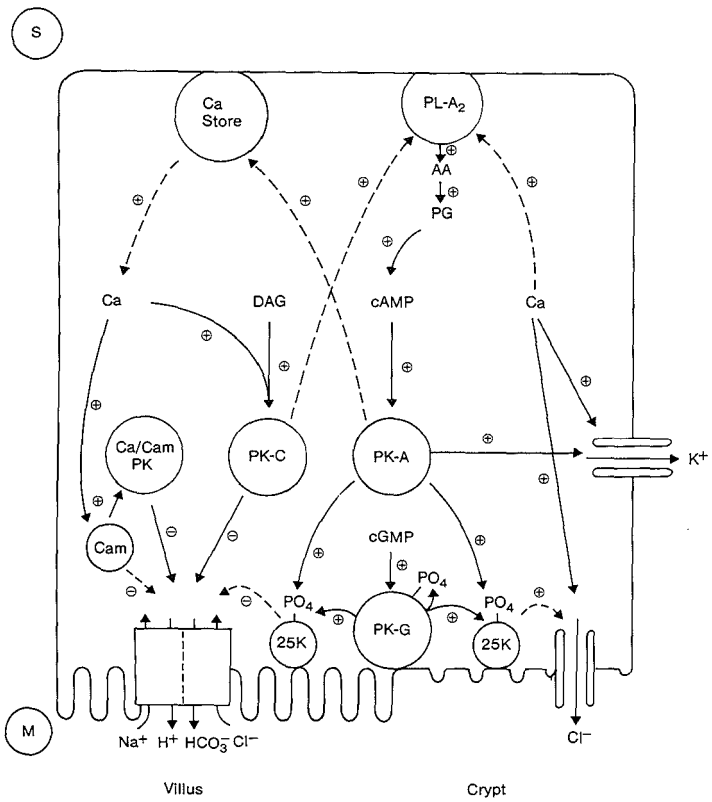

Figure 2.
Hypothetical scheme for the mechanism of action of second messengers in the enterocyte.

Ca^{2+}/CaM-PK: Ca^{2+}/CaM dependent protein kinase
PK-A : cAMP dependent protein kinase
CaM : calmodulin
$PL-A_2$: phospholipase A_2
AA : archidonic acid
PG : prostaglandins
---- : hypothetical effects
S : serosa
: channel

PK-C : Ca^{2+}/phospholipid dependent protein kinase
PK-G : cGMP dependent protein kinase
25 K : substrate protein for PK-G and PK-A
The generation of prostagandins in response to DAG and Ca^{2+} is probably not located in the enterocyte itself but in the subepithelial layer
——: established effects
M : mucosa

been established for any of the phosphoproteins identified in the in vitro or in vivo phosphorylation studies.

As mentioned before the various second messengers are likely to affect similar or identical transport systems. It is therefore likely that the regulatory pathways activated by each messenger converge at the level of the transport protein itself or at an earlier step. In the first case it is possible that: (i) cyclic nucleotide-dependent phosphorylation of the transporter may increase its affinity for Ca^{2+} in analogy to cAMP regulation of phosphorylase kinase [65] and Ca^{2+}-activated K^+ channels [66] or (ii) the second messengers may provoke the phosphorylation of a common or different site on the transport protein (cf. the 136 kDa, 28 kDa or 25 kDa substrate proteins). In the second case (i) the cyclic nucleotides, DAG or Ca^{2+} may phosphorylate a common regulatory protein which then modulates the action of the salt transporter (cf. phospholamban which regulates the activity of the Ca^{2+}-ATPase and is cophosphorylated by cAMP- and Ca^{2+}/calmodulin-dependent kinases [63]) or (ii) only one of the messengers directly modulates the transport protein and the other second messengers merely generate this second messenger, which then functions as third messenger (cf. the increase of intracellular Ca^{2+} levels by cAMP in chicken enterocytes [47] or the generation of cAMP by prostaglandins which are induced by a rise in intracellular Ca^{2+} in colon [42].) A combination of these mechanisms or even more complicated models are also conceivable (cf. Ref. 58).

In recent measurements of intracellular free Ca^{2+} levels in HT-29 cells, a human colon carcinoma cell line displaying Ca^{2+}- and cAMP-activated apical Cl^- channels, no rise in Ca^{2+} after administration of 8-Br-cAMP was observed [67]. As mentioned earlier for another colonic cell line, the T84 cells, and for rabbit colon in vitro the Ca^{2+}-ionophore A23187 was unable to reproduce all effects of 8-Br-cAMP on ion transport systems. These studies indicate that at least in colonic crypts the action of cAMP is not mediated by Ca^{2+}. In chicken villus enterocytes however, Semrad et al.[47] have demonstrated a rise in intracellular Ca^{2+} in response to 8-Br-cAMP; moreover, the inhibition of Na^+/H^+ exchange by cAMP could be blocked by a low concentration of the calmodulin antagonist calmidazolium and could be mimicked by the Ca^{2+}-ionophore. In view of these results it is conceivable

that in this cell type cAMP modulates NaCl uptake indirectly by exploiting Ca^{2+} as a third messenger.

In mammalian small intestine the interactions between the various second messengers are far from clear. Studies showing inhibition of the cAMP-induced rise in I_{sc} by calmodulin antagonists like trifluoperazine (TFP) and chlorpromazine have been interpreted recently as an argument against the involvement of calmodulin in cyclic nucleotide-induced Cl^- secretion since the concentration of the antagonist needed to fully block the secretory process were too high to claim any specificity for calmodulin [68]. Transport studies carried out with relatively low concentrations of these calmodulin antagonists point to a possible role of calmodulin in the regulation of basal NaCl absorption in rabbit ileum [68]. The finding by Hardcastle et al. that cAMP only induces secretion in rat jejunum in the presence of extracellular Ca^{2+} [38] is at variance with results from Donowitz group, who was unable to demonstrate a Ca^{2+} dependency of cAMP-provoked Cl^- secretion in rabbit ileum [68].

The postulated mechanism of action of the second messengers discussed in the foregoing are summarized in Fig. 2. In view of the apparent occurrence of species differences in signal transduction mechanisms, the validity of regulatory models as the one shown in Fig. 2 should be verified experimentally for each species.

USE OF BRUSH BORDER MEMBRANES IN STUDIES OF SALT TRANSPORT REGULATION

Studies on isolated membranes are widely used to gain a better understanding of the mechanism and regulation of transport processes across biological membranes [69,70]. Isolated membranes offer a more simple model of study, lacking many of the complex interactions in a living cell, but are also mor suitable for experimental manipulation. In addition they can be studied under in vitro conditions which are optimal for assaying specific enzyme and transport systems in the membrane.

The apical membrane of the villus enterocyte can be isolated in two forms: (i) open brush border caps (BB) and (ii) closed brush border membrane vesicles (BBMV) (see Fig. 3) . The BB which can be obtained by the vibration of everted intestine in a hypotonic EDTA buffer [71] are

composed of intact microvilli stabilized by a cytoskeletal core which in turn is connected to a terminal web mainly composed of actin and myosin. Because the intracellular side of the membrane is readily accessible for membrane impermeable cofactors like ATP, GTP or purified kinases, this preparation is very suitable for the biochemical analysis of the apical membrane including studies of protein phosphorylation [58], regulatory aspects of guanylate cyclase [24] and to cytoskeletal structure and function [72]. The "open" BB are however not suitable for ion transport experiments; in contrast the BBMV mainly consisting of tightly sealed membranes in the right-side out orientation are ideal preparations for the in vitro characterization of transport mechanisms. They are readily generated by freeze-thawing of intestinal tissue and purified by differential precipitation with divalent cations (Ca^{2+} or Mg^{2+}) [73]. These BBMV still contain cytoskeletal material and encapsulated cytosolic factors [60]. Recently Hopfer described a procedure to obtain almost pure apical membranes by treatment of BB with a high concentration of the chaotropic salt KSCN [74]. These BBMV are virtually depleted of cytoskeletal elements and were used by Wright to visualize the Na^+ glucose transporter on SDS-PAGE gels [75].

Sofar the following transport pathways for Na^+ and Cl^- have been identified in intestinal BBMV : (i) a Na^+-dependent glucose carrier which is very sensitive to membrane potentials [76,77] and various Na^+-dependent aminoacid cotransporters [77], (ii) an amiloride inhibitable Na^+/H^+ exchanger displaying a K_m for Na^+ of approx. 5 mM [78,79], (iii) a Cl^-/HCO_3^- exchanger showing a K_m for Cl^- of approx. 5 mM and is inhibitable by SITS and DIDS [5,6], (iv) non saturable conductive pathways for Na+ and Cl^- [80,81] and (v) a Na^+-Cl^--cotransporter [82] which may however represent a combination of the Na^+/H^+ exchanger and the Cl^-/HCO_3^- exchanger coupled by a pH gradient. Using rabbit jejunal vesicles as a model system, Wright et al. have calculated that in the absence of glucose, amino acids and a pH gradient the contribution of the Na^+ conductance was 14 times that of the saturable Na^+/H^+ exchange at 150 mM NaCl [81]. A number of studies have already provided evidence for a regulation of these Na^+ and Cl^- transport pathways in BBMV by intravesicular factors. Aronson et al. showed that the Na^+/H^+ exchange can be modulated by intravesicular protons [83]. Kahn et al. reported a small inhibition of the Na^+/H^+ exchanger in rabbit renal BBMV following

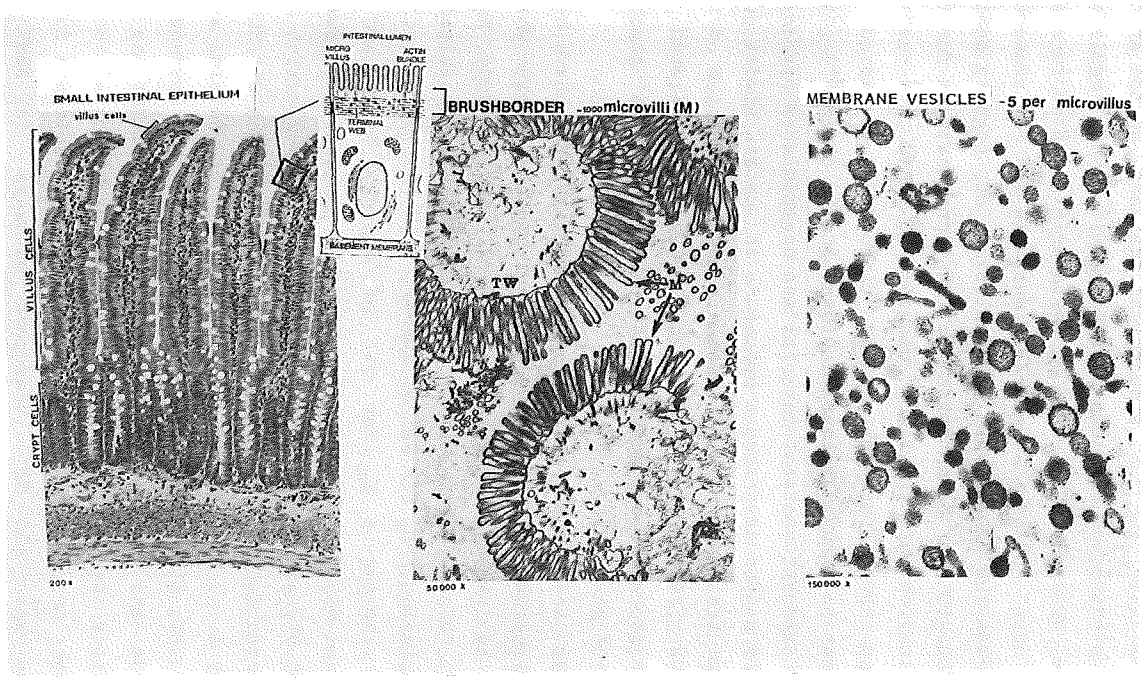

Figure 3.
Morphology of the small intestine and the brush border membrane.

entrapment of cAMP and ATP [45], while inclusion of DAG and ATP (presumably provoking protein kinase-C action) led to a small stimulation of this exchanger [84]. In rabbit ileal BBMV, an incubation for 4 hours with extravesicular calmodulin was found to inhibit NaCl uptake in the presence of micromolar Ca^{2+} [51], and in rat jejunal BBMV entrapment of cAMP and ATP caused an increase in Cl^- permeability as judged by the stimulation of Na^+-dependent glucose uptake only in the presence of a NaCl gradient [85]. Nevertheless, the present knowledge of all the effects of second messengers on Na^+ and Cl^- transport in mammalian small intestinal BBMV is yet far from complete.

SCOPE OF THIS THESIS

In this study we tried to gain a better understanding of the molecular mechanism involved in the regulation of Na^+ and Cl^- transport systems in the apical membrane of the enterocyte. We therefore examined the effects of the second messengers cAMP, cGMP, Ca^{2+} and DAG on Na^+ and Cl^- influx in isolated intestinal BBMV under various conditions and studied the characteristics of lipid phosphorylation and breakdown in isolated BB and BBMV. This so-called "PI-cycle" has been implicated in the generation of second messengers for hormones, neurotransmitters and growth factors but may also play a more direct role in the regulation of epithelial transport systems.

Chapter II is concerned with the characterization of the PI-cycle in BB and BBMV and the effects of Ca^{2+} and GTP analogues on PI turnover. Chapter III deals with measurements of Na^+ and Cl^- permeabilities in BBMV isolated from rat small intestine and their modulation by intravesicular Ca^{2+} levels. A sensitive method for the determination of Cl^-/anion exchange in isolated membrane vesicles and a characterization of this exchange in ileal and jejunal BBMV are presented in Chapter IV. In Chapter V the effects of prephosphorylation of BBMV with ATP and cAMP, cGMP, Ca^{2+} or phorbolester *in vitro* and with cholera toxin *in vivo*, on vesicular Cl^-/anion exchange and Na^+/H^+ exchange are described. Finally in Chapter VI a general discussion of the foregoing chapters and an outline for future research is presented.

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CHAPTER II

PHOSPHOINOSITIDE METABOLISM IN INTESTINAL BRUSH BORDER MEMBRANES. Stimulation of inositoltrisphosphate formation by guanine nucleotides and calcium

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SUMMARY

The potential role of polyphosphoinositide (PPI) metabolism as a signal transduction mechanism in the apical membrane of a polarized epithelial cell was evaluated by examining the formation and breakdown of PPI in rat intestinal brush border caps (BB) and cytoskeletal-free brush border membrane vesicles (BBMV) prelabeled by intraluminal injection of [^{3}H]inositol *in vivo* or by [γ-^{32}P]ATP *in vitro*. BB isolated in the absence of Ca^{2+} and MgATP were found to contain proportionately high amounts of phosphatidylinositol 4,5-bisphosphate (PIP_2; appr. 14% of total phosphoinositides) and were enriched in lipid kinases converting phosphatidylinositol (PI) into phosphatidylinositol 4-phosphate (PIP_1) and PIP_2, and diacylglycerol (DAG) into phosphatidic acid (PA). In the absence of Ca^{2+} and ATP, the turnover of PPI was relatively slow ($t_{1/2}$ [^{32}P]PIP_2 ≈ 7 min; $t_{1/2}$ [^{3}H]PIP_2 ≈ 10 min) and resulted mainly from dephosphorylation by endogenous phosphomonoesterase(s). In the presence of 10^{-4} M Ca^{2+} however, endogenous pools of PPI were rapidly depleted by the action of an intrinsic PPI-specific phospholipase C ($t_{1/2}$ [^{32}P]PIP_2 ≈ 20 s; $t_{1/2}$ [^{3}H]PIP_2 ≈ 8 min). The different half-life of freshly phosphorylated [^{32}P]PIP_2 and the bulk of [^{3}H]inositol-labeled PIP_2 may indicate the existence of various subpools of PPI in the microvillus membrane. In the presence of β-glycerophosphate which prevents rapid hydrolysis of inositol phosphates (IP) by alkaline phosphatase, Ca^{2+}-promoted breakdown of [^{3}H]PIP_2 was parallelled by an equivalent release of [^{3}H]IP_3 which, in the absence of the IP_3-ase inhibitor 2,3-diphosphoglycerate, was degraded further into IP_2 and IP_1 by the action of a Mg^{2+}-and Ca^{2+}-dependent IP_3-specific phosphomonoesterase and a Ca^{2+}-activated IP_2-ase associated with the BB. In the presence of nonhydrolyzable GTP analogs, the Ca^{2+} dose-response curve for IP release was shifted from millimolar to submicromolar concentrations. At pCa 6, IP release was activated up to 14-fold by 200 μM guanosine 5'-[3-O-thio]triphosphate (GTPγS) and reached similar levels as observed at 10^{-4} M Ca^{2+} alone. Less potent activation was effected by guanyl-5'-yl $\beta\gamma$-8-imidodiphosphate (Gpp(NH)p; 8-fold) or GTP (3-fold). Exposure of the microvillus membrane to pertussis toxin *in vivo* or to several Ca^{2+}-mobilizing hormones *in vitro* did not result in inhibition or further activation of IP release from the BBM. The activity of PI- and

PIP-kinase and of the Ca^{2+}- and guanine nucleotide-sensitive phospholipase C was latent in BBMV but could be unmasked by the membrane-permeabilizing agent alamethicin. These data are consistent with a topographical distribution of at least part of the PPI cycling in the entrocyte at the interior of the intestinal microvilli and its activation at physiological Ca^{2+} levels by a G protein distinct from G_i. Its possible involvement in the neurohormonal regulation of growth, differentiation and transport functions of the enterocyte deserves further exploration.

INTRODUCTION

Accelerated turnover of inositol phospholipids has been established as a major transduction mechanism for a number of cellular effectors, including hormones, neurotransmitters and growth factors (1-3). Occupancy of surface receptors triggers the hydrolytic breakdown of phosphatidylinositolbisphosphate (PIP_2) trhough the activation of a specific phosphodiesterase (phospholipase C). The resulting diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) have been shown to function as second messengers, with DAG activating Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C) and IP_3 causing release of Ca^{2+} from intracellular stores (1,2,4). Agonist-sensitive pools of polyphosphoinositides (PPI) are commonly replenished by increased PI synthesis and phosphorylation of PI and PIP by PI-kinase and PIP-kinase (1-3).

Recent evidence has indicated a pivotal role of PIP_2 metabolites as intracellular regulators of salt and water transport across the intestinal epithelium: (i) addition of IP_3 to EDTA- or saponin-permeabilized enterocytes caused a rapid and reversible release of Ca^{2+} from a non-mitochondrial compartment (5-7); Ca^{2+} has been shown to trigger net ion and water secretion by activating apical Cl^- channels (8) and basolateral K^+ channels (9) in the intestinal crypt cell and by inhibiting coupled transport of Na^+ and Cl^- in the absorptive villus cell possibly through interaction with calmodulin (CaM) or a CaM-dependent protein kinase (10,11); (ii) phorbolesters, by activation of protein kinase C, potentiate the action of Ca^{2+} on Na^+-Cl^- cotransport, presumably by inhibiting a Na^+/H^+ exchanger in the apical membranes (12); as shown in the human colon carcinoma cell line HT-29, they may also provoke VIP and α-adrenergic

receptor inactivation (13,14); (iii) measurements of cytosolic free Ca^{2+} levels in HT-29 and T84 colonocytes (14,15) and chicken enterocytes (16,17) have provided evidence for intracellular Ca^{2+} mobilization in response to various agonists of intestinal secretion; for some secretagogues, e.g. carbachol, serotonin and neurotensin, a parallel increase of IP formation has been demonstrated in [^{3}H]inositol labeled epithelial cells (18,19).

In view of the asymmetrical distribution of transmembrane signalling enzymes (e.g. adenylate and guanylate cyclase) among the brush border membrane (BBM) and basolateral membrane (BLM) of the polarized enterocyte (20,21) and the exclusive localization of most hormone receptors at the basolateral pool of the epithelial cells (22), receptor-activated IP_3 and DAG formation is expected to be confined to this subcellular region.

However, the present in vitro study of PI metabolism in intestinal brush border caps (BB) and cytoskeletal-free brush border membrane vesicles (BBMV) provides evidence for a secregation of most enzymes of the PI cycle to the apical membrane of the villus cell, including a PI- and PIP-kinase and a PPI-specific phosphodiesterase. The release of IP_3 from the BBM was triggered by Ca^{2+} and non-hydrolysable GTP analogs and appeared insensitive to pertussis toxin, suggesting the involvement of a GTP-binding protein distinct from G_i (23). Although the physiological activators of the PPI cycle in the BBM remain to be identified, the local generation of a protein kinase C activator (DAG) and a Ca^{2+} mobilizing signal (IP_3) in the intestinal microvilli is likely to play a role in the regulation of ion transport systems localized in the same subcellular compartment.

EXPERIMENTAL PROCEDURES

Preparation of BB and BBMV

Male specific-pathogen-free Wistar rats weighing 200-230 g, fed ad libitum, were used throughout the present study. Under light diethyl ether anaesthesia the small intestine was removed and rinsed twice with 20 ml of icecold saline (0.9% NaCl). All further steps were performed at 0-4°C. Everted segments of rat jejunum were mounted on metal rods attached to a vibration apparatus (Vibro-Mixer model E1 from Chemap A.G., Hännedorf ZN, Switzerland) as described previously (24). Intact brush borders were

released by mechanical vibration (50 Hz, amplitude 1.5 mm) for 25 min in Tris-buffered EDTA (2.5 mM, pH 7.0) and isolated by low speed centrifugation (10 min, 1500 g) following the removal of nuclear aggregates with glasswool as described by Harrison and Webster (25). The BB pellet was washed at least four times by resuspension in 20 ml Tris-EDTA buffer and homogenization in a Teflon glass Potter-Elvehjem homogenizer (20 strokes). The purity of the BB fraction was routinely monitored by phase contrast microscopy and the determination of marker enzymes as described previously (26). Highly purified microvillus membranes were prepared by treatment of isolated BB with a chaotropic agent (0.52 M KSCN) as described in detail by Hopfer et al. (27). As judged by enzyme determinations, protein staining pattern of SDS-PAGE gels, and a $^{125}I^{-}$-calmodulin radioimmunoassay, BBMV isolated by this procedure were virtually depleted of terminal web and microvillus core proteins, e.g. actin, myosin, the 110 kDa cross filament (28) and calmodulin (29), and showed an enrichment of 35-40 fold for alkaline phosphatase and 80-100 fold for sucrase as compared to mucosal homogenates (results not shown).

Phosphorylation and dephosphorylation of BBM lipids

BB and BBMV were resuspended in 0.1 M Hepes/Tris buffer pH 7.0 (5-10 mg/ml protein) and diluted 5-fold into a phosphorylation mix containing 5 mM $MgCl_2$, 25 mM β-glycerophosphate, 5 mM β-mercaptoethanl, 0.2 mg/ml soy bean trypsin inhibitor, 20 μM [γ-^{32}P]ATP (10 Ci/mmol) and, where indicated, 2 mM EGTA, 2 mM EGTA/Ca^{2+} buffer (pCa 4) and 0.3 mg/ml alamethicin (total volume 0.05 ml). Phosphorylation was carried out for 1 min at 30°C and was either terminated by addition of 0.2 ml 1 M HCl or by diluting the phosphorylated suspension 10-fold with 20 mM Hepes/Tris buffer pH 7.0 containing 2 mM EGTA or 2 mM EGTA/Ca^{2+} buffer (pCa 4) in order to study lipid dephosphorylation (at this point residual ATP levels as determined by luciferase assay (30) were reduced to less than 0.1 μM as a result of endogenous ATPase activity). At various time points following dilution, 0.2 ml aliquots were removed and mixed with 0.05 ml 5 M HCl.

[^{3}H]inositol labeling of BBM lipids

Using a surgical procedure described earlier in full detail (31), a jejunal segment (≈ 20 cm) of rat small intestine was injected *in situ* with 3 ml 0.9% NaCl containing 75 μCi *myo*-[2-^{3}H]inositol (15 Ci/mmol).

Three hours later, the abdomen was reopened under ether narcosis and the intestine was excised and used for BB isolation as described above. The incorporation of [^{3}H]inositol into the BB lipids amounted to about 0.13 μCi/mg protein (average of 16 experiments).

Metabolism of [^{3}H]inositol lipids in BBM

[^{3}H]inositol prelabeled BB (100-200 μg protein) were incubated for 10 min (unless indicated otherwise) at 30°C in 0.1 ml of the phosphorylation mix described above, occasionally replenished with 0.1 M KCl and various other additions as indicated in the legends. Incubations were terminated by addition of trichloroacetic acid (final concentration 10%) and standing in ice for 15 min. Following centrifugation the protein and lipid pellet was extracted with 0.5 ml chloroform/methanol/12 N HCl (200:100:0.75) prior to lipid analysis and the supernatant was extracted 5 times with 1 ml of diethyl ether and subsequently neutralized with 1 ml 20 mM Hepes/-Tris buffer pH 8.0 prior to determination of inositol and inositol phosphates.

Determination of inositolphosphate monoesterase activities

Non-radioactive BB were incubated under identical conditions as described above for [^{3}H]inositol labeled BB except that 0.1 M [^{3}H]inositol 1,4,5-trisphosphate (IP_3; 1 Ci/mmol) was included in the phosphorylation mix. Breakdown of [^{3}H]IP_3 by BB hydrolases was monitored by analysis of [^{3}H]inositol and [^{3}H]inositolphosphates in BB supernatants following trichloroacetic acid precipitation.

Extraction and separation of lipids

Lipid extraction was started by further addition of 0.5 ml chloroform/methanol (2:1 v.v) to 0.2 ml of the acidified samples. Following rigorous mixing and centrifugation the upper phase was removed and the lower phase containing the lipids was washed three times with 0.5 ml methanol/0.6 M HCl (1:1) and separated on K-oxalate impregnated Silica gel 60 high-performance thin-layer chromatography plates (HPTLC plates, layer thickness 25 μm, Merck) developed with chloroform/acetone/methanol/glacial acetic acid/H_2O (40:15:13:12:7 by vol) as described in detail by Van dongen et al. (32). ^{3}H-labeled lipids were visualized by treating the plates with EN^{3}HANCE spray followed by 2-4 days of exposure to Kodak X-ray

film (SB-5), using a Kodak X-OMAT enhancing screen, whereas ^{32}P-labeled spots were detected autoradiographically by direct exposure of the dried Silica plate to the X-ray film. Quantification of the radioactivity in each spot was performed by scraping and liquid scintillation counting in 10 ml Instagel.

Separation of inositolphosphates

Separation of inositol phosphates was achieved by anion-exchange chromatography on 0.2 ml Dowex 1 X-10 column (200-400 mesh) in the formate form (33). The eluents (1.5 ml) used were: (a) water (inositol); (b) 0.1 M formic acid, 0.2 M ammonium formate (IP_1); (c) 0.1 M formic acid, 0.4 M ammonium formate (IP_2); (d) 0.1 M formic acid, 1 M ammonium formate (IP_3). Radioactivity in the eluates was quantitated by liquid scintillation counting.

Other biochemical assays

The phosphor content of the phospholipid spots collected from the HPTLC plates was determined as described by Bartlett (34). Amounts of proteins were determined by the method of Lowry et al. (35). Free Ca^{2+} concentration of the Ca^{2+}/EGTA buffers were calculated as described by Bers (36) and verified with a Ca^{2+} electrode (Radiometer).

Materials

[γ-^{32}P]ATP (3000 Ci/mmol) and [^{3}H]inositol 1,4,5,-trisphosphate (1 Ci/mmol) were purchased from Amersham, myo-[2-^{3}H]inositol (15 Ci/mmol), and EN^{3}HANCE spray came from NEN. Reference phospholipids were obtained from Sigma and GTPγS and Gpp(NH)p from Boehringer. Silica gel 60 HPLTC plates were purchased from Merck. Alamethicin was a generous gift from Dr. Grady (Upjohn Company, New York, N.Y., U.S.A.). Pertussis toxin was obtained from List Biological Laboratories (Campbell, CA, U.S.A.). All other chemicals were of the highest purity available from commercial sources.

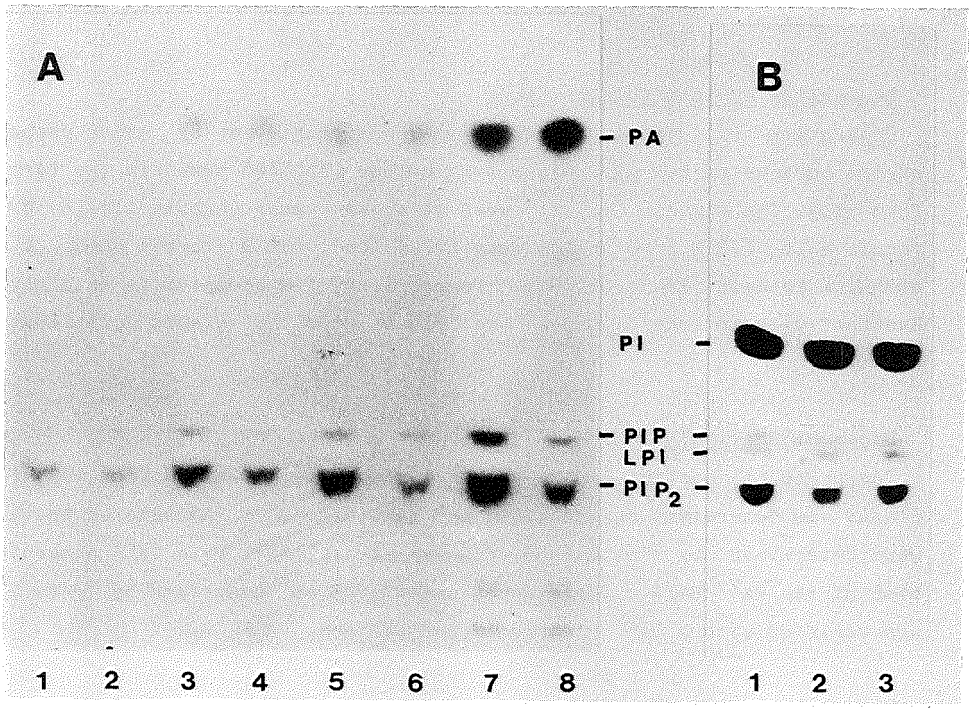

Figure 1.
[^{32}P]phosphorylation and [^{3}H]inositol labeling of lipid in rat intestinal BBM.
Panel A: Autoradiograph of ^{32}P labeled phospholipids. Rat intestinal BB (appr. 100 µg protein) or BBMV (appr. 20 µg protein) were prepared and phosphorylated with [γ-^{32}P]ATP under standard conditions in the absence or presence of 2 mM EGTA alone, 2 mM EGTA/Ca^{2+} buffer (pCa 4) and alamethicin (0.3 mg/ml). Membrane lipids were extracted and separated on HPTLC and their radioactivity was visualized by autoradiography as described under Experimental Procedures. Lane 1-4: BBMV; lane 5-8: BB; lane 1,3,5,7: EGTA; lane 2,4,6,8: EGTA/Ca; lane 1,2,7,8: - alamethicin; lane 3-6: + alamethicin.
Panel B: Autoradiograph of [^{3}H]inositol-labeled lipids. Rat intestinal BB (appr. 100 ug) prelabeled by <u>in vivo</u> exposure of jejunal segements to intraluminal [^{3}H]inositol were either anlyzed directly (lane 1) or incubated for 10 min at 30°C in 20 mM Hepes/Tris (pH 7.0), 0.1 M KCl, 25 mM β-glycerophoshate, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol and 0.2% trypsin inhibitor in the presence of 2 mM EGTA alone (lane 3) or a 2 mM EGTA/Ca^{2+} buffer (pCa 4; lane 2). Membrane lipids were extracted and separated on HPTLC, and their radioactivity was made visible by autoradiography as described under Experimental Procedures. Data shown are representative for at least 6 different experiments.

RESULTS

Phosphorylation of BB and BBMV

Exposure of isolated rat intestinal BB for 1 min to [γ-^{32}P]ATP under conditions favoring endogenous protein phoshorylation in earlier studies (37) resulted in the exclusive incorporation of ^{32}P into three lipid components of the membrane, i.e. PIP, PIP_2 and PA (Fig. 1A, lane 7). Upon vesiculation of the BBM and removal of terminal web and cytoskeletal elements by KSCN treatment, both PI- and PIP-kinase activities became latent but could be partially restored by disruption of the membrane barrier by the pore-forming agent alamethicin (Fig. 1A, lanes 1 and 3). The loss of DAG-kinase activity and an incomplete restoration of endogenous PIP and PIP_2 labeling in BBMV was observed also in alamethicin-treated "open" BB (cf. Fig. 1A, lane 7 versus lane 5) and apparently reflects an unwanted side-effect of the membrane-pertubing action of alamethicin (in contrast, endogenous protein phosphorylation in BB was not disturbed by alamethicin, cf. ref. 38). The proportion of ^{32}P incorporated into PI compared to PIP, ranging from 0.2 to 2.0, appeared rather variable among separate BB preparations but was independent of large variations in ATP concentration (0.5-100 uM) suggesting a similar K_m^{ATP} for both PI- and PIP-kinases under our experimental conditions.

The additions of pure exogenous PI and PIP to BB or alamethicin-permeabilized BBMV caused a further (up to 10-fold) increase of ^{32}P-incorporation into both substrates indicating that, in the absence of exogenous lipids, the lipid kinases apparently operate far below their V_{max} (results not shown). Inclusion of Ca^{2+} (pCa 4) in the phosphorylation assay drastically reduced the ^{32}P-labeling of PIP and PIP_2 but not of PA in both BB and BBMV (Fig. 1A, compare lane 7 and 8 or 3 and 4). Although a direct inhibitory effect of Ca^{2+} on PI- and PIP-phoshorylation through interaction with the lipid kinases or their substrates cannot be completely ruled out, the Ca^{2+} effect is readily explained by the rapid breakdown of freshly phosphorylated PIP and PIP_2 by a Ca^{2+} activatable phosphodiesterase as will be shown later in [^{3}H]inositol labeling experiments (Table 2).

Inositolphosphate monoesterase activities in isolated BB

The generation of IP_1, IP_2 and IP_3 in BB supernatants may in principle result from the action of a single or multiple forms of a BB-bound phospholipase C on all three inositol lipids (i.e. PI, PIP and PIP_2, respectively) or from the action of a single PIP_2- (and perhaps PIP-) specific phosphodiesterase followed by the breakdown of IP_3 to IP_2 or even further to IP_1 and inositol catalyzed by intrinsic inositolphosphate monoesterases associated with the BB. A substrate specificity study of intrinsic phospholipase C activities in the BBM therefore requires prior information regarding the interconversion of inositolphosphates in intestinal BB preparations. Under the basal incubation conditions shown in

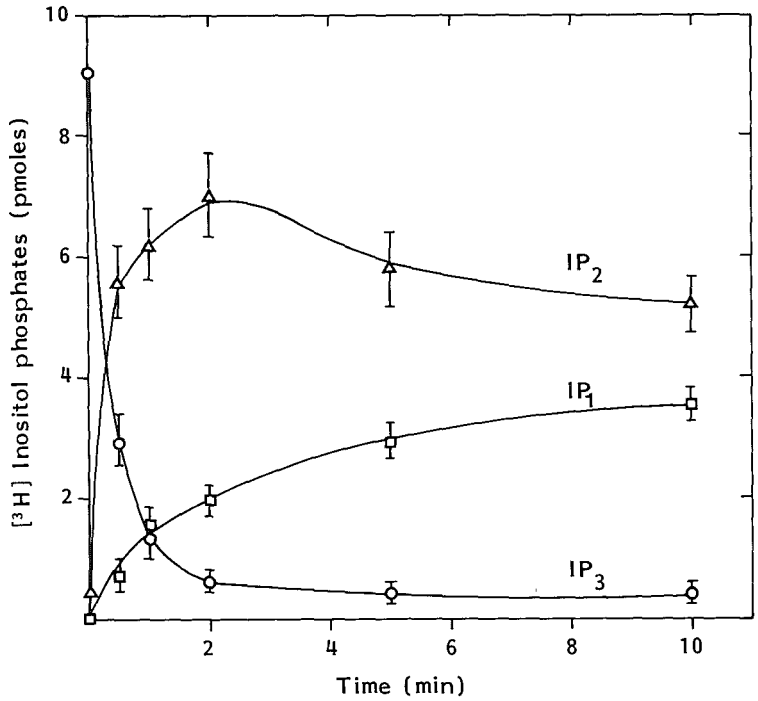

Figure 2

Metabolism of exogenous [^{3}H]IP_3 by isolated intestinal BB. BB (appr. 100 µg protein) isolated from rat small intestine were incubated at 30°C in the presence of 20 mM Hepes/Tris, pH 7.0, 0.1 M KCl, 25 mM β-glycerophosphate, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 2 mM EGTA/Ca^{2+} (pCa 4.0), 0.2% trypsin inhibitor and 0.1 µM [^{3}H]inositol-1,4,5-trisphosphate (total volume 0.1 ml). At various time points, incubations were terminated by addition of 10% trichloroacetic acid, and water-soluble [^{3}H]inositolphosphates were analyzed as described in Experimental Procedures. ○ = IP_3, △ = IP_2 and □ = IP_1. Data are means ± S.E., n=4.

TABLE 1.
INOSITOLPHOSPHATES MONOESTERASE ACTIVITIES IN ISOLATED BB

Rat intestinal BB (appr. 100 µg protein) were incubated in triplicate for 10 min at 30°C in the presence of 20 mM Hepes/Tris, pH 7.0, 25 mM β-glycerophosphate, 0.1 M KCl, 5 mM β-mercaptoethanol, 0.2% trypsin inhibitor, 2 mM EGTA, 0.1 µM [^{3}H]inositol 1,4,5-trisphosphate, and the additions specified in the Table (total volume 0.1 ml). The incubations were terminated by addition of 10% trichloroacetic acid. [^{3}H]inositolphosphates were separated and quantitated as described in Experimental Procedures. Data are means ± S.E., n=3.

Incubation conditions	[^{3}H]inositolphosphates (pmol)			
	Inositol	IP_1	IP_2	IP_3
No incubation	< 0.05	< 0.05	0.5±0.1	9.0±0.5
Basal conditions:	< 0.05	0.7±0.2	3.1±0.3	5.8±0.5
- β-glycerophosphate	9.6±0.5	0.1±0.1	0.1±0.1	0.1±0.1
+ 2 mM EGTA/Ca^{2+} (pCa 4)	< 0.05	2.4±0.3	4.0±0.4	3.1±0.3
+ 2,3-DPG (20 mM)	< 0.05	< 0.05	0.5±0.2	8.8±0.3
+ 2,3-DPG (20 mM) + 2 mM EGTA/Ca^{2+} (pCa 4)	< 0.05	0.5±0.2	1.5±0.3	7.0±0.5
+ Mg^{2+} (5 mM)	< 0.05	2.3±0.3	6.7±0.6	0.5±0.2
+ Mg^{2+} (5 mM) + 2 mM EGTA/Ca^{2+} (pCa 4)	< 0.05	3.5±0.4	5.2±0.5	0.4±0.2
+ 2,3-DPG (20 mM) + Mg^{2+} (5 mM)	< 0.05	0.7±0.2	3.1±0.4	5.3±0.5
+ 2,3-DPG (20 mM) + Mg (5 mM) + 2 mM EGTA/Ca (pCa 4)	< 0.05	1.7±0.3	3.1±0.4	4.0±0.4

Table 1, a 10 min exposure of exogenous [^{3}H]IP_3 to isolated BB resulted in its complete degradation to [^{3}H]inositol in the absence but not in the presence of a large excess of β-glycerophosphate (25 mM). By acting as a competitive substrate for alkaline phosphatase in BBM, this compound has been previously shown to prevent excessive degradation of ATP in endogenous protein phosphorylation experiments (37,38), and is apparently also able to partially counteract the dephosphorylation of inositolphosphates by this non-specific phosphomonoesterase. Subsequent experiments done in the presence of β-glycerophosphate (Table 1, Fig. 2) provided additional evidence for the existence of (a) a phosphomonoesterase relatively specific for IP_3 which is activated by Mg^{2+} (5 mM) and Ca^{2+} (10^{-4} M) and inhibited by 2,3-diphosphoglycerate 2,3-DPG, and (b) a phosphomonoesterase acting on IP_2 and stimulated by Ca^{2+} (10^{-4} M). A phosphomonoesterase specific for IP_1 was either absent or completely inhibited by β-glycerophosphate. Under conditions selected in later studies for examing Ca^{2+}-activation of phospholipase C in [^{3}H]inositol labeled BB (i.e. in the

presence of Mg^{2+} and Ca^{2+} and the absence of 2,3-DPG), IP_3 was rapidly converted to IP_2 (t 1/2 ≈ 15 sec), which in turn was much slower and incompletely degraded to IP_1 (Fig. 2).

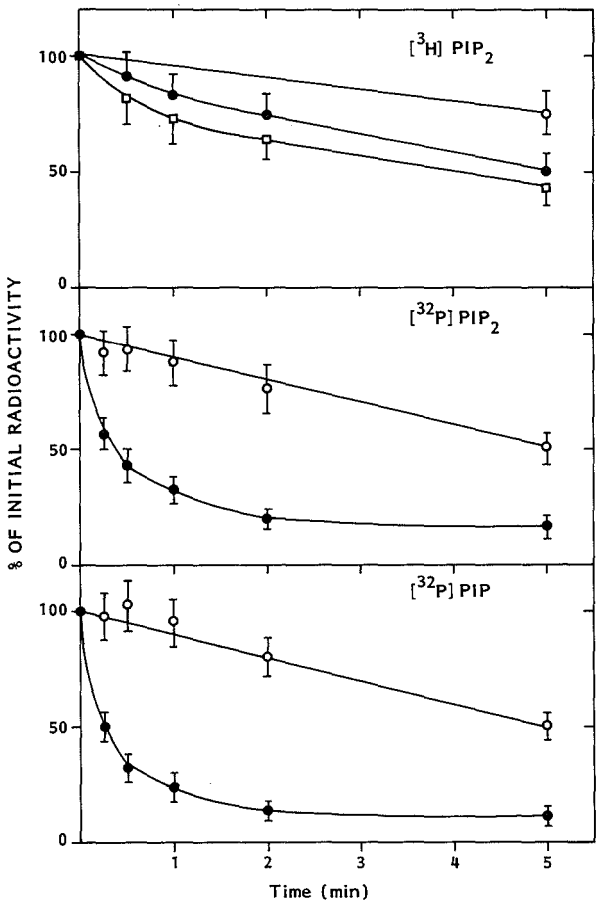

Figure 3
Metabolic conversion of [^{3}H]inositol- and ^{32}P-labeled polyphosphoinositides in isolated intestinal BB. Rat intestinal BB (appr. 100 µg protein) prelabeled by in vivo exposure of jejunal segments to intraluminal [^{3}H]inositol (upper panel) or phosphorylated for 1 min with 10 µM [γ-^{32}P]ATP followed by a 10-fold dilution in the phosphorylation buffer (middle and lower panel) were incubated at 30°C in the presence of 20 mM Hepes/Tris, pH 7.0, 0.1 M KCl, 25 mM β-glycerophosphate, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.2% trypsin inhibitor and 1 mM EGTA (o), 1 mM EGTA/Ca^{2+} (pCa 4) (•) or 1 mM EGTA/Ca^{2+} (pCa 3) (□) (total volume 0.1 ml). At various time points incubations were terminated by addition of 10% trichloroacetic acid and the radioactivity in PIP_2 (upper and middle panel) and PIP (lower panel) was determined as described under Experimental Procedures. Data are means ± S.E., n=3.

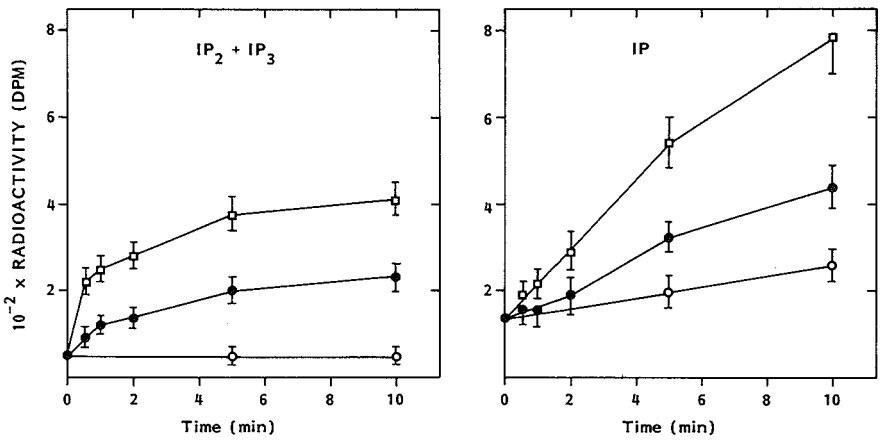

Figure 4
Time dependency of Ca^{2+}-provoked release of [^{3}H]inositolphosphates from intestinal BB.
Rat intestinal BB (appr. 100 µg protein) prelabeled *in vivo* with [^{3}H]inositol (see Experimental Procedures) were incubated at 30°C in 20 mM Hepes/Tris, pH 7.0, 0.1 M KCl, 5 mM β-glycerophosphate, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.2% trypsin inhibitor, and in the additional presence of 1 mM EGTA (o), 1 mM EGTA/Ca^{2+} pCa 4 (●) or 1 mM EGTA/Ca^{2+} pCa 3 (□); total volume 0.1 ml . The incubations were terminated at various time points by addition of 10% trichloroacetic acid. Radioactivities in IP_3 and IP_2 (left panel) and IP_1 (right panel) were determined as described under Experimental Procedures. Data are means ± S.E., n=3.

Metabolic conversion of phosphoinositides in BB prelabeled with [γ-^{32}P]ATP or [^{3}H]inositol

When isolated BB were first phoshorylated for 1 min with [γ-^{32}P]ATP and further phosphorylation was blocked by a drastic reduction of ATP levels below 0.1 µM (see Experimental Procedures), the spontaneous breakdown of ^{32}P-labeled PIP and PIP_2 was relatively slow in the absence of Ca^{2+} ($t_{1/2}$ ≈ 7 min) but was accelerated drastically at 10^{-4} M Ca^{2+} (t 1/2 ≈ 20 sec; see Fig. 3, middle and lower panel). In the absence of ATP, the loss of [^{32}P]PIP and [^{32}P]PIP_2 (labeled presumably at position 4 and 5 of the inositol ring) may either reflect the dephosphorylation of PIP and PIP_2 by a Ca^{2+}-sensitive phosphomonoesterase (producing $^{32}P_i$ and non-radioactive

PI and PIP_2) or the cleavage of both polyphosphoinositides by a Ca^{2+}-activatable phosphodiesterase (producing $[^{32}P]IP_2$, $[^{32}P]IP_3$ and DAG). A further discrimination between both possibilities using ^{32}P-prelabeled BB was hampered by (a) the incomplete separation of $[^{32}P]IP_3$ and residual trace amounts of $[\gamma\text{-}^{32}P]ATP$ by the anion exchange columns and (b) the high activity of the IP_3 phosphomonoesterase rapidly converting $[^{32}P]IP_3$ into ^{32}P and non-radioactive IP_2. We therefore preferred to use $[^3H]$inositol prelabeled BB to study the metabolism of the phosphoinositides in more detail.

Due to the high turnover rate of protein and lipid components of intestinal BB (cf. ref. 39), a 3 h exposure of intestinal epithelium in vitro to luminally injected $[^3H]$inositol appeared sufficient to label the phosphoinositide pools in the membrane close to equilibrium; the relative amounts of total PI (25±5 nmol/mg protein; n=5) and PIP (3.5±1.0 nmol/mg protein; n=5) in isolated BB as calculated from the phosphor content of each phospholipid class (see Experimental Procedures) corresponded closely with the relative incorporation of $[^3H]$inositol into PI and PIP_2 (respectively accounting for 82±5% and 14.0±3.9% of total incorporation). Only 0.5-1% of the total $[^3H]$inositol incorporation into BB lipids was recovered in PIP and the residual $[^3H]$inositol activity (1-4%) resided in lyso PI. The amount of PIP in BB appeared too low to allow an accurate quantitation on the basis of its phosphor content.

As shown in Table 2, freshly isolated suspensions of BB kept for 15-30 min in ice already contained detectable amounts of IP_1, IP_2 and IP_3. A further incubation of the BB for 10 min at 30°C in the absence of Ca^{2+} led to an increase of $[^3H]IP_1$ and $[^3H]IP_2$ and a decrease of $[^3H]IP_3$ which almost equalled the rise in $[^3H]IP_2$. Concurrently, the total $[^3H]PIP_2$ content of the BBM was reduced by about 33% (Table 2, Fig. 1B). The amount of $[^3H]$PIP was also decreased but this decrease could not be quantitated accurately because of the low amount of radioactivity involved and the presence of lyso PI in close vicinity of the PIP spot. It should be noted that, despite the presence of β-glycerophosphate which prevented the further breakdown of the $[^3H]$inositolphosphates into $[^3H]$inositol (Table 2), the total increase of $[^3H]$inositolphosphates was about 4-fold lower than the loss of $[^3H]PIP_2$ observed in the same experiment (Table 2). It follows that, in the absence of Ca^{2+} the dephosphorylation of PIP_2 to PIP, rather than its breakdown by an endogenous phospholipase C, is the major

catabolic pathways for PIP_2 in the isolated BB. Since the loss of $[^3H]PIP_2$ is not accompanied by an equivalent increase in $[^3H]PIP$ (Fig. 1B), the product PIP is apparently further dephosphorylated at the same rate by a PIP phospmonoesterase. In the presence of 10^{-4} M Ca^{2+} however, the $[^3H]PIP_2$ pool became rapidly depleted (about 60% loss during 10 min incubation at 30°C) and the breakdown products were almost fully recovered in the $[^3H]$inositolphosphate fractions (mainly IP_1 and IP_2; Table 2). In the presence of Ca^{2+} therefore, the major route for PIP breakdown appears to shift from dephoshorylation to a phosphodiesteratic cleavage by a Ca^{2+}-activatable PIP_2-specific phospholipase C, resulting in the release of $[^3H]IP_3$, which, in the absence of 2,3-DPG is rapidly degraded to IP_2 and IP_1 by phosphomonoesterases associated with the BB (cf. Table 1). By repeating the experiment in the presence of the IP_3-phosphomonoesterase inhibitor 2,3-DPG, it could be shown that the $[^3H]IP_2$ accumulating in the presence of Ca^{2+} originated almost exclusively from IP_3 breakdown and not from dephosphorylation of PIP_2 followed by phosphodiesteratic cleavage of PIP (cf. the IP_3/IP_2 ratio in Table 1 and 2, bottom line). Unfortunately,

TABLE 2.
METABOLISM OF $[^3H]$INOSITOL-LABELED MEMBRANE LIPID IN ISOLATED BB.

Rat intestinal BB (appr. 100 µg protein) prelabeled with $[^3H]$inositol *in vivo* (see Experimental Procedures) were incubated for 10 min at 30°C in the presence of 20 mM Hepes/Tris, pH 7.0, 25 mM β-glycerophosphate, 0.1 M KCl, 5 mM β-mercaptoethanol, 5 mM $MgCl_2$, 0.2% trypsin inhibitor and the additions specified in the Table (total volume 0.1 ml). The incubations were terminated by addition of 10% trichloroacetic acid. Radioactivity in the polyphosphoinositides and inositolphoshates was determined as described in Experimental Procedures. Data are means ± S.E., n=3.

Incubation conditions	$[^3H]$inositolphosphates (dpm x 10^{-2})				$[^3H]$phosphoinositides* (dpm x 10^{-2})	
	IP_1	IP_2	IP_3	Σ IP	PI	PIP_2
No incubation	0.8±0.1	0.5±0.1	1.7±0.2	3.0±0.3	97±8	24±4
2 mM EGTA	3.0±0.4	2.1±0.2	0.3±0.1	5.4±0.6	100±11	18±2
2 mM EGTA/Ca^{2+} (pCa 4)	5.0±0.7	11.1±1.3	0.6±0.1	16.6±2.0	101±12	11±2
2 mM EGTA + 20 mM 2,3-DPG	1.9±0.3	1.3±0.2	1.2±0.2	4.4±0.5	105±12	18±2
2 mM EGTA/Ca (pCa 4) + 20 mM 2,3-DPG	2.6±0.3	2.7±0.4	2.7±0.3	8.0±1.0	101±10	17±2

*The radioactivity in the PIP spots was only slightly above background and did not change significantly by variation of the incubation conditions (data not shown).

2,3-DPG also partially prevented PIP_2 hydrolysis in the presence (but not in the absence) of Ca^{2+} and may therefore exert an additional inhibitory action on phospholipase C (Table 2). Mg^{2+} deprivation which also led to IP_3-ase inactivation (Table 1) likewise resulted in a further increase of the IP_3/IP_2 ratio (up to 8.5) in line with a direct breakdown of PIP_2 by a diesterase in the presence of Ca^{2+}.

A further comparison between the data in Table 1 and 2 obtained under similar incubation conditions (i.e. Mg^{2+} and Ca^{2+} present) additionally suggests most of the all $[^3H]IP_1$ formed during incubation of [^{3}H]inositol labeled membranes (i.e. appr. 30% of total inositolphosphates) originates from breakdown of $[^3H]IP_3$ rather than [^{3}H]PI, arguing against a substantial contribution of a PI-specific phospholipase C to IP_1 and DAG formation in the isolated BB. In view of the similar turnover rate of ^{32}P-labeled PIP and PIP_2 in the presence of Ca^{2+} noted in Fig. 3, and assuming that the Ca^{2+}-activated breakdown of freshly ^{32}P-labeled and [^{3}H]inositol labeled polyphosphoinositide pools are catalyzed by the same enzyme, the endogenous phosholipase seems equally active on the endogenous pool of PIP and PIP_2 in the isolated BB. There is however a large discrepancy between the half-life of freshly phosphorylated $[^{32}P]PIP_2$ (t 1/2 ≈ 20 s, Fig. 3 middle panel) and [^{3}H]inositol-labeled PIP_2 (t 1/2 ≈ 8 min; Fig. 3, upper panel) which supposedly reflects the total pool of PIP_2 in the BBM. Moreover, the biphasic time course of Ca^{2+}-triggered [^{3}H]IP release shown in Fig. 4, i.e. a fast initial release (≈ 30 s) followed by a slower and almost linear further increase of inositolphosphate formation, provides additional evidence for the existence of PIP_2 subpools in the rather homogeneous BB preparation. The minor pool is apparently located in close vicinity of the PIP kinase and seems highly susceptible to the action of a Ca^{2+}-sensitive phosphodiesterase (or, less likely, a Ca^{2+}-activated PIP phosphatase), whereas the major pool accounts for most of the [^{3}H]inositol labeling and is less sensitive to phosphodiesterase action. From a comparison of the turnover rates of $[^{32}P]PIP$ and [^{3}H]inositol-labeled PIP measured in the absence of Ca^{2+} (Fig. 3, lower panel; Table 2, + EGTA; Fig. 1B), it becomes also clear that PIP exists in different subpools, i.e. a freshly phosphorylated pool which, in the absence of ATP, turns over very slowly (Fig. 3, lower panel) and a fraction produced by a Ca^{2+}-independent dephosphorylation of at least 30% of the $[^3H]PIP_2$ pool (Table 2, data + EGTA) which was not recovered as [^{3}H]PIP or [^{3}H]inositolphosph-

ates and must therefore have been converted back to PI much more rapidly than the newly formed PI kinase product.

Activation of polyphosphoinositide-specific phospholipase C in BB by Ca^{2+} and stable GTP analogs

A dose response curve for Ca^{2+}-activation of BB-bound phospholipase C measured in the presence of physiological levels of Mg^{2+} and KCl is presented in Fig. 5. In the absence of other effectors, enzyme activation was started at 10^{-5} M Ca^{2+} and half-maximal activation was reached at

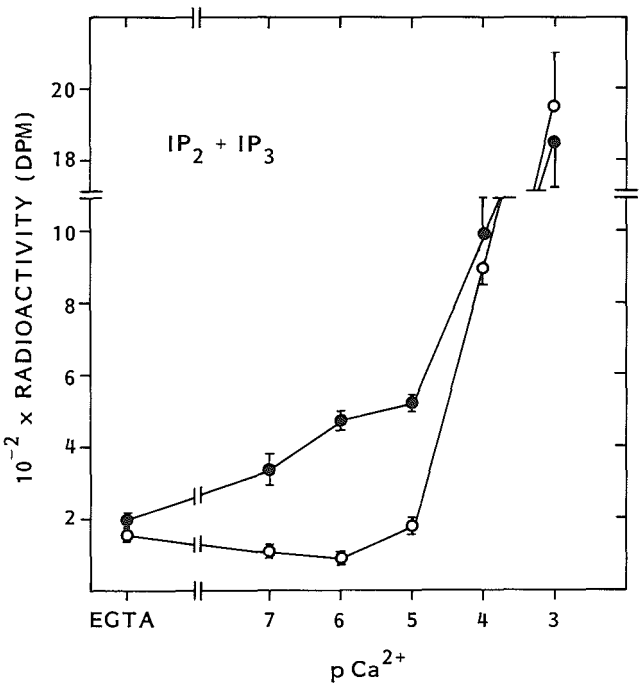

Figure 5
Activation of phospholipase C in intestinal BB by Ca^{2+} and GMP-PNP.
Rat intestinal BB (appr. 100 µg protein) prelabeled in vivo with [^{3}H]inositol (see Experimental Procedures) were incubated for 10 min at 30 C in 20 mM Hepes/Tris, pH 7.0, 0.1 M KCl, 25 mM β-glycerophosphate, 5 mM MgCl, 5 mM β-mercaptoethanol, 0.2% trypsin inhibitor and 1 mM EGTA/Ca^{2+} buffer of various pCa; o, no further addition; • + 100 µM Gpp(NH)p. The incubations were terminated by addition of 10% trichloroacetic acid and radioactivities in IP_3 and IP_2 were determined as described under Experimental Procedures. Data are corrected for the amount of [^{3}H]IP_2 + IP_3 present in BB prior to incubation and represent means ± S.E., n=3.

10^{-6} M Ca^{2+}. Addition of an optimal concentration (200 μM) of the non-hydrolyzable GTP analog Gpp(NH)p, a strong stimulation of $[^3H]IP_2$ and IP_3 release (appr. 8-fold) was seen at physiological Ca^{2+} levels (10^{-7}-10^{-5} M; maximal at 10^{-6} M)but not above or below this physiological range (Fig. 5, Table 3). Another stable GTP analog, GTPγS, was almost twice as effective (appr. 14-fold stimulation) whereas GTP even at 10-fold higher concentrations was a weak activor (2-3-fold activation, Table 3). These results suggest that the phospholipase C in BB is synergistically activated by Ca^{2+} and a G protein which may couple the lipase to unknown intra- or extracellular effectors. Phospholipase C regulation by a G factor could be demonstrated also in highly purified BBMV provided that alamethicin was

TABLE 3.
ACTIVATION OF A POLYPHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C IN INTESTINAL BB AND BBMV BY CA^{2+}, GTP AND STABLE GTP ANALOGUES

Rat intestinal BB (appr. 100 μg protein) prelabeled with $[^3H]$inositol *in vivo*, and BBMV (appr. 10 μg protein), prepared from these BB by a KSCN treatment (see Experimental Procedures), were incubated for 10 min at 30°C in the presence of 20 mM Hepes/Tris pH 7.0, 25 mM β-glycerophosphate, 0.1 M KCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.2% trypsin inhibitor and the additions specified in the Table (total volume 0.1 ml, EGTA concentration was kept at 2 mM). The interior of the BBMV was made accesssible by the additon of 0.2% alamethicin. The incubation was terminated by addition of 10% trichloroacetic acid. Radioactivity in the combined IP_2 and IP_3 fraction was determined as described in Experimental Procedures and corrected for the radioactive IP_2 and IP_3 present before incubation. Data are means ± S.E. (n=3).

Incubation conditions	Increase of $[^3H]$ radioactivity in IP_2 and IP_3 (dpm x 10^{-3}.mg^{-1} protein)	
	BB	BBMV
EGTA	0.6±0.3	11.0±3.2
EGTA/Ca^{2+} (pCa 6)	0.7±0.4	15.2±4.1
EGTA + Gpp(NH)p (0.2 mM)	0.7±0.3	n.d.
EGTA/Ca^{2+} (pCa 6) + Gpp(NH)p (0.2 mM)	5.7±0.7	n.d.
EGTA + GTPγS (0.2 mM)	1.5±0.5	13.6±4.0
EGTA/Ca^{2+} (pCa6) + GTPγS (0.2 mM)	9.9±1.5	30.3±6.5
EGTA + GTP (2 mM)	0.6±0.3	n.d.
EGTA/Ca^{2+} (pCa 6) + GTP (2 mM)	2.3±0.5	n.d.
EGTA/Ca^{2+} (pCa 6) + GTP (2 mM) + carbachol (10^{-4} M)	2.4±0.5	n.d.
EGTA/Ca^{2+} (pCa 6) + GTP (2 mM) + bradykinin (10^{-5} M)	2.2±0.5	n.d.

present as a membrane-permeabilizing agent (Table 3). However, the basal rate of IP_2 and IP_3 release expressed on a protein basis was much higher and the GTPγS activation considerably lower compared to BB caps, which could be attributed largely to the membrane pertubing action of alamethicin observed also in BB under similar conditions (results not shown).

Attempts to show a direct in vitro activation of the enzyme in the presence of GTP by secretagogues known to promote PIP_2 turnover in intact enterocytes and multiple other cell types, i.e. carbachol and bradykinin, were unsuccessful, presumably as a consequence of the orientation of functional receptors for both compounds at the BLM of the enterocyte. The simultaneous injection of both [^{3}H]inositol and relatively high amounts of purified pertussis toxin (1 μg/ml) into the jejunal segement, known to catalyze ADP-ribosylation and inactivation of the inhibitory G protein (G_i) in other tissues (23), did not impair the GTPγS activation of phospholipase C in intestinal BB isolated from the toxin pretreated segment (results not shown). It seems therefore unlikely that the G protein activating the BB-bound form of phospholipase C is identical to G_i.

DISCUSSION

Most studies of receptor-mediated PPI turnover in mammalian tissues, including the enterocyte (18,19), have been carried out with intact or permeabilized cells, precluding an assessment of the contribution of separate membrane domains to the generation of intracellular messengers. More recent studies showing effects of hormones and guanine nucleotides on PIP_2 hydrolysis in isolated membranes have been performed mostly with crude particulate fractions of non-polarized cell types, e.g. platelets (40,41), neutrophils (42), astrocytoma cells (43), or a mixture of canalicular and basolateral membranes, eg. liver cells (44). In the present study, circumstantial evidence for a substantial contribution of the apical membrane of a polarized epithelial cell to Ca^{2+}- and nucleotide-dependent PPI hydrolysis was obtained by in vitro analysis of PPI metabolism in the microvillus membrane of rat enterocytes isolated in two configurations: (a) open brush border caps (BB) consisting of intact microvilli stabilized by a cytoskeletal core; in this preparation the

inner side of the membrane is readily accessible for membrane-impermeable factors, e.g. ATP, GTP, EGTA; and (b) brush border membrane vesicles obtained by KSCN-treatment of BB and consisting of pure microvillus membranes sealed in a right-side-out orientation and virtually free of cytoskeletal elements and contamination with BLM (26,27).

In agreement with very early observations of Gurr et al. (39), in vivo labeling of PI in rat intestinal mucosa was facilitated by the rapid synthesis and breakdown of lipids in the enterocyte, allowing complete equilibration of [^{3}H]inositol with endogenous PI and PPI pools in the BBM within 3 h following its injection into the intestinal lumen. As an additional advantage, the BBM of rat enterocytes has been shown highly-enriched in PI (appr. 22% of total lipid phosphor) in comparison with basolateral membranes (45). The distribution of [^{3}H]inositol label in phosphoinositides (appr. 82% PI, 0.8% PIP and 14% PIP_2) in the intestinal BB was almost proportional to the amount of phosphor in each lipid class and resembled the labeling pattern of liver plasma membranes (44). The proportion of PPI in vivo might be even higher considering the PPI phosphomonoesterase activities present in the BB and the isolation of BB in MgATP-free medium. The inclusion of ATP in the isolation medium was required for regeneration of PIP_2 in membranes of several other cell types, e.g. platelets (41) and GH_3 cells (46), but was not considered in our study in view of its known destabilizing action on the BB cytoskeleton (47). To our knowledge, the only comparable data on PPI levels in BBM have been reported by Khalifa et al. for dog kidney BBMV (48). The pool size of phosphoinositides found in this study (appr. 41% PI, 26% PIP and 33% PIP_2) differs widely from our data which may reflect tissue or species variation or methodological differences in membrane isolation technique or phosphoinositide separation (cf. the possible interference of lyso PI with the PIP determination).

Our study of lipid (de)phosphorylation and IP release in BB and BBMV has provided clear evidence for the association of the major enzymes of the PPI cycle with the BB structure, i.e. a PI-, PIP-, and DAG-kinase, a PPI-specific and Ca^{2+}/guanine nucleotide-sensitive phosphodiesterase, and specific phosphomonoesterases catalyzing the dephosphorylation of PIP_2, PIP, IP_3 and IP_2. In suspensions of open BB, but not in intact cells, the analysis of PPI turnover was further complicated by a rapid and non-specific dephosphorylation of all IP (including IP_1) by the action of

alkaline phosphatase extruding from the external surface of the microvilli. As shown earlier for ATP hydrolysis (37,38), degradation of IP by this enzyme could be effectively counteracted by excessive amounts of an alternative substrate, i.e. β-glycerophosphate. Considering the non-vesiculated structure of the intact BB and the continuous exposure of the microvillus interior to large volumes (≈ 0.5 l) of hypotonic EDTA during BB isolation, the association of the enzymes of the PPI cycle with BB is unlikely to result from entrapment of cytosolic enzymes or from a redistribution of non-BB protein following cell disruption. Moreover, at least three enzymes, i.e. PI- and PIP-kinase and the phosphodiesterase were shown to copurify with cytoskeletal-free BBMV gernerated by homogenization of BB in the presence of a chaotropic agent (0.52 M KSCN), arguing strongly against a cytoskeletal or cytosolic origin of these proteins. The remote possibility that all enzyme activities were associated with redisual tags of BLM still adhering to the BB structure upon isolation could be virtually ruled out by the observations that: (a) BBMV prepared by the KSCN procedure were 50-100 fold enriched in BBM markers, and are almost devoid of BLM markers, e.g. adenylate cyclase (27); (b) all three enzyme activities in BBMV were latent and became detectable only upon disruption of the microvillus barrier with alamethicin; this finding is in line with the right-side-out orientation of BBMV and provides evidence for an exclusive localization of the enzymes, including the putative G protein regulating the phosphodiesterase, at the microvillus interior; (c) the PIP_2 pool possibly present in contaminating BLM is unlikely to account for the massive breakdown of $[^3H]PIP_2$ in BB observed upon activation of intrinsic phospholipase C with 10^{-4} M Ca^{2+} or 10^{-6} M Ca^{2+} plus GTPγS (i.e. 60% loss after 10 min at 30°C, cf. Fig. 5 and Table 2); (d) finally, preliminary results of a similar study performed with intestinal BLM have shown a similar or lower activity of PPI cycle enzymes towards endogenous substrates as compared to isolated BB (A.B. Vaandrager, unpublished results).

The tight association of the lipid kinases with the intestinal BBM is consistent with their membranal localization found in most other cell types (1,2). Our experiments do not exclude however an additional linkage to membrane-associated components of the cytoskeleton, as has been demonstrated for the particulate form of cGMP-dependent protein kinase in the intestinal BB (26,38,49) and the PIP kinase in the erythrocyte (50).

The localization of the PPI-specific phosphodiesterase at the interior of the microvilli apparently in intimate contact with a PIP_2 pool in the membrane abolished the need for detergents or exogenous substrates for characterization of its enzymic properties as required in some other studies (51). In spite of the relative homogeneity of the intestinal membranes, compared to membrane preparations used in other studies, the different half-lives of ^{32}P-labeled and [^3H]inositol labeled PIP_2 observed both in the presence and absence of Ca^{2+}, are compatible with the existence of subpools of PIP_2 in the BBM. The minor pool of freshly phosphorylated and dephosphorylated PIP_2, in comparison with the bulk of [^3H]PIP_2, clearly displayed a kinetic advantage in the subsequent hydrolytic cleavage by endogenous phospholipase C and PIP_2 phosphomonoesterase, respectively. It is conceivable therefore that the PIP kinase and the phospholipase C, as well as the PIP_2-and PIP-phosphomonoesterase (assuming that the latter enzymes are not identical), occur in supramolecular complexes promoting rapid consecutive conversions of PIP and PIP_2 and restricting the exchange of reaction intermediates with the major PPI pools in the membrane. Evidence for the utilization of distinct pools of PPI that are readily depleted and resynthesized for signal transduction has been obtained also in other cell types but is usually ascribed, with the exception of erythrocyte ghosts (52), to a compartmentation of phosphoinositides in different subcellular regions (53) or the localization of an inert pool of PI on the external surface of the plasma membrane (54).

From our measurements of PPI turnover and IP formation in the isolated BB it is evident that in the absence of Ca^{2+}, the metabolic conversion of PIP_2 and PIP is rather slow and results mainly from the action of endogenous phosphomonoesterase(s). In addition, the BB contains a low activity of a PI-specific and Ca^{2+}-independent form of phospholipase C apparently responsible for the low rate of IP_1 (and presumably DAG) formation measured under basal incubation conditions (cf. Table 2). In view of its Ca^{2+}-independency, this BB enzyme is apparently different from the Ca^{2+}-activated phosphoinositide-selective phospholipase C identified in the cytosol of a number of tissues including intestinal mucosa (55) and recently detected also in renal BLM (56). Above a threshold level of 10^{-5} M Ca^{2+}, the breakdown of PIP_2 and PIP increased gradually to reach a maximum at about 10^{-3} M Ca^{2+}. The Ca^{2+}-dependent and PPI-specific phospho-

lipase C which is responsible for this degradation was shown to become active also at physiological values of pCa in the presence of the non-hydrolyzable GTP analogs Gpp(NH)p or GTPγS. Such a synergistic action between Ca^{2+} and guanine nucleotide analogs leading to a leftward shift in the dose-response curve for Ca^{2+} activation of PPI hydrolysis has been recently observed in permeabilized cells and isolated membranes of many other tissues (1,2,42,57) and is diagnostic for the involvement of a GTP-binding protein mediating activation of PPI turnover and IP/DAG release in the intestinal microvilli. The identity of the G protein however remains uncertain. In mast cells (58) and neutrophils (59) the agonist-induced activation of phospholipase C is blocked by ADP-ribosylation of G_i-related proteins by pertussis toxin, but in many other systems, ADP-ribosylation of G_i is without effect (44,60,61). In platelets and other cells, a number of guanine nucleotide binding proteins with lower M_r values than the classical G proteins have been recently identified (62,63). Another membrane-bound G protein, P21 (the product of the ras gene) has also been implicated in phosphoinositide metabolism (64) but its possible presence in BBM has not been verified sofar. Luminal exposure of intestinal mucosa in vivo to excessive concentrations of pertussis toxin (1 μg/ml) under conditions in which cholera toxin caused a profound activation of adenylate cyclase in the BLM of the enterocytes (31) did not affect the GTPγS activation of phospholipase C in isolated BB. Although this result argues against a role for a pertussis toxin substrate in the activation mechanism, conclusive evidence pro or against such involvement awaits a further characterization of G proteins in the intestinal BB by immunological techniques and in vitro incubations with preactivated pertussis toxin and [^{32}P]NAD. Similar studies performed by Dominguez et al. with intestinal BBMV from the rabbit have recently identified a 45 kDa BB protein as the major substrate for a GTP-dependent ADP ribosylation by cholera toxin (65). In spite of the present lack of experimental proof for their hypothesis that the ADP-ribosylated G protein in the BBM could become translocated subsequently to the BLM and used for activation of adenylate cyclase, it is tempting to speculate that the putative G factor regulating PPI metabolism in the intestinal BB might likewise arise by translocation of a G protein in the serosal border triggered by the interaction of agonists with the basolateral membrane. Although present evidence for a transfer of G proteins from a donor to a recipient membrane is restricted

to in vitro experiments with isolated membranes (66), such a translocation would be in line with (a) the hydrophilicity of their α subunits (67), (b) their recent implication in the regulation of cellular functions situated distally from the hormone receptor e.g. ion channel regulation (68); exocytosis (69,70), and (c) the predominant localization of receptors for intestinal secretagogues, including bradykinin and carbachol, at the BLM (22; cf. also Table 3). The polarized enterocyte seems to offer an interesting intact cell model to confirm or disprove a translocation of G proteins experimentally.

Alternatively, the G protein in the intestinal BB might function as a coupling factor between receptors for agonists on the microvillar surface and the phospholipase C at the microvillar interior. Sofar only a few receptors for agonists have been identified at the apical border of the intestinal epithelial cells, i.e. insulin (71) and adenosine (22) receptors in rabbit and human colonocytes and receptors for bacterial toxins, e.g. cholera toxin and heat-stable E.coli toxin (ST_A) on the BBM of mammalian enterocytes (49). Our original hypothesis that PPI turnover in the intestinal BB might play a role in the coupling mechanism between the ST_A receptor and guanylate cyclase in the microvillus membrane was rejected in view of our observation that the toxin was unable to activate or potentiate PPI breakdown or to affect PI and PIP phosphorylation in isolated BB under conditions in which guanylate cyclase was strongly activated (Ref. 72 and A.B. Vaandrager, unpublished results). Sofar none of the other agonists have been tested for their possible effects on PPI turnover in isolated BB or intact enterocytes and colonocytes.

Apart from its possible regulation by a G protein at basal intracellular Ca^{2+} levels, the phospholipase C in the BBM is also activatable by Ca^{2+} alone at concentrations exceeding 10^{-5} M (Fig. 3). In view of this sensitivity to elevated levels of Ca^{2+}, the PPI cycle in the microvilli could in principle function as an amplification mechanism for Ca^{2+} signals elicited by agonists interacting with receptors at the BLM. Moreover, the DAG produced by splitting of PPI in the BBM may directly interact with the pool of protein kinase C localized in the same membrane and presumably involved in ion transport regulation, i.e. inhibition of Na^+/H^+ exchange (12). Such a mechanism might circumvent the need for BLM → BBM translocation of either DAG or protein kinase C locally produced/activated by PPI cycling in the BLM. Recent measurements of intracellular Ca^{2+} concentra-

tions in monolayers of colonocytes with fluorescent Ca^{2+} probes have shown that most secretagogues, with the exception of Ca^{2+}-ionophores, are unable to elevate cytoplasmic Ca^{2+} above the micromolar level (14,15). It remains possible, however, that under certain conditions the local concentration of Ca^{2+} at the interior of the microvilli may exceed the threshold level for phospholipase C activation.

Other intracellular factors besides Ca^{2+} and G proteins which have been reported to modulate PPI cycling in other systems include: (a) protein kinase C, acting as feedback inhibitor at the levels of PIP kinase (73) or phospholipase C (74); (b) cAMP-dependent protein kinase (75,76) and insulin kinase (77), supposedly stimulating the PPI cycle by acting as a PI-kinase or by phosphorylating a regulatory protein of PI- and PIP-kinases, e.g. phospholamban (77). The latter study, however, has been recently critisized on methodological grounds (78); (c) cGMP-dependent protein kinase in platelets, acting as an inhibitor of the PPI cycle (79); in this cell type and in neutrophils, cAMP-dependent protein kinase is also inhibiting the cycle possible through inhibition of PI kinase (80,81). In the intestine, direct evidence for a role of any of these factors in regulation of the PPI cycle in the BB is sofar lacking. In chicken enterocytes however, but not in cultured human colonocytes (14,15), cAMP and cGMP have been shown to inhibit Na^+/H^+ exchange in the apical membrane indirectly by mobilization of intracellular Ca^{2+} (17,82). Considering the predominant association of cGMP-dependent protein kinase and a 25 kDa proteolipid, serving as a cosubstrate for cGMP- and cAMP-dependent protein kinase, with the BBM of mammalian enterocytes (49,72), it is tempting to postulate a connection between this regulatory system and the PPI cycle in the same membrane which would offer an elegant explanation for the rise in intracellular Ca^{2+} brought about by cyclic nucleotides. Measurements of PPI metabolism in intact enterocytes are needed to substantiate this model.

Finally, the possibility also exists that the enzymes and substrates of the PPI cycle localized in the BBM do not actually participate in PPI cycling in the intact microvilli but merely serve as a reservoir for replenishment of the components of the PPI cycle in the BLM. This possibility can only be ruled out be identification of agonists capable of changing PPI metabolism in the enterocyte by interaction with apical

membrane receptors. Studies along these lines are presently carried out in our laboratory.

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CAPTER III

MODULATION OF SALT PERMEABILITIES OF INTESTINAL BRUSH BORDER MEMBRANE VESICLES BY MICROMOLAR LEVELS OF INTERNAL CALCIUM

A. Bas Vaandrager, Matty C, Ploemacher and Hugo R, de Jonge (1986)
Biochim. Biophys. Acta 856, 325-336

SUMMARY

A possible modulation of ion permeabilities of rat intestinal brush border membrane vesicles by Ca^{2+}, a putative second messenger of salt secretion, was explored by three independent methods: (1) measurements of [^{3}H]glucose accumulation driven by a Na^+ gradient; (2) stopped-flow spectrophotometry of salt-induced osmotic swelling; (3) $^{86}Rb^+$, $^{22}Na^+$ and $^{36}Cl^-$ flux measurements. Cytoskeleton-deprived membrane vesicles were prepared from isolated brush borders by thiocyanate treatment. Intravesicular Ca^{2+} levels were varied by preincubating vesicles in Ca/EGTA buffers in the presence of the Ca^{2+}-ionophore A23187. At $Ca_{free} > 10^{-5}$ M, initial Na^+-dependent glucose uptake in the presence of a 0.1 M NaSCN gradient (but not in its absence) was inhibited by about 50 per cent as compared to EGTA alone ($ED_{50} \approx 10^{-6}$ M Ca^{2+}). By contrast, initial rates of $^{22}Na^+$ uptake and reswelling rates of vesicles exposed to a NaSCN gradient were increased at least 2-fold by 10^{-5} M Ca_{free}. Both observations are compatible with a Ca^{2+}-induced increase of the Na^+ permeability of the vesicle membrane. The modulation of ion transport was fully reversible and critically dependent on internal Ca^{2+}, suggesting a localization of Ca^{2+}-sensor sites at the inner surface of the microvillus membrane. As shown by radiotracer and osmotic swelling measurements, micromolar Ca^{2+} additionally increased the flux rate of K^+, Rb^+, Cl^- and NO_3^- but did not change the membrane permeability for small uncharged molecules, including glucose and mannitol. The effect of Ca^{2+} on ion permeabilities could be blocked by Ba^{2+} (10^{-3} M) or Mg^{2+} (10^{-2} M), but not by amiloride (10^{-3} M), apamin (2.10^{-7} M), trifluoperazine (10^{-4} M) or quinine (5.10^{-4} M). At present it is unclear whether Ca^{2+} activates a nonselective cation and anion channel or multiple highly selective channels in the vesicle membrane.

INTRODUCTION

Studies of stimulus-secretion coupling in intestinal epithelium have revealed a crucial role for cyclic AMP, cyclic GMP and Ca^{2+} as modulators of transepithelial Na^+ and Cl^- transport [1-4]. The major cyclic nucleotide- and Ca^{2+}-sensitive ion transport systems have been localized in the apical membrane of the enterocyte and consist of (i) an electroneutral Na^+-Cl^- cotransport system in the brush border of the mature villus cell, presumably composed of separate Na^+/H^+ and Cl^-/HCO_3^- exchangers coupled by circular proton movements [5-8] and (ii) an electrogenic Cl^- channel, apparently enriched in intestinal crypt cells [1,9]. The molecular nature of the transporters and of the signal transduction mechanism is largely unknown. An intermediate step in the coupling mechanism shared by both cyclic nucleotides is the cophosphorylation of a 25 kDa proteolipid in the microvillus membrane by the type II-isoenzyme of cyclic AMP-dependent protein kinase and by a unique isoenzyme of cyclic GMP-dependent protein kinase previously discovered in the intestinal brush border [3,4,10-13]. High-affinity receptors for Ca^{2+} in the brush border region may include calmodulin, associated with the cross-filaments [14] and with a Ca^{2+}-dependent protein kinase [3,4,15]; a Ca^{2+}/phospholipid-dependent protein kinase [3,4]; polyphosphoinositides of the lipid bilayer [16]; intrinsic Ca^{2+}-activatable phospholipases [17]; and Ca^{2+} regulatory sites on the transport protein itself.

Studies of ion transport modulation at the level of brush border membrane vesicles, avoiding the complexity of the intact cell, in principle allow a more detailed analysis of the kinetic and regulatory properties of the ion channels and carriers in the apical membrane. A preliminary characterization of ion transport pathways in intestinal brush border membrane vesicles have sofar provide direct evidence for the occurrence of (1) Ca^{2+}-calmodulin inhibition of Na^+-Cl^- cotransport in rabbit brush borders [18]; (2) allosteric regulation of the Na^+/H^+ exchanger by intravesicular pH [19]; (3) Cl^-/OH^- exchange in rat ileal but not in jejunal vesicles [6,7]; (4) selective conductance pathways for Na^+, K^+ and Cl^- [20]; (5) various cotransport systems for Na^+ and non-electrolytes, e.g. glucose [21]; and (6) activation of an anion conductance in rat brush border membrane vesicles loaded with cyclic AMP and an ATP-regenerating

system [13]. The latter study carried out at millimolar levels of Ca^{2+} prompted us to investigate a possible modulation of ion transport pathways by intravesicular Ca^{2+} alone and varied within a physiological concentration range. In order to avoid complications arising from interaction of Ca^{2+} with the microvillar cytoskeleton [22], most experiments were carried out with brush border membrane vesicles deprived of their cytoskeleton by KSCN exposure as described by Hopfer et al. [23]. The results provide evidence for a fully reversible modulation of both cation and anion permeabilities of the vesicle membrane by intravesicular high-affinity Ca^{2+}-receptors apparently different from calmodulin, Ca^{2+}/phospholipid-dependent protein kinase or phospholipase.

MATERIALS AND METHODS

Materials. D-[1(n)-^{3}H]glucose (15.7 Ci/mmol), ^{22}NaCl (11 Ci/mmol), ^{86}RbCl (0.48 Ci/mmol) and H^{36}Cl (0.35 mCi/mmol) were purchased from Amersham International. Valinomycin and Ca^{2+}-ionophore A23187 were obtained from Boehringer. Phlorizin came from Roth, trifluoperazine from Röhm Pharma, apamin and quinine from Sigma. Amiloride was a gift from Merck, Sharp and Dohme. All other chemicals were analytical grade.

Animals. Adult male Wistar rats weighing 300-350 g and fed normal laboratory chow were used throughout this study.

Preparation of vesicles. Each batch of brush border membrane vesicles originated from jejunal and ileal segments of small intestine freshly obtained from 2-3 rats. Under light ether anaesthesia, the intestine was removed and rinsed three times with 20 ml icecold 0.9% NaCl. All further steps were performed at 0-4°C. Intact brush borders were obtained by mechanical vibration (50 Hz, amplitude 1.5 mm) of everted intestine for 25 min in Tris-buffered EDTA (2.5 mM), removal of nuclear aggregates with glasswool and low speed centrifugation as described by Harrison and Webster [24]. Removal of terminal web and cytoskeletal elements, and vesiculation of the microvillus membrane was effected by exposing the brush border to 0.52 M KSCN as described by Hopfer et al. [23]. Vesicles isolated according to this procedure (hereafter referred to as "KSCN-brush border membrane vesicles") are virtually depleted of cytoskeletal proteins (e.g. actin, myosin, the 110 kDa cross filament [22] and calmodulin [14])

as judged by protein staining patterns of SDS-acrylamide gels and ^{125}I-calmodulin radioimmunoassay (results not shown).

Alternatively, intestinal villus cells were released by mechanical vibration and microvillus vesicles were generated by a freeze-thaw technique as described previously [13,25]. Vesicles obtained by this method (hereafter referred to as "Mg-brush border membrane vesicles") were purified by differential Mg^{2+} precipitation and a washing step as described in Ref. 25. For most experiments, vesicles were finally resuspended in buffer A (10 mM Hepes/Tris, 0.3 M mannitol, pH 7.0) by means of a Potter-Elvehjem homogenizer.

Radiotracer flux measurements. Initial rates of [^{3}H]glucose uptake into the vesicles in the presence of an inwardly directed gradient of Na^+ (0.1 M) and the half-filling time for [^{3}H]glucose uptake under isotope equilibrium exchange conditions were determined as described previously [25] using a semi-automatic rapid mixing and stopping apparatus (constructed according to Kessler et al.; [26]) and nitrocellulose filters (Millipore, pore size 0.45 um) to separate vesicles from the medium.

The uptake of $^{22}Na^+$, $^{86}Rb+$ and $^{36}Cl^-$ radiotracers was measured by mixing 50-200 μl of a brush border membrane vesicle suspension in buffer A (1-2 mg protein per ml) with an equivolume of Na^+, K^+ or Cl^- salts (0.2 M) in buffer A containing 1-4 uCi of the various isotopes. With various time intervals, samples of the mixture (50 μl) were loaded on minicolumns (0.6 ml of packed resin) of Dowex AG 50W-X8 (Tris form, 50-100 mesh) or Dowex AG 1-X8 (gluconate form, 50-100 mesh) to promote rapid and quantitative binding of labeled cations or anions present in the vesicle medium. Vesicles were quickly eluted with 1 ml icecold buffer A and processed for liquid scintillation counting (^{36}Cl, $^{86}Rb^+$) or γ-emission spectroscopy ($^{22}Na^+$). The ion exchange separation technique was found superior to Millipore filtration in respect to reproducibility, radioactivity of blanks obtained in the absence of vesicles (5% of vesicle uptake), and separation time (less than 15 s) limiting the efflux of radiotracers during the separation phase.

Light-scattering measurements. Osmotically induced volume changes in brush border membrane vesicles were detected by measuring the changes in light-scattering intensity at 470 nm (slit width 15 nm) at right angle to the incident beam using an Aminco DW2 spectrophotometer operated in the dual beam mode. Brush border membrane vesicles suspended in buffer B (10

mM Hepes/Tris, 0.1 M mannitol, pH 7.0) to a concentration of 0.4-0.6 mg protein per ml were mixed at 25°C in a stopped-flow apparatus (Aminco) with an equivolume (100 µl) of buffer B containing 0.1 M salt or 0.2 M additional mannitol. The light-scattering signal was plotted graphically and recorded simultaneously by a data storage system (Dasar) for subsequent mathematical analysis [27].

Manipulation of intravesicular Ca^{2+} levels. Intra- and extravesicular Ca^{2+} levels were equilibrated by incubating brush border membrane vesicles at 0°C for at least 15 min in the presence of 1 mM EGTA/Ca buffers and 20 µM Ca^{2+}-ionophore A23187 (cf. Ref. 13). Free Ca^{2+} concentrations of the buffers were calculated as described by Bers [28] and verified with a Ca^{2+}-electrode (Radiometer).

Biochemical assays. Protein was determined by the procedure of Lowry et al. [29], using bovine serum albumin as a standard.

Data evaluation. All values shown in the figures and tables represent means of triplicate experiments with a single batch of vesicles and the outcome was representative of at least two other batches of freshly isolated vesicles. The significance of the differences between two conditions was calculated by the (unpaired) Student's t-test.

RESULTS

Effects of Ca^{2+} on Na^+-driven glucose uptake. In brush border membrane vesicles, the initial rates of Na^+-dependent glucose transport are linearly related to the electrochemical driving force for Na^+, which in turn is a function of the membrane potential and the Na^+ concentration gradient across the membrane [30]. The transient accumulation of [^{3}H]glucose by the Na^+-symport carrier could therefore be used as a diagnostic tool to analyze Ca^{2+}-induced permeability changes of the vesicle membrane towards Na^+ and anions.

As shown in Fig. 1, the initial rates of glucose-uptake expressed per mg of protein, but not the glucose "overshoot" defined as: $[glucose]_{max}/[glucose]_{equilibrium}$, was considerably higher in KSCN- as compared to Mg-brush border membrane vesicles, apparently due to the removal of cytoskeletal proteins by the KSCN treatment (cf. Refs. 23,31). Raising the intra- and extravesicular free Ca^{2+} levels in KSCN-vesicles

from 10^{-7} to 10^{-5} M led to a 50% reduction in the initial rate of glucose uptake and in glucose overshoot driven by a NaSCN gradient, but did not affect the equilibrium concentration of intravesicular glucose. Under isotope equlibrium conditions, however, the half-time (t 1/2) for maximal uptake of labeled glucose (determined according to Ref. 25) appeared insensitive to variations in Ca^{2+} levels (9.6±1.8 s at pCa 7.0; 9.4±2.0 s at pCa 5.0; n=4) arguing against a direct effect of Ca^{2+} on the glucose carrier itself. In contrast to these findings in KSCN-vesicles, Na^{+}-dependent glucose uptake in Mg-brush border membrane vesicles appeared unresponsive over a wide range of free Ca^{2+} concentrations (10^{-7}-10^{-3} M; Fig. 1).

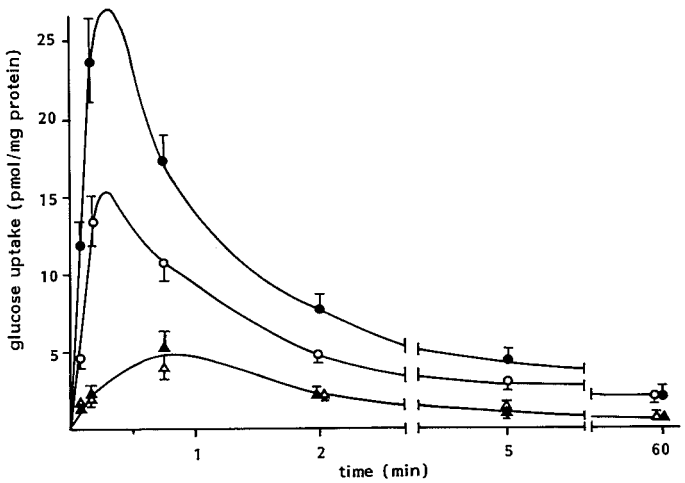

Figure 1.
Na^{+}-dependent glucose transport in intestinal brush border membrane vesicles plotted as a function of time.
KSCN-vesicles (o,●) or Mg-vesicles (△,▲) in buffer A were preincubated for 15 min at $0^{o}C$ in the presence of Ca^{2+}-ionophore A23187 (20 µM) and 1 mM EGTA/Ca mixtures buffered at pCa 7.0 (●,▲) or pCa 5.0 (o,△). Na^{+}-driven glucose uptake was measured in the presence of 1.0 µM [^{3}H]glucose plus 0.1 M NaSCN (see Methods). Vertical bars indicate S.E.

Half-maximal inhibition of glucose uptake in KSCN-vesicles by Ca^{2+} was reached at 10^{-6} M (Fig. 2). If Ca^{2+}-depleted vesicles, preloaded with 0.2 mM EGTA during the vesiculation step and incubated in a medium containing 0.2 mM EGTA and Ca^{2+}-ionophore, were exposed to Ca^{2+} (10^{-3} M), full inhibition of glucose transport was reached without a detectable lag phase (Table I). In the absence of Ca^{2+}-ionophore, however, a similar inhibition was obtained only after 30 min, confirming the low basal Ca^{2+} permeability observed earlier in Mg-brush border membrane vesicles loaded with a Ca^{2+}-probe [13]. The acceleration of Ca^{2+}-inhibition in the presence of ionophore clearly demonstrates that the Ca^{2+}-inhibitory site is localized at the interior of the vesicles.

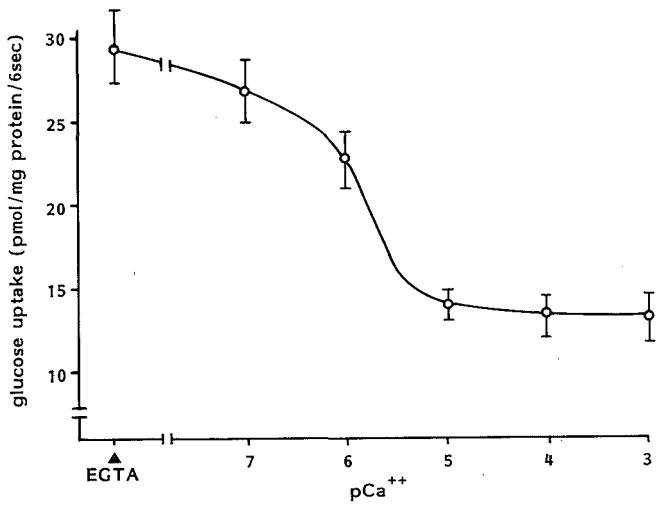

Figure 2.
Na^+-dependent glucose transport in KSCN-brush border membrane vesicles plotted as a function of pCa.
KSCN-vesicles in buffer A were preincubated for 15 min at 0°C in the presence of Ca^{2+}-ionophore A23187 (20 µM) and 1 mM EGTA/Ca buffers covering a broad range of pCa. Na^+-driven glucose uptake was measured at 0.1 min in the presence of 1.0 µM [^{3}H]glucose plus 0.1 M NaSCN (see Methods). Vertical bars indicate S.E.

TABLE I

INHIBITION OF Na^+-DEPENDENT GLUCOSE TRANSPORT IN KSCN-BRUSH BORDER MEMBRANE VESICLES BY Ca^{2+}: RAPID ONSET IN THE PRESENCE OF Ca^{2+}-IONOPHORE

Na^+-driven glucose uptake was measured at 0.1 min in the presence of 1.0 µM [^{3}H]glucose plus 0.1 M NaSCN (see Methods) following preincubation of KSCN-vesicles (prepared in buffer A containing 0.2 mM EGTA) for various times at 25°C in the presence or absence of Ca^{2+}-ionophore A23187 (20 µM) and 1 mM Ca^{2+}. In case of preincubation 0 min, Ca^{2+} was added to the radiotracer mixture instead of to the vesicle suspension

Preincubation time (min)	Addition of Ca^{2+} (1 mM)	[^{3}H]glucose uptake (pmol/0.1 min/mg protein)	
		- A23187	+ A23187
0	-	21±2	21±2
0	+	23±2	11±1*
0.5	+	21±2	10±1*
2.5	+	20±2	11±1*
30	+	13±1	11±1*

Data are means of triplicate experiments ± S.E. *P 0.05 compared to data in the absence of ionophore.

TABLE II

INHIBITION OF Na^+-DEPENDENT GLUCOSE TRANSPORT IN KSCN-BRUSH BORDER MEMBRANE VESICLES BY Ca^{2+}: DEMONSTRATION OF ITS REVERSIBILITY

Na^+-driven glucose uptake was measured at 0.1 min in the presence of 1.0 µM [^{3}H]glucose plus 0.1 M NaSCN (see Methods) following preincubation of KSCN-vesicles in buffer A plus Ca^{2+}-ionophore A23187 (20 µM) for 15 min at 0°C in the presence or absence of 1 mM EGTA/Ca buffers

Preincubation conditions	[^{3}H]glucose uptake (pmol/0.1 min/mg protein)
No additions	9±1
EGTA/Ca (pCa 5.0)	9±1
EGTA/Ca (pCa 7.0)	17±2*
EGTA/Ca (pCa 7.0) followed by 1 mM Ca^{2+} (1 min)	9±1$^+$

Data are means of triplicate experiments ± S.E. *P 0.05 compared to pCa 5.0; $^+$P 0.05 compared to pCa 7.0

TABLE III

Na^+-DEPENDENT GLUCOSE TRANSPORT IN INTESTINAL BRUSHBORDER MEMBRANE VESICLES; EFFECT OF ANION REPLACEMENT, VOLTAGE CLAMPING AND Ca^{2+}

Na^+-driven glucose uptake was measured at 0.1 min in the presence of 1.0 μM [^{3}H]glucose plus 0.1 M Na^+-anion (see Methods). KSCN-vesicles in buffer A were preincubated for 30 min at 0°C in the presence of the Ca^{2+}-ionophore A23187 (20 μM) plus 1 mM EGTA/Ca buffer (pCa 5.0 or 7.0). Voltage clamping was achieved by loading the vesicles during preincubation (60 min, 0°C) with 50 mM K gluconate plus 10 μM valinomycin (final concentration of K gluconate in transport medium: 25 mM). Mg-vesicles in buffer A were preincubated and voltage clamped in the absence of Ca buffers.

Anion	Voltage clamping	[^{3}H]glucose uptake (pmol/0.1 min/mg protein)		
		KSCN-vesicles		Mg-vesicles
		pCa=7.0	pCa=5.0	-
SCN^-	-	23 ±2	12 ±1*	4.5±0.5
	+	21 ±1	15 ±2*	2.5±0.3
NO_3^-	-	14 ±1	8.1±0.7*	1.7±0.2
	+	15 ±2	16 ±2	1.8±0.2
Cl^-	-	4.0±0.5	2.7±0.3*	0.5±0.1
	+	11 ±1	11 ±1	1.5±0.2
Gluconate$^-$	-	2.4±0.3	2.4±0.3	n.d.
	+	9.0±1.1	9.2±1.0	n.d.

Data are means of triplicate experiments ± S.E. *P 0.05 compared to pCa 7.0; n.d., not determined.

As shown in Table II, KSCN-brush border membrane vesicles preincubated in the absence or presence of EGTA/Ca buffer (pCa 5.0) plus Ca^{2+}-ionophore displayed similar rates of glucose uptake, suggesting that free Ca^{2+} levels at the interior of freshly isolated KSCN-vesicles were at least in the micromolar range. Such a level was anticipated in view of the rather high concentrations of Ca^{2+} (5.10^{-6}-10^{-5} M) measured in buffer A. The reversibility of Ca^{2+}-triggered transport inhibition is demonstrated in the same table by showing that initial exposure of KSCN-brush border membrane vesicles to an EGTA/Ca buffer (pCa 7.0) plus ionophore led to a 2-fold increase of the glucose uptake rate whereas subsequent titration with excess Ca^{2+} (10^{-3} M) again lowered the transport rate to a value seen in the absence of Ca^{2+} buffers.

Table III shows a comparison of the effects of anion replacement and voltage clamping on initial rates of Na^+-driven glucose uptake in KSCN-brush border membrane vesicles, exposed to 10^{-7} or 10^{-5} M free Ca^{2+}, and

in Mg-vesicles (no Ca^{2+} buffers added). Clamping of the vesicle membrane at a slightly negative potential (inside) was accomplished by preincubation in the presence of potassium gluconate plus valinomycin and diluting the vesicle suspension 2-fold in the transport assay. The replacement of a monovalent anion by a more permeable species is expected to result in a change in membrane potential (inside negative) leading to acceleration of Na^+-driven glucose transport (cf. Ref. 30). The anion permeability sequence of conductance pathways suggested by the anion replacement data in Table III (first and third column; no voltage clamping) was similar for Ca^{2+}-poor KSCN-vesicles and Mg^{2+}-vesicles ($P_{SCN^-} > P_{NO_3^-} > P_{Cl^-} > P_{gluconate^-}$). In the clamped condition, Na^+-driven glucose uptake was slightly decreased (NaSCN), strongly increased (NaCl, sodium gluconate) or maintained at the same level ($NaNO_3$). This behaviour is consistent with a permselectivity sequence $P_{SCN^-} > P_{NO_3^-} > P_{Na^+} > P_{Cl^-} > P_{gluconate^-}$ creating an inside negative (NaSCN, $NaNO_3$) or inside positive (NaCl, sodium gluconate) diffusion potential across the vesicle membrane. Interestingly, a shift in pCa from 7.0 to 5.0 substantially lowered the rate of glucose uptake in response to a NaSCN, $NaNO_3$ and NaCl gradient but failed to affect glucose transport rates in the voltage clamped condition (except in case of NaSCN, possibly due to incomplete clamping in the presence of the highly permeable SCN^- ion). Both observations are diagnostic for a Ca^{2+}-induced increase of the P_{Na^+}/P_{anion} ratio of the vesicle membrane leading to membrane depolarization. Such a change may originate from an increase of P_{Na^+} or a decrease of P_{anion}. Less indirect measurements of cation and anion permeabilities were needed to discriminate between both possibilities.

Effect of Ca^{2+} on $^{22}Na^+$, $^{86}Rb^+$ and $^{36}Cl^-$ uptake. In the presence of amiloride, an inhibitor of the Na^+/H^+ antiporter [19], and in the absence of Na^+-cotransport, sodium mainly enters the brush border membrane through conductance pathways [7,20]. Using SCN^- as the counterion, the initial uptake of $^{22}Na^+$ into KSCN-brush border membrane vesicles under these conditions showed a 2-fold increase in response to 10^{-5} M Ca^{2+} (Fig. 3). Considering the Ca^{2+} inhibition of NaSCN-driven glucose uptake shown above (Figs. 1,2; Table III), its effect on $^{22}Na^+$ uptake seems more consistent with an increase of P_{Na^+} than with an increase of P_{SCN^-}. As shown in Fig. 4, Ca^{2+} caused an additional increase of the influx of $^{86}Rb^+$ measured in the presence of KSCN or KCl gradient, without changing equilibrium uptake.

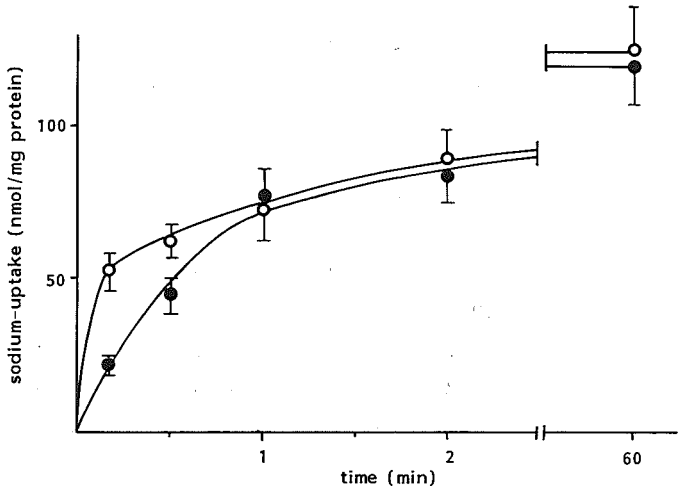

Figure 3.
Uptake of $^{22}Na^{+}$ in KSCN-brush border membrane vesicles plotted as a function of time. KSCN-vesicles in buffer A were preincubated for 15 min at 0°C in the presence of 1 mM amiloride, Ca^{2+}-ionophore A23187 (20 µM) and either 1 mM EGTA alone (●) or 1 mM EGTA/Ca buffered at pCa 5.0 (○). Vesicular $^{22}Na^{+}$ uptake in the presence of 0.1 M NaSCN was measured at 25°C as a function of time by a method involving rapid removal of $^{22}Na^{+}$ from the medium by cation exchange on minicolumns of Dowex AG 50W-X8 as described in Methods. Vertical bars indicate S.E.

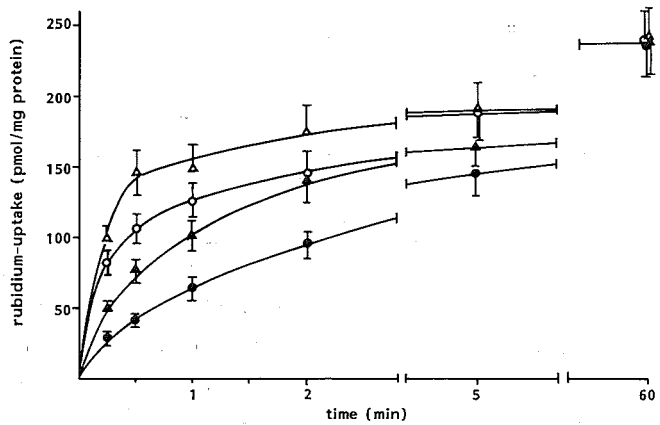

Figure 4.
Uptake of $^{86}Rb^{+}$ in KSCN-brush border membrane vesicles plotted as a function of time. KSCN-vesicles in buffer A were preincubated for 15 min at 0°C in the presence of Ca^{2+}-ionophore A23187 (20 µM) and either 1 mM EGTA alone (▲,●) or 1 mM EGTA/Ca buffered at pCa 5.0 (△,○). Vesicular $^{86}Rb^{+}$ uptake in the presence of 0.1 M KSCN (△,▲) or 0.1 mM KCl (○,●) was measured at 25°C as a function of time by a method involving rapid removal of $^{86}Rb^{+}$ from the medium by cation exchange on minicolumns of Dowex AG 50W-X8 as described in Methods. Vertical bars indicate S.E.

Since K^+ and Rb^+ usually share the same transport system (cf. Ref. 7), these experiments are indicative for a Ca^{2+}-triggerd increase of the K^+ permeability of the brush border membrane. In the presence of 0.1 M KCl, Ca^{2+} was also able to increase the initial rate of $^{36}Cl^-$ uptake in KSCN-brush border membrane vesicles (Table IV). This increase could not be explained entirely by an increase of P_{K^+}, since the K^+-ionophore valinomycin was unable to abolish the effect of Ca^{2+} on $^{36}Cl^-$ uptake. The results of radiotracer experiments taken together point to a general enhancement of both cation and anion permeabilities of the vesicle membrane by micromolar Ca^{2+}.

Effect of Ca^{2+} on salt-induced osmotic swelling. To further assess the influence of Ca^{2+} on the permeability of KSCN-brush border membrane vesicles for monovalent ions and uncharged solutes, osmotic volume changes of a vesicle suspension in response to hypertonic salt or mannitol solutions were measured by monitoring rapid changes in light-scattering. For a restricted range of vesicle osmolarities (below 1 osM), a linear relationship between scattered light intensity and vesicle volume has been established with apical membranes from various sources [27,32]. A fast increasing phase in scattering intensity (less than 0.5 s), corresponding to shrinkage of the vesicles as a result of water outflow, is followed by

TABLE IV
UPTAKE OF $K^{36}Cl$ INTO KSCN-BRUSH BORDER MEMBRANE VESICLES: EFFECTS OF Ca^{2+} AND K^+-IONOPHORE

KSCN-vesicles were preincubated for 15 min at 0°C in the presence of Ca^{2+}-ionophore A23187 (20 µM) and either 1 mM EGTA or 1 mM EGTA/Ca^{2+} buffer (pCa 5.0) plus or minus the K^+-ionophore valinomycin (10 µM). Vesicular $^{36}Cl^-$ uptake in the presence of 0.1 M KCl was measured at 25°C as a function of time by a method involving rapid removal of $^{36}Cl^-$ from the medium by anion exchange on minicolumns of Dowex AG1-X8 as described in Methods

Preincubation condition	$^{36}Cl^-$ uptake (nmol/mg protein) 0.25 min	0.5 min
EGTA	4.4±0.5	8.3±0.8
EGTA/Ca (pCa 5.0)	11.3±1.2*	17 ±2.0*
EGTA + valinomycin	8.1±0.9	15 ±1.4
EGTA/Ca (pCa 5.0) + valinomycin	15.3±1.4$^+$	22 ±2.0$^+$

Data are means of triplicate experiments ± S.E. *P 0.5 compared to EGTA alone; $^+$P 0.05 compared to EGTA + valinomycin

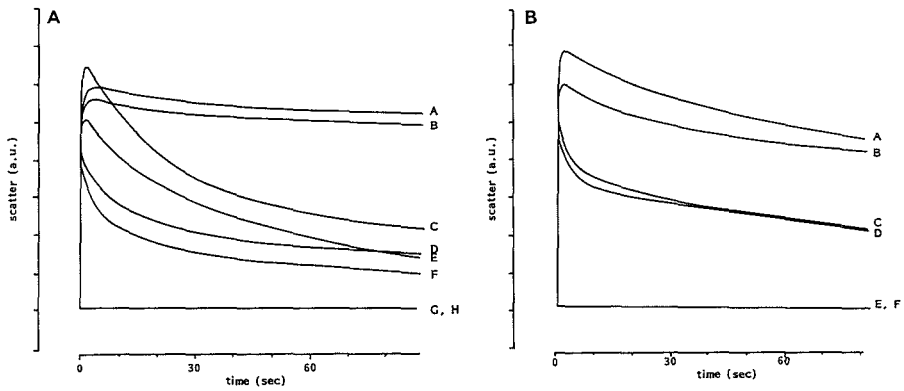

Figure 5.
Effect of Ca^{2+} on osmotic volume changes of KSCN-brush border membrane vesicles in response to salt or mannitol gradients.
KSCN-vesicles in buffer B were preincubated for 15 min at $0^{o}C$ in the presence of Ca^{2+}-ionophore A23187 (20 µM) and either 1 mM EGTA alone (-) or 1 mM EGTA/Ca buffered at pCa 5.0 (+). Shrinkage-reswelling of vesicles in response to 0.05 M salt or 0.1 M mannitol in buffer B was monitored by registration of changes in light-scatter (plotted as arbitrary units) in a stopped-flow apparatus attached to a spectrophotometer as described in Methods. The plots shown were representative for at least two other batches of vesicles.
Conditions in Fig. 5A: A (-, mannitol); B (+, mannitol); C (-, KSCN); D (+, KSCN); E (-, NaSCN), F (+, NaSCN); G (-, buffer B alone); H (+, buffer B alone).
Conditions in Fig. 5B: A (-, KCl); B (-, NaCl); C (+, KCl); D (+, NaCl); E (-, buffer B alone; F (+, buffer B alone).

a slower reswell phase, dependent on solute entry into the intravesicular space. Therefore the velocity of the reswell phase in response to a salt gradient is a function of the total permeability (P) of the vesicle membrane to the least permeable cation or anion.

The results of the osmotic experiments, shown in Figs. 5 and 6, confirm and replenish the glucose transport and radiotracer studies.
(1) In the presence of a highly permeable anion (SCN^{-}), initial rates of reswelling in response to Na^{+} or K^{+} gradient were dramatically increased

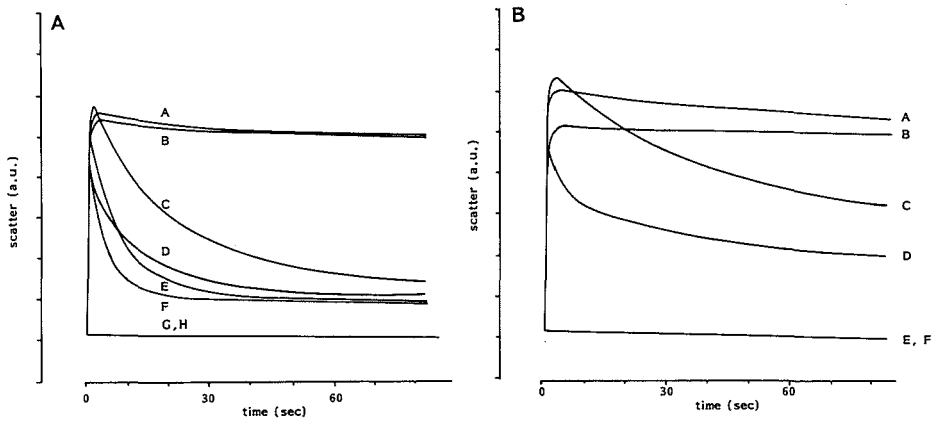

Figure 6.
Effect of Ca^{2+} on osmotic volume changes of KSCN-brush border membrane vesicles in response to K+-salts in the presence of K^+-ionophore.
KSCN-vesicles in buffer B were preincubated for 15 min at 0°C in the presence of the K^+-ionophore valinomycin (10 µM) and the Ca^{2+}-ionophore A23187 (20 µM) and either 1 mM EGTA alone (-) or 1 mM EGTA/Ca buffered at pCa 5.0 (+). Shrinkage-reswelling of vesicles in response to 0.05 M K^+-salt or 0.1 M mannitol in buffer B was monitored by registration of changes in light-scatter (plotted as arbitrary units) in a stopped-flow apparatus attached to a spectrophotometer as described in Methods. The plots shown are representative for at least two other batches of vesicles.
Conditions in Fig. 6A: A (-, mannitol); B (+, mannitol); C (-, NO_3^-); D (+, NO_3^-); E (-, SCN^-); F (+, SCN^-); G (-, buffer B alone); H (+, buffer B alone).
Conditions in Fig. 6B; A (-, gluconate$^-$); B (+, gluconate$^-$); C (-, Cl^-); D (+, Cl^-); E (-, buffer B alone); F (+, buffer B alone).

at 10^{-5} M Ca^{2+} as compared to EGTA alone (Fig. 5A) confirming a Ca^{2+}-triggered increase of P_{Na^+} and P_{K^+}. Apparently, P_{Na^+} is slightly higher than P_{K^+} under both conditions.
(2) Ca^{2+} did not simply increase the leakiness of the membrane to low-molecular weight solutes, as demonstrated by the lack of Ca^{2+} effects on the permeability for mannitol (Figs. 5A and 6A; curve A versus B) and its minor effect on the permeability for gluconate (measured in the presence of K^+ plus valinomycin; Fig. 6B, curve A versus B).

(3) As shown by comparison of the reswell curves A and B in Fig. 5B with curves C and E in Fig. 5A, the P_{Cl^-} of the brush border membrane vesicles must be much lower than P_{SCN^-}, and P_{Na^+} or P_{K^+} are apparently also larger than P_{Cl^-}, in agreement with the permselectivity sequence suggested by the glucose uptake study. The reswell rates in response to NaCl and KCl gradients were strongly increased in the presence of Ca^{2+} (Fig, 5B, curves C and D), suggesting an activating effect of Ca^{2+} on P_{Cl^-}.

(4) If it is assumed that reswelling rates in the presence of K^+ plus valinomycin mainly reflect the anion permeability of the vesicle membrane, the anion permselectivity sequence suggested by comparison of curves C and E in Fig. 6A and curves C and A in Fig. 6B was again similar to the electrogenic conductance sequence concluded from the glucose transport measurements, i.e. $P_{SCN^-} > P_{NO3^-} > P_{Cl^-} > P_{gluconate^-}$. Moreover, Ca^{2+} caused a profound acceleration of the reswell process in the presence of Cl^- (Fig. 6B, curve D versus C) and NO_3^- (Fig. 6A, curve D versus C), directly confirming a Ca^{2+}-provoked increase of the anion permeability of the vesicle membrane. A partial selectivity of the Ca^{2+}-sensitive anion permeability was suggested by the much smaller Ca^{2+}-induced changes observed in the presence of SCN^- (Fig. 6A, curve F versus E) and gluconate$^-$ (Fig. 6B, curve B versus A).

Blockade of the Ca^{2+} effect by Mg^{2+} and Ba^{2+}. Using Na^+-dependent glucose transport as a probe to monitor Ca^{2+} modulation of vesicular ion permeability, various compounds were tested for their ability to antagonize this Ca^{2+} effect (Table V). Amiloride, a blocker of Na^+ channels in colon epithelium and of Na^+/H^+ exchange in ileal brush border membrane vesicles appeared ineffective. Specific blockers of Ca^{2+}-activated K^+ channels, i.e. quinine and apamin, and trifluoperazine, a calmodulin antagonist, were likewise incapable of counteracting Ca^{2+} inhibition of glucose uptake (Table V) or Ca^{2+}-triggered $^{36}Cl^-$ uptake (results not shown). In contrast, Ba^{2+} ions at a rather low concentration (10^{-3} M) completely antagonized the effect of Ca^{2+} on glucose uptake (Table V) as well as on $^{36}Cl^-$ and $^{86}Rb^+$ influx and salt-induced osmotic swelling (not shown). Another bivalent cation, Mg^{2+}, only partially inhibited Ca^{2+} modulation at 10^{-3} M (30-50% inhibition) but was completely inhibitory at 10^{-2} M. Most likely, this Mg^{2+} "brake" (cf. Ref. 33) is also responsible for the lack of Ca^{2+} effects on ion permeabilities in Mg-brush border membrane vesicles since (i) KSCN-vesicles transiently exposed twice to 10

mM Mg^{2+} in order to mimic the isolation conditions of Mg-vesicles, had lost their Ca^{2+}-sensitivity; and (ii) extraction of residual Mg^{2+} from Mg^{2+}-vesicles by incubation for 30 min at room temperature in the presence of 5 mM EDTA and 20 μM A23187, unmasked a similar Ca^{2+}-sensitivity of the Na^{+}-dependent glucose transport process as found routinely in KSCN-vesicles (results not shown). The dependence on Ca^{2+}/Mg^{2+}-ionophore for sensitization is diagnostic for a localization of the Mg^{2+}-inhibitory site at the vesicle interior.

TABLE V

EFFECT OF VARIOUS COMPOUNDS ON THE INHIBITION OF Na^{+}-DEPENDENT GLUCOSE TRANSPORT IN KSCN-BRUSH BORDER MEMBRANE VESICLES BY Ca^{2+}

Na^{+}-driven glucose uptake was measured at 0.1 min in the presence of 1.0 μM [^{3}H]glucose plus 0.1 M NaSCN (see Methods) following preincubation of KSCN-vesicles in buffer A for 15 min at 0°C in the presence of Ca^{2+}-ionophore A23187 (20 μM) and either 1 mM EGTA or 1 mM EGTA/Ca buffer (pCa 5.0). Compounds to be tested were present during preincubation.

Compound	Concentration (M)	[^{3}H]glucose uptake (pmol/0.1 min/mg protein)		
		EGTA	EGTA/Ca pCa 5.0	% inhibition of Ca^{2+} effect
-	-	23±2	13±1	0
Amiloride	10^{-3}	24±3	13±1	0
Apamin	2.10^{-7}	23±1	14±1	0
Ba^{2+}	10^{-3}	22±2	23±2*	100
	3.10^{-4}	23±2	18±2*	50
Mg^{2+}	10^{-3}	23±2	17±2*	40
	10^{-2}	21±2	22±2*	100
Quinine	5.10^{-4}	24±3	13±1	0
Trifluoperazine	10^{-4}	24±3	12±1	0

Data are means of triplicate experiments ± S.E. *P 0.05 compared to control (-).

DISCUSSION

By using three independent techniques to characterize salt permeabilities of rat intestinal brush border membrane vesicles prepared by the thiocyanate method, a Ca^{2+}-triggered increase of both cation and anion permeabilities of the vesicle membrane by micromolar levels of Ca^{2+} has been clearly demonstrated. On the basis of Na^+-dependent glucose uptake measurements, the following conclusions could be drawn: (i) Ca^{2+} apparently acts through a high-affinity receptor ($K_D \approx 10^{-6}$ M; Fig. 2) localized at the interior of the vesicle membrane (Table I); (ii) in view of the depletion of cytoskeletal elements in KSCN-vesicles, interaction of Ca^{2+} with Ca^{2+}-binding proteins of the microvillar core e.g. villin [22] seems unlikely; (iii) the action of Ca^{2+} was fully reversible (Table II), arguing against the involvement of a Ca^{2+}-activatable phospholipase (rapid resynthesis of phospholipids is hampered by a lack of energy source, e.g. ATP or CTP, at the vesicle interior); (iv) the Ca^{2+} effect on transport is blocked partially by 10^{-3} M Mg^{2+} and completely by 10^{-3} M Ba^{2+} or 10^{-3} M Mg^{2+}, suggesting that these bivalent cations compete with Ca^{2+} for a common binding site (cf. the effect of $Ba2^+$ on Ca^{2+}-activated K^+ channels in renal apical membranes; Ref. 34). The Mg^{2+}-brake may also explain why Ca^{2+} modulation of glucose or ion transport has not been observed earlier by us and others in Mg-brush border membrane vesicles; depletion of Mg^{2+} with EDTA and the Ca^{2+}/Mg^{2+}-ionophore A23187 however resensitized the ion channels to micromolar levels of Ca^{2+}. Because the physiological level of free Mg^{2+} at the interior of the microvilli is unknown (probably around 1-2 mM), the efficacy of the Mg^{2+}-brake in the intact enterocyte is difficult to assess; (v) no evidence was found of a direct effect of Ca^{2+} on the Na^+-glucose symporter; moreover, Ca^{2+} did not induce a general leakiness of the vesicle membrane to small molecules, e.g. glucose (Fig. 1, equilibrium uptake) or mannitol (Figs. 5 and 6); (vi) the reduction of Ca^{2+} effects on Na^+-driven glucose transport under voltage clamped conditions (Table III) indicated a major effect of Ca^{2+} on Na^+ and/or anion conductance pathways in the vesicle membrane (increase of P_{Na^+} or decrease of P_{anion}). Because the radiotracer and osmotic swelling experiments provided evidence for a Ca^{2+}-provoked increase of both anion and

cation permeabilities, the inhibitory effect on glucose uptake can only result from an increase of P_{Na^+}, apparently not fully compensated by a concomitant rise in P_{anion} (Ca^{2+}-triggered permselectivity change).

Osmotic shrinkage-swelling experiments (Fig. 5 and 6) in principle enabled us to study the effects of Ca^{2+} on both carrier-mediated and conductance pathways for ions, avoiding complications arising from ion binding to the vesicle membrane. As shown earlier for gastric apical membrane vesicles [27], salt-induced reswelling rates measured in Ca^{2+}-depleted KSCN-brush border membrane vesicles could be fitted to a double exponential (A.B. Vaandrager, unpublished data), suggesting a functional heterogeneity of the vesicle population. Upon exposure to Ca^{2+}, the first phase, in contrast to the second (slow) phase, was accelerated dramatically under conditions that either Na^+ and K^+ (Fig. 5A) or NO_3^- and Cl^- (Figs. 6A and B) were the rate-limiting ions. The fall in peak height of the shrinkage-swelling curve additionally indicated that the salt permeability in the presence of Ca^{2+} was no longer orders of magnitude different from the water permeability of the vesicle. Although a quantitative description of the Ca^{2+} effect in terms of rate constants and ion permeability coefficients is hampered by the functional heterogeneity of the vesicle population, the data do suggest the existence of nonselective cation- and anion channels unmasked by micromolar Ca^{2+} and presumably confined to a subpopulation. By comparison with Ca^{2+} inhibition of Na^+-dependent glucose uptake, a specific function of the brush border membrane, at least the change in Na^+ conductance is apparently localized in a subpopulation of vesicles orginating with certainty from the intestinal microvilli.

It should be pointed out that, independent of the intravesicular Ca^{2+} level, the permeability sequence of Na^+ and Cl^- ions observed in both KSCN- and Mg-brush border membrane vesicles ($P_{Na^+} > P_{Cl^-}$) was clearly opposite to the sequence reported for rabbit intestinal brush border membrane vesicles prepared by the Ca^{2+}-precipitation method [20]. The reason for this discrepancy is not clear but may be related to the species difference or to the different vesicle isolation procedure.

At present we can only speculate about the molecular nature of he high-affinity Ca^{2+} receptor. Considering its insensitivity to quinine, apamin and trifluoperazine, the receptor does not seem to belong to the major class of Ca^{2+}-activatable K^+ channels (quinine- and apamin-inhibitable)

neither can it be identical to calmodulin. This does not disqualify calmodulin as a potential regulator of vesicular ion transport, e.g. neutral Na^+-Cl^- cotransport [18], because (i) the residual calmodulin content in KSCN-treated vesicles was extremely low (2.10^{-5} M) compared to Mg-vesicles (5.10^{-4} M; cf. Ref. 14); (ii) the conditions in our ion transport experiments (0.05-0.1 M salt) were unfavorable for the detection of saturable non-conductance pathways [7,20]. More complicated mechanisms of Ca^{2+} regulation, e.g. involving a Ca^{2+}-dependent phosphorylation process [2-4], can be excluded in view of a lack of ATP in KSCN-vesicles. Instead, the possible presence of Ca^{2+}-regulatory sites on the channels itself might well explain the survival of Ca^{2+} regulation in "empty" brush border membrane vesicles, its reversibility and its inhibition by Mg^{2+} and Ba^{2+}. Alternatively, the Ca^{2+} sensor site could be identical to a high-affinity Ca^{2+} binding component of the lipid bilayer, e.g. polyphosphoinositides [16,35], recently identified in intestinal brushborders and KSCN-vesicles (Chapter II, this thesis).

To our knowledge, no physiological equivalent of a Ca^{2+}-triggered nonselective cation channel has been assigned to the apical membrane of the enterocyte. Nonselective cation channels displaying a similar Ca^{2+}-sensitivity have been characterized in cardiac [36], neuroblastoma [37] and pancreatic acinar cells by a patch-clamp technique [38] but their relationship to the cation conductance in intestinal brush border membrane vesicles is unclear. A Ca^{2+}-opened cation channel found in the apical membrane of rat colonocytes seemed rather specific for K^+ [39]. In some tissues, silent conductive channels for K^+ and Cl^- may be activated by intracellular Ca^{2+} to promote KCl efflux in response to osmotic swelling [40]. However, the existence of a similar mechanism of volume regulation in the enterocyte has not been demonstrated sofar. In contrast, a Ca^{2+}-triggered anion channel probably enriched in the apical membrane of the intestinal crypt cell, has been postulated as a major target for intestinal secretagogues acting through intracellular Ca^{2+} signals [1-4]. Although the vesicles used in the present study mainly originate from mature villus cells, it could be speculated that a silent anion channel in the brush border membrane may become reactivated by the removal of inhibitory factors during the vesicle isolation procedure.

Regardless of its possible physiological significance, the finding of Ca^{2+}-activatable and Mg^{2+}-inhibitable conductance pathways for cations and

anions in intestinal brush border membrane vesicles may have important implications for other vesicle studies: first, dependent on the intravesicular Mg^{2+} level, the channels may become spontaneously activated by trace amounts of Ca^{2+} in the medium, leading to changes in permeability of the vesicle membrane and to diminished and variable rates of Na^+-driven cotransport processes; secondly, in the absence of a Mg^{2+}-brake, a possible inhibitory effect of Ca^{2+} on a Na^+-Cl^- cotransport system in the brush border membrane [1,8] could become masked by a concomitant opening of Na^+ and Cl^- conductance pathways; finally, a better knowledge of the effect of physiological Ca^{2+} levels on ion transport characteristics of "empty" brush border membrane vesicles is a prerequisite for the subsequent study of Na^+ and Cl^- transport regulation by other physiological regulatory factors (e.g. ATP, cyclic nucleotides, calmodulin, protein kinases) entrapped at the vesicle interior during vesiculation [13] or by osmotic shock [41]. Studies along this line are in progress in our laboratory.

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CHAPTER IV

A SENSITIVE TECHNIQUE FOR THE DETERMINATION OF ANION EXCHANGE ACTIVITIES IN BRUSH BORDER MEMBRANE VESICLES.

Evidence for two exchangers with different affinities for HCO_3^- and SITS in rat intestinal epithelium

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submitted to Biochim. Biophys. Acta

SUMMARY

A large percentage (up to 70%) of $^{36}Cl^-$ influx in brush border membrane vesicles from rat small intestine under isotope equilibrium conditions was found to be mediated by SITS inhibitable anion exchange. This Cl^- anion exchange could be measured 10-15 times more sensitive by determining the uptake of trace amounts of $^{125}I^-$ driven by a large Cl^- gradient (in > out) generated by passing the vesicles through an anion-exchange column. Voltage clamping of the vesicle membrane with K^+ and valinomycin did not effect the chloride driven $^{125}I^-$ uptake, showing that the "overshooting" I^- uptake was not mediated by an electrical diffusion potential, as might be generated by the Cl^- gradient in the presence of a chloride channel.
The Cl^-/anion exchange was further characterized in brush border membrane vesicles from both rat ileum and jejunum by studying the inhibitory action of various anions on the Cl^- driven I^- uptake. NO_3^-, Cl^- SCN^- and formate at 2 mM could inhibit Cl^-/I^- exchange for more than 80%. The ileal brush border membrane vesicles displayed a clear heterogeneity with respect to the inhibitory action of SO_4^{2-}, SITS and HCO_3^- on Cl^-/I^-exchange. Approximately 30% of the Cl^-/I^- exchange was insensitive to SO_4^{2-} and showed a relatively low sensitivity to SITS (IC_{50} $\approx$ 1 mM) but could be inhibited for 80% by 2 mM HCO_3^-. Presumably this exchange represents the "classical" Cl^-/OH^- or Cl^-/HCO_3^- exchange. The other 70% showed a high sensitivity to SO_4^{2-} (IC_{50} $\approx$ 0.5 mM) and SITS (IC_{50} $\approx$ 2.5 μM) but was less sensitive to HCO_3^-. This part of the exchange activity showed inhibition characteristics very similar to the Cl^-/I^- exchange in the jejunal vesicles. The latter process was also inhibited for 80% by 2 mM oxalate. As discussed in this paper both exchangers may be involved in the electroneutral transport of NaCl across the apical membrane of the small intestinal villus cell.

INTRODUCTION

The active transport of sodium and chloride across the epithelium of the small intestine can be modulated by intracellular second messengers like cAMP, cGMP and Ca^{2+}-ions [1-4]. According to the model proposed by Field [5] the major cyclic nucleotide- and Ca^{2+}-sensitive ion transporters are (i) an electroneutral Na^+-Cl^- cotransport system, located in the apical membrane of the mature villus cell and (ii) an electrogenic Cl^- channel, presumably enriched in the intestinal crypt cell. The molecular nature of the transporters and of the signal transduction mechanisms involved is largely unknown. Phosphorylation studies with isolated brush borders and brush border membrane vesicles derived from intestinal villus cells provided evidence for the cophosphorylation of a 25 kDa proteolipid by an endogenous cAMP-dependent protein kinase and a unique isoenzyme of cGMP-dependent protein kinase [3,4-6]. A Ca^{2+}/calmodulin- and a Ca^{2+}/ phospholipid-dependent protein kinase and specific substrate proteins for these kinases were also detected in brush border membranes [3,4]. Transport studies with brush border membrane vesicles from intestinal villus cells so far demonstrated the existence of (i) non-saturable electrogenic pathways for Na^+ and Cl^- [7-9] and (ii) an electroneutral Na^+/H^+ [10,11] and a Cl^-/OH^- or Cl^-/HCO_3^- exchanger [7,12] presumably coupled by circular proton movements, but failed to demonstrate unambiguously the presence of a true Na^+-Cl^- cotransporter [8,13]. Both exchangers are potential targets for second messengers, inhibiting salt transport across villus epithelium. Prior to a detailed study of second messenger regulation of anion exchange at the level of isolated brush border membrane vesicles, we first developed a simple and sensitive method to characterize Cl^-/anion exchange activity in apical membrane vesicles from rat ileum and jejunum. The method exploits a large chemical gradient of Cl^- across the membrane (in >> out) to energize an overshoot of $^{125}I^-$, mediated through a Cl^-/anion exchanger. The results provided evidence for the existence of two different types of Cl^- anion exchangers, a SITS- and SO_4^{2-} sensitive exchanger found in both jejunum and ileum, and a HCO_3^- sensitive exchanger present only in the ileum. Both exchangers may be involved in electoneutral NaCl transport.

MATERIALS AND METHODS

Materials. $H^{36}Cl$ (0.45 mCi/mmol) and $Na^{125}I$ (2000 Ci/mmol) were obtained from Amersham. Valinomycin was purchased from Boehringer and 4- acetamido-4´isothiocyanatostilbene-2,2´-disulfonate (SITS) from Sigma. All other chemicals were analytical grade.

Methods.

Preparation of vesicles. Each batch of brush border membrane vesicles originated from 30 cm long segments of ileum or jejunum or from a 90 cm combined segment from small intestine freshly obtained from 3-4 adult male Wistar rats, weighing 300-350 g. Brush border membrane vesicles were generated from isolated villus cells by a freeze-thawing technique and purified by differential Mg^{2+} precipitation and a washing step as described previously ("Mg-brush border membrane vesicles") [14]. Alternatively brush border membrane vesicles were prepared from isolated brush borders [9] by treatment with 0.52 M KSCN as described by Hopfer [15]. Vesicles isolated by this procedure are virtually devoid of cytoskeletal proteins [9,15]. The vesicles were finally resuspended in buffer A (300 mM mannitol, 20 mM HEPES/Tris) pH 7.0 or pH 8.2 and loaded with salts by preincubation for 1 h at 0° C.

Transport studies. The uptake of $^{36}Cl^-$ was measured by mixing 50 µl of a vesicle suspension in buffer A (pH 7.0) containing 25 mM KCl or Kgluconate (3-5 mg protein/ml) with 250 µl of buffer A (pH 7.0) containing 25 mM $K^{36}Cl$ (3 µCi). With various time intervals 50 µl samples were loaded on minicolumns (0.6 ml of packed resin) of Dowex AG1-X8 anion exchanger (gluconate form, 50-100 mesh) and quickly eluted with 1 ml icecold buffer A. Radioactivity in the eluate was detected by liquid scintillation counting.

$^{125}I^-/Cl^-$ exchange was measured by a modification of the procedure described by Garty et al. [16] for the detection of cation channels in a heterogeneous population of vesicles. 100 µl of the brush border membrane vesicle suspension in buffer A (pH 7.0 or 8.2) preloaded with 100 mM Cl^- (K^+ or choline) were applied to a similar minicolumn of Dowex anion exchanger (0.7 ml of packed resin) as used in the $^{36}Cl^-$ uptake studies, and the column was eluted with 450 µl of buffer A. The last 300 µl of the eluate which contained approx. 90% of the vesicles were collected and

mixed with 25 µl of $Na^{125}I$ (0.5 µCi; 1 nM final concentration) one minute after the the column loading. Exact timing was essential to avoid differences in intravesicular Cl^- concentration due to the rapid efflux of this ion following the generation of a steep chemical gradient. At various time points extravesicular $^{125}I^-$ was removed by loading 50 µl of mixture on a second Dowex minicolumn and eluting this column with buffer A exactly as described for the $^{36}Cl^-$ uptake measurements. In a typical experiment for the analysis of inhibition of the I^-/Cl^- exchanger, 300 µl vesicle suspension eluted from the first column was split into 3 aliquots (100 µl) and mixed with 25 µl Kgluconate (final concentration 85 mM) additionally containing the inhibiting anion and $Na^{125}I$. The ^{125}I uptake was terminated by column filtration 60 and 90 s after the mixing at which time points the $^{125}I^-$ uptake was at its maximum in the absence of inhibitors. Cl^-/I^- specific exchange was defined as the difference between the $^{125}I^-$ uptake in the presence or absence of a Cl^- gradient.

Protein was measured by the method of Lowry [17] with bovine serum albumin as a standard.

RESULTS

The presence of 25 mM Cl^- at the interior of rat brush border membrane vesicles obtained by differential Mg^{2+} precipitation caused a 2.5-3 fold stimulation of $^{36}Cl^-$ uptake at 15 s as compared to intravesicular gluconate (Fig.1) or mannitol (not shown). SITS (4 mM), an inhibitor of anion exchange, completely abolished this stimulation (Fig.1). This shows that intestinal brush border membrane vesicles contain an active anion exchange mechanism, which can account for up to 70% of the $^{36}Cl^-$ uptake into the vesicles under isotope equilibrium conditions and displays a low sensitivity to gluconate. To increase the sensitivity of the anion exchange assay, we tried to establish a large Cl^- gradient across the vesicle membrane ($[Cl^-]_{in} >> [Cl^-]_{out}$) by passing the vesicles, preloaded with 100 mM Cl^-, through a Dowex anion exchange column in order to replace the extravesicular Cl^- by gluconate. In view of the low specific radioactivity of the $^{36}Cl^-$ isotope however, this approach required the presence of millimolar concentrations of Cl^- in the extravesicular medium and therefore severely limited the magnitude of the Cl^- gradient needed to

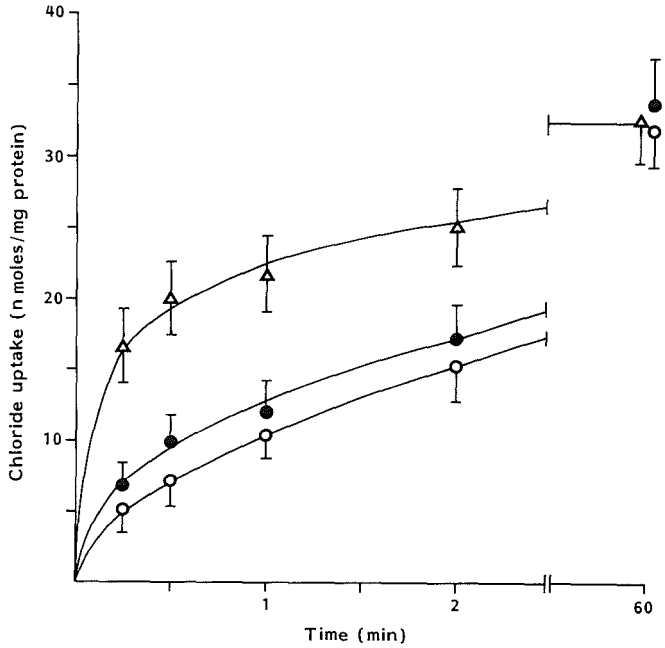

Figure 1
Stimulation of $^{36}Cl^-$ uptake in intestinal brush border membrane vesicles by internal Cl^-.
Intestinal brush border membrane vesicles in buffer A, pH 7.0 were preincubated for 60 min at 0^o with valinomycin (10 μM) and 25 mM K Gluc (•) or 25 mM KCl (o,Δ). $^{36}Cl^-$ (25 mM outside) uptake was measured as described in MATERIALS AND METHODS at 25^oC as a function of time. o, 4 mM SITS was present during both preincubation (15 min) and incubation. Vertical bars indicate S.E., (n=3).

energize the overshooting uptake of $^{36}Cl^-$. The maximum overshoot seen in the presence of 5 mM external Cl^- was only 2-3 fold (not shown); however a 10-15 fold overshoot could be attained by replacing $^{36}Cl^-$ by nanomolar concentrations of $^{125}I^-$, which also has a high affinity for most anion exchangers studied [18,12] (Fig. 2). The overshoot gradually declined after 2 min presumably as a result of the collapse of the Cl^- gradient due to Cl^- efflux. If however the vesicles were preloaded with gluconate instead of Cl^-, uptake at 1 min and equilibrium uptake of $^{125}I^-$ were low (generally less than 10% of the overshoot observed in Cl^--loaded vesicles; results not shown), indicating that the overshoot could not be simply explained by excessive binding of $^{125}I^-$ to the exterior of the vesicle

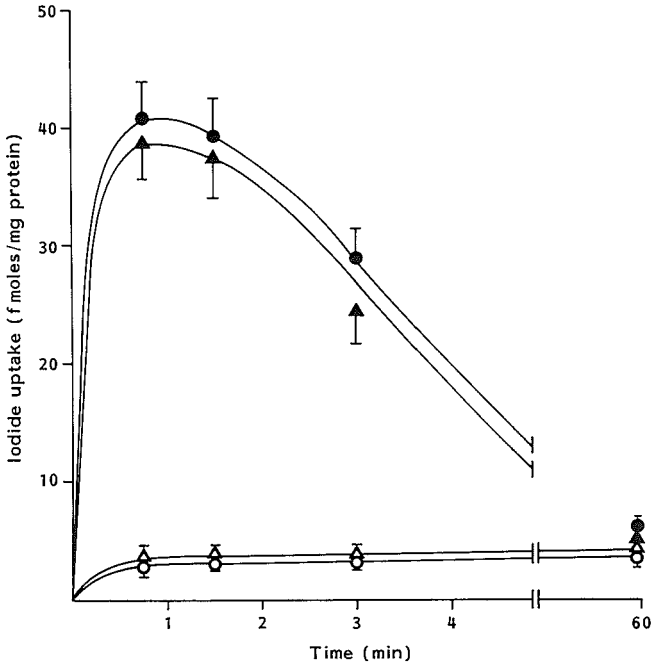

Figure 2
Cl^- driven $^{125}I^-$ uptake in brush border membrane vesicles plotted as a function of time.
Intestinal brush border membrane vesicles in buffer A pH 7.0 were preincubated for 1 h at 0°C with 75 mM choline Cl and 25 mM KCl. After replacement of the extravesicular Cl^- by gluconate on a Dowex AG-1 anion exchange column, $^{125}I^-$ (1 nM) uptake was measured at 25°C as a function of time (see MATERIALS AND METHODS). ●, no addition; ▲, + valinomycin (10 µM) present during preincubation; △ + SITS (4 mM) present in both preincubation and incubation; o, no Cl^- gradient present. Vertical bars indicate S.E., (n=3).

membrane in the absence of competing Cl^- and subsequent replacement of the bound isotope by Cl^- which is gradually released from the Cl^--loaded vesicles into the extravesicular medium. The observation that voltage clamping of the vesicles with K^+ and valinomycin had no effect on the I^- uptake in the presence of a Cl^- gradient (Fig. 2) argues against electrogenic coupling of I^- and Cl^- transport by a membrane potential as might be generated by a large Cl^-/K^+ conductance ratio of the vesicle membrane (cf. Ref. 16). The stimulation of I^- uptake by a Cl^- gradient must therefore represent an electroneutral Cl^-/I^- exchange, which is in agreement with the inhibitory effect of 4 mM SITS.

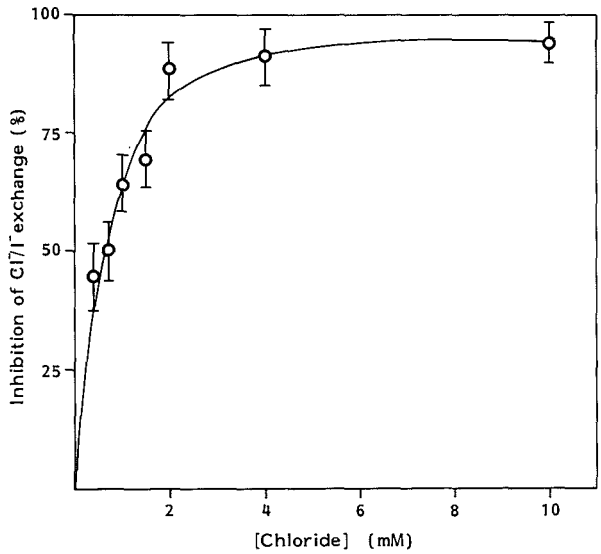

Figure 3
Effect of external Cl^- on Cl^- driven $^{125}I^-$ uptake in intestinal brush border membrane vesicles. Inhibition of $^{125}I^-$ uptake was measured in brush border membrane vesicles from rat small intestine suspended in buffer A pH 8.2 in the presence of a Cl^-gradient (100 mM KCl inside, 85 mM gluconate outside) at 25°C between 60 and 90 s as described in MATERIALS AND METHODS. KCl was mixed with the $^{125}I^-$ before the addition of the vesicles. K gluconate was added at a concentration to establish a constant concentration of salt (85 mM) on the outside. Vertical bars indicate S.E., (n=3).

In order to further characterize this I^-/Cl^- exchange process present in brush border membrane vesicles we measured the Cl^- driven I^- uptake in the presence of various anions in the extravesicular medium. Because the I^- uptake was only linear during the first seconds we instead determined the percentage of inhibition of Cl^- driven I^- uptake at the time points when I^- uptake was maximal (between 1 and 2 min). Apparently the $^{125}I^-$ influx by the I^-/Cl^- exchanger is then balanced by the $^{125}I^-$ efflux. Considering the low internal concentration of $^{125}I^-$ in comparison with Cl^-, the assumption is made that (i) $^{125}I^-$ efflux rates are linearly related to intravesicular $^{125}I^-$ levels and (ii) $^{125}I^-$ efflux from the vesicles is mediated through transport pathways different from the Cl^-/I^-exchanger and is therefore unlikely to be sensitive to external anions. Therefore the $^{125}I^-$ uptake between 1 and 2 min can be taken as an indicator of the activity of the Cl^-/I^- exchanger at these time points. If we additionally assume a competitive interaction between $^{125}I^-$ and the second anion for the extravesicular binding site on the Cl^-/anion exchanger, inhibition of

the $^{125}I^-$ uptake by the non-radioactive anion can be described by the following equation: $IC_{50} = (1 + \frac{S}{K_m}) K_i$ [17] in which IC_{50} is the concentration of anion causing 50% inhibition. S represents the concentration of external $^{125}I^-$, K_m is the Michaelis Menten constant for carrier-mediated I^- uptake, and K_i is the inhibitory constant for the competing anion. Since S << K (as evidenced by the lack of effect of addition of 100 nM cold I^- on $^{125}I^-$ uptake; not shown), the IC_{50} approaches the K_i under our conditions. Therefore the IC_{50} can be used as an indicator for the affinity of the inhibiting anion for the external site of the exchanger. In order to stabilize the HCO_3^- concentration during the assay all studies involving inhibitors were performed at pH 8.2. $^{125}I^-$ uptake in the absence of competing anions was not significantly different at pH 7.0 and pH 8.2 (compare Figs. 2 and 6). In Fig. 3 it is shown that extravesicular chloride inhibits the Cl^-/I^- exchange in a concentration dependent manner ($IC_{50} \approx 0.5$ mM). Data on inhibition by other anions measured separately in jejunal and ileal vesicles are summarized in Table I. Externally added SCN^-, NO_3^-, I^-, Cl^-, formate and oxalate could strongly inhibit the I^-/Cl^- exchange at 2 mM concentrations while acetate and phosphate were much less

TABLE I
EFFECTS OF VARIOUS ANIONS ON Cl^- DRIVEN $^{125}I^-$ UPTAKE IN ILEAL AND JEJUNAL BRUSH BORDER MEMBRANE VESICLES

$^{125}I^-$ uptake in brush border membrane vesicles from rat ileum and jejunum suspended in buffer A pH 8.2 was measured in the presence of a Cl^- gradient (100 mM KCl inside, 85 mM KGluc outside) at 25°C, between 60 and 90 s as described in MATERIALS AND METHODS. K-salts of various anions were mixed with the $^{125}I^-$ to reach a final concentration of 2 mM. Data are means of triplicate experiments ± S.E. n.d., not determined.

Anion	% inhibition of Cl^-/I^- exchange	
	Jejunal vesicles	Ileal vesicles
gluconate	0	0
formate	77±5	82±5
acetate	4±15	25±10
oxalate	82±5	n.d.
HPO_4^{2-}	18±10	n.d.
SO_4^{2-}	78±5	64±6
HCO_3^-	25±8	55±7
NO_3^-	97±3	95±3
Cl^-	90±4	91±3
SCN^-	100±3	100±3
I^-	100±3	100±3
SITS	100±3	92±4

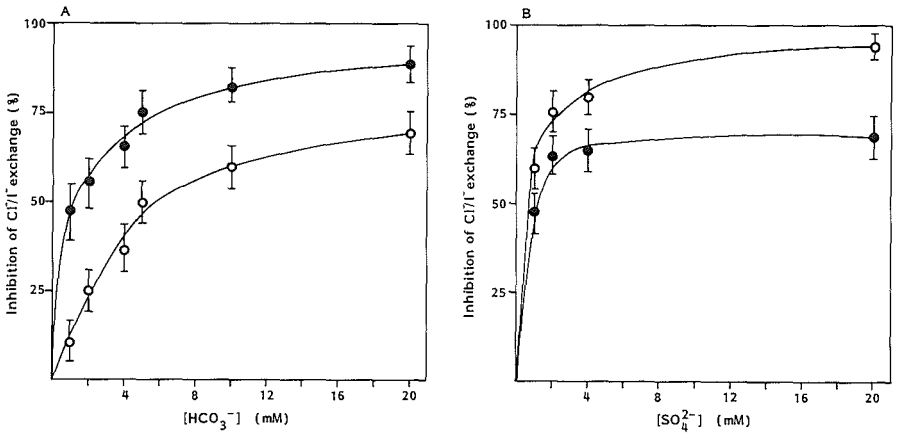

Figure 4
Effects of SO_4^{2-} and HCO_3^- on Cl^- driven $^{125}I^-$ uptake in ileal and jejunal brush border membrane vesicles. Inhibition of $^{125}I^-$ uptake was measured in brush border membrane vesicles from ileum (•) or jejunum (o) in buffer A pH 8.2 in the presence of a Cl^-gradient (100 mM KCl inside, 85 mM Kgluconate/anion outside) at 25°C between 60 and 90 s as described in MATERIALS AND METHODS. $KHCO_3$ (A) or K_2SO_4 (B) were mixed with the $^{125}I^-$ before the addition of the vesicles to reach the final concentration as stated in the figures. The concentration of Kgluconate was varied to keep the total salt concentration on the vesicle outside constant (85 mM). Vertical bars indicate S.E., (n=3).

inhibitory. Interestingly 2 mM of HCO_3^-caused a more than two times larger inhibition in ileal compared to jejunal brush border membrane vesicles (55% versus 25% respectively). SO_4^{2-} was more effective in inhibiting I^-/Cl^- exchange in vesicles from jejunum. The concentrations of HCO_3^- needed for half-maximal inhibition of the Cl^-/I^- exchange was 1.8 mM in vesicles from ileum but 6 mM in jejunal vesicles (Fig. 4), indicating a difference in the affinity of the Cl^-/anion exchanger for HCO_3^- between these two segments of the small intestine. In contrast, the dose dependency of the Cl^-/I^- exchange inhibition by SO_4^{2-} (Fig. 5) indicates that the difference in inhibition between vesicles from ileum and jejunum is due to a difference in the maximal percentage of inhibition but not to a difference in the IC_{50} for SO_4^{2-} (0.5-0.6 mM for both types of vesicles). Approximately 30% of the Cl^-/I^- exchange in ileal brush border membrane vesicles could not be inhibited by relatively large concentrations (20 mM) of SO_4^{2-}, raising the possibility that Cl^-/I^- exchange in ileal vesicles is performed by at least two different exchangers, one of which has a very low affinity for SO_4^{2-}. This possibility is corrobated by the biphasic

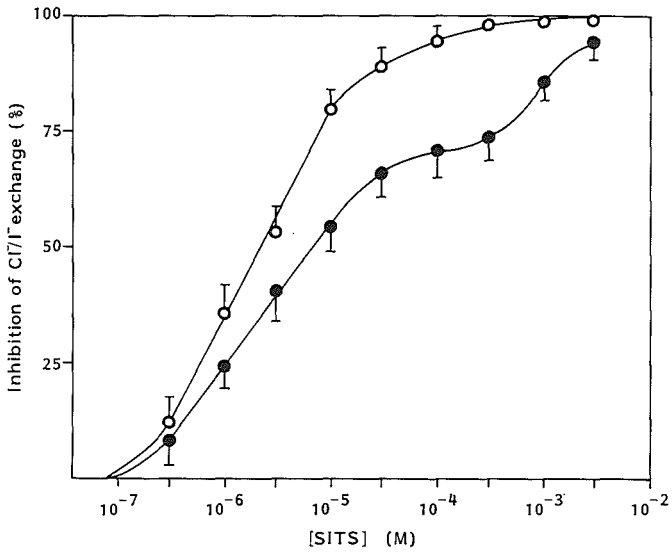

Figure 5
Effect of SITS on Cl^- driven $^{125}I^-$ uptake in rat ileal and jejunal brush border membrane vesicles. Inhibition of $^{125}I^-$ uptake was measured in brush border membrane vesicles from rat ileum (•) or jejunum (o) in buffer A pH 8.2 in the presence of a Cl^- gradient (100 mM KCl inside, 85 mM K gluconate outside) at 25° C between 60 and 90 s as described in MATERIALS AND METHODS. SITS was mixed with the $^{125}I^-$ before the addition of the vesicles to reach a final concentration as stated in the figure. Vertical bars indicate S.E., (n=3).

character of the inhibition curve of Cl^-/I^- exchange by SITS in ileal, but not in jejunal vesicles (Fig. 5). It can be established from Fig. 5 that virtually the Cl^-/I^- exchange activity in jejunal and 70% of the exchange in ileal vesicles is extremely sensitive to SITS ($IC_{50} \approx 2.5\ \mu M$), whereas the residual part of the Cl^-/I^- exchange in ileal vesicles is only half-maximally inhibited by 1 mM SITS. Table II shows that the 30% of Cl^-/I^- exchange activity in ileal brush border membrane vesicles which is not inhibited by low concentrations of SITS (0.1 mM) is also insensitive to SO_4^{2-} at a concentration (2 mM) which largely inhibits the SO_4^{2-} sensitive part of the Cl^-driven I^- uptake. This indicates that the SO_4^{2-} and the highly SITS sensitive Cl^-/I^- exchangers in ileal vesicles are identical. The finding that 2 mM HCO_3^- in combination with either 0.1 mM SITS or 2 mM

TABLE II
EFFECT OF SITS, SO_4^{2-} AND HCO_3^- ON THE Cl^- DRIVEN $^{125}I^-$ UPTAKE IN ILEAL BRUSH BORDER MEMBRANE VESICLES

$^{125}I^-$ uptake in brush border membrane vesicles from rat ileum suspended in buffer A, pH 8.2 was measured in the presence of a Cl^- gradient (100 mM KCl inside, 85 mM K Gluc outside) at 25°C between 60 and 90 s as described in MATERIALS AND METHODS. K-salts of the anions were mixed with the $^{125}I^-$ reach the final concentrations as stated in the Table. Data are means of triplicate experiments ± S.E.

Anion	% inhibition of Cl^-/I^- exchange
2 mM SO_4^{2-}	65±6
2 mM HCO_3^-	57±6
0.1 mM SITS	70±5
2 mM SO_4^{2-} + 2 mM HCO_3^-	90±4
2 mM SO_4^{2-} + 0.1 mM SITS	71±5
2 mM HCO_3^- + 0.1 mM SITS	93±4

SO_4^{2-} can inhibit the Cl^-/I^-exchange in ileal vesicles up to 93% (Table II) additionally indicates that the component of Cl^-/I^- exchange, which is insensitive to relatively low concentrations of SITS or SO_4^{2-} has a high affinity for HCO_3^- ($IC_{50} \approx 0.5$ mM). Fig. 6 shows that the activities of the Cl^-/I^- exchange per mg of protein in ileal and jejunal vesicles are only slightly different. It also shows that ileal and jejunal vesicles are both capable of HCO_3^-/I^- exchange when preloaded with $KHCO_3$ instead of KCl. However the activity of HCO_3^-/I^- exchange per mg protein is larger in ileal than in jejunal vesicles which is in line with the previous demonstration of a high affinity Cl^-/HCO_3^- exchange only in ileal vesicles.

Similar results as described above were obtained with brush border membrane vesicles derived from brush border caps by a thiocyanate treatment (not shown). These cytoskeleton-depleted vesicles could also be useful in future studies of the regulatory properties of Cl^-/anion exchange. In a previous study these KSCN-brush border membrane vesicles were successfully used to analyze a regulation of ion permeabilities by Ca^{2+} [9].

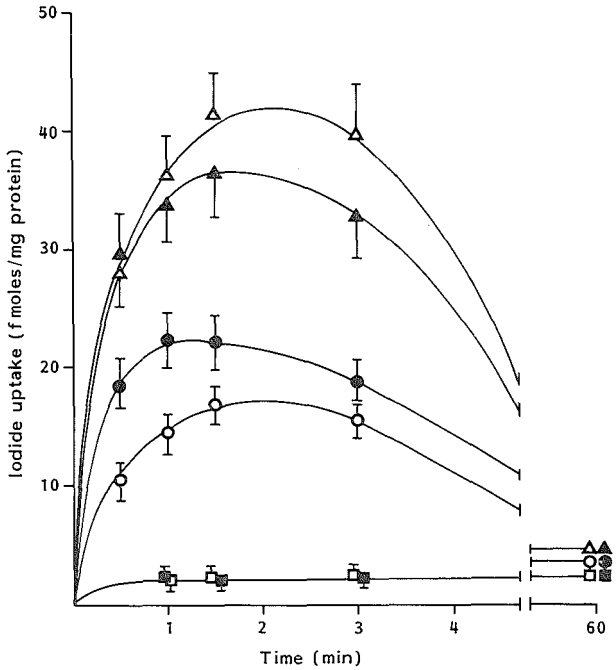

Figure 6
$^{125}I^-$ uptake in ileal and jejunal brush border membrane vesicles by an outwardly directed Cl^- or HCO_3^- gradient.
Brush border membrane vesicles from ileum (●,▲,■) or jejunum (○,△,□) were preincubated in buffer A pH 8.2 plus 100 mM KCl (△,▲,□,■) or 100 mM HCO_3^- (○,●). After replacement of extravesicular anions by gluconate on Dowex AG-1 columns, (except □,■), $^{125}I^-$ uptake was measured as described in MATERIALS AND METHODS at 25°C as a function of time. □,■, no Cl^- gradient. Vertical bars indicate S.E., (n=3).

DISCUSSION

Coupled NaCl transport in small intestine and particularly in ileum is presumably mediated by a Na^+/H^+ antiport in combination with Cl^- anion exchange [7,12,13]. In the present study we obtained evidence for a highly active anion exchange process in brush border membrane vesicles from rat small intestine, which was responsible for up to 70% of the $^{36}Cl^-$ uptake under isotope equilibrium condition (25 mM Cl^- on both sides of the

membrane). By generating a large chemical gradient for Cl^- across the vesicle membrane and by the use of trace amounts of $^{125}I^-$ we could increase the sensitivity of the assay for the anion exchange activity 10-15 fold; $^{125}I^-$ may accumulate far above its chemical equilibrium in exchange for intravesicular Cl^-, without interfering with the magnitude of the gradient. As mentioned in the Results section this method could in theory also detect Cl^- channels in the vesicles in the presence of an impermeable cation (cf. also Ref. 16).

Because most anion exchangers which transport Cl^- also have an affinity for I^- [12,18], this method seems generally applicable for detection of Cl^-/anion exchangers in isolated membrane vesicles.

Extravesicular Cl^- inhibited the Cl^- driven I^- uptake in intestinal brush border membrane vesicles in a concentration-dependent manner. The IC_{50} for Cl^- was approximately 0.5 mM. This is much lower than the K_m for the Cl^-/HCO_3^- exchanger (3.5 mM) or Cl^-/oxalate exchanger (4.0 mM) in rabbit ileum [12,20] and the K_i for the external binding site of the anion exchanger from red blood cells (4.3 mM in the presence of 100 mM Cl^- inside) [21]. It is not clear whether this discrepancy is caused by different properties of the exchangers or by methodological differences. Other anions like NO_3^-, SCN^-, I^- and SITS at relatively low concentration (2 mM) could also inhibit the Cl^-/I^- exchange for more than 90%, in both ileal and jejunal vesicles confirming a general affinity of Cl^-/anion exchangers for these ions [18]. In the inhibition studies with SO_4^{2-}, HCO_3^- and SITS evidence could be obtained for the existence of at least two different Cl^-/anion exchangers in brush border membrane vesicles from rat ileum: (i) a Cl^-/anion exchanger with relatively high affinity for SO_4^{2-} ($IC_{50} \approx 0.5$ mM) and SITS ($IC_{50} \approx 2.5$ uM) and (ii) a Cl^-/anion exchanger with a high affinity for HCO_3^- ($IC_{50} \approx 0.5$ mM) but a low affinity for SITS ($IC_{50} \approx 1$ mM) and SO_4^{2-} ($IC_{50} > 20$ mM). Considering the similar affinities for SO_4^{2-} and SITS found for the first type of Cl^-/I^- exchanger in the ileum and the jejunal exchanger it seems likely that both exchangers are very similar or identical proteins. The second type found predominantly in ileum is presumably identical to the classical $Cl^-HCO_3^-$or Cl^-/OH^- exchanger which was detected earlier by the use of pH or HCO_3^- gradients in a mixture of ileal and jejunal brush border membrane vesicles from rat [7] and ileal vesicles from rabbit [12]. The Cl^-/HCO_3^- exchanger from rabbit ileum was likewise insensitive to SO_4^{2-} and was only half-

maximally inhibited by 1 mM SITS [12]. The absence of a Cl^-HCO3^- exchanger in jejunal vesicles is in agreement with the failure of Cassano et al. [22] and Gunther et al. [8] to detect Cl^-/OH^- exchange in brush border membrane vesicles from rat and rabbit jejunum respectively and with the in vivo studies on salt transport in human ileum and jejunum by Turnberg who found evidence for Cl^-/OH^- exchange in the ileum and not in the jejunum [23,24]. Because the SO_4^{2-} sensitive Cl^-/anion exchange in jejunal vesicles can be largely inhibited by 2 mM oxalate, this anion exchange may be similar to the Cl^-/oxalate exchange described by Knickelbein et al. [20] in brush border membrane vesicles from rabbit ileum, which could also be dissected kinetically from Cl^-/HCO_3^- exchange in the same preparation.

The role of the classical Cl^-/HCO_3^- exchanger in combination with a Na^+/H^+ antiporter in the neutral transport of NaCl in ileum is generally accepted. However, in our experiments the SO_4^{2-} and SITS sensitive but HCO_3^- insensitive anion exchanger accounts for the major part of the Cl^-/I^- exchange activity in ileal vesicles and for all exchange activity in jejunal vesicles. Moreover, Liedtke and Hopfer [7] have demonstrated SITS inhibition of NaCl uptake in rat jejunum where the classical Cl^-/HCO_3^- exchanger is apparently absent. Based on these results it seems likely that the HCO_3^- insensitive exchanger is also involved in transepithelial transport of Cl^-. We can think of three different mechanisms by which this type of exchanger may function in such a process:

i By catalyzing Cl^-/HCO_3^- exchange, as discussed by Turnberg [23]. Although the carrier has a low affinity for HCO_3^- it may accept HCO_3^- if present at high concentrations as demonstrated in the present study by the presence of HCO_3^-/I^- exchange in jejunal vesicles (Fig. 6).

ii By catalyzing Cl^-/formate exchange as proposed by Karmski et al. for the uptake of NaCl in the proximal tubule of the kidney [25]. Formate has a similar function as HCO_3^- because it may pass the membrane in the protonated form. The high affinity of the Cl^-/anion exchanger for this anion is evident from the 80% inhibition of Cl^-/I^- exchange in jejunal vesicles by 2 mM formate (Table I).

iii By catalyzing the exchange of Cl^- for another anion which can be subsequently exchanged for OH^-. In brush border membrane vesicles from rabbit ileum for example Knickelbein et al. [20] found evidence for both an oxalate/OH^- or SO_4^{2-}/OH^- exchange and a Cl^-

/oxalate exchange which was inhibited by SO_4^{2-}. Combination of these carriers may under the right conditions result in a Cl^-/OH^- exchange. Because most anion exchangers have a broad substrate specificity it is not unlikely that a number of other anions in addition to oxalate and SO_4^{2-} can mediate the net uptake of Cl^- in a similar way.

In view of its simplicity and high sensitivity the technique for measuring Cl^-/anion exchange described in this paper may find general application in in vitro studies of anion exchange mechanisms in plasma membrane vesicles and, in combination with an efficient procedure for the entrapment of regulatory factors inside brush border membrane vesicles described recently [26,27], may be advantageously used to investigate a possible modulation of anion exchange activities by intracellular messengers (Ca^{2+}, cGMP, cAMP) in the apical membrane of mature enterocytes.

ACKNOWLEDGEMENTS

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CHAPTER V

EVALUATION OF A ROLE FOR cAMP-, cGMP-, AND Ca^{2+}-DEPENDENT PROTEIN PHOSPHORYLATION IN THE REGULATION OF $Na^{+}/H+$ AND Cl^{-}/ANION EXCHANGE IN RAT INTESTINAL BRUSH-BORDER MEMBRANE VESICLES

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(submitted to Biochim. Biophys. Acta)

SUMMARY

The second messengers cAMP, cGMP, Ca^{2+} and diacylglycerol have been postulated to inhibit an electroneutral Na^+-Cl^- cotransport system located in the apical membrane of the intestinal villus cell and presumably consisting of separate Na^+/H^+ and $Cl^-HCO_3^-$ exchangers, through a mechanism involving protein phosphorylation. In an attempt to reconstitute second messenger regulation of this transport system in isolated membranes, brush-border membrane vesicles from rat small intestine were phosphorylated either by encapsulation of ATP, creatine phosphate and second messengers during vesiculation of intestinal microvilli effected by a freeze-thaw procedure, or by preincubation of unsealed brush-border caps with the phosphorylating system followed by a vesiculation procedure based on KSCN treatment. In both procedures a change in the phosphorylation state of intravesicular membrane proteins was confirmed by demonstrating prephosphorylation of specific endogenous substrates for cGMP-dependent protein kinase in cGMP plus ATP-loaded vesicles by means of back-titration with [γ-^{32}P]ATP. However, neither the Na^+/H^+ exchange activity as measured by the acridine orange technique or by amiloride-sensitive $^{22}Na^+$ uptake, nor the Cl^-/anion exchange activity measured as overshooting $^{125}I^-$ uptake driven by an outwardly directed Cl^- gradient was significantly changed by cyclic nucleotide-, Ca^{2+}- or phorbolester (PMA)-dependent phosphorylation. The induction of a state of secretion by intraluminal injection of cholera toxin in vivo was equally ineffective in provoking a change in Na^+/H^+ or Cl^-/anion exchange activity in brush-border membrane vesicles isolated from the toxin-exposed intestinal segment. It was found however that free Mg^{2+} (5mM) or the calmodulin antagonist trifluoperazine (50 μM) even in the absence of Ca^{2+} could inhibit amiloride-sensitive $^{22}Na^+$ uptake upto 75% and thereby complicates the interpretation of vesicle studies exploring the regulation of Na^+/H^+ exchange by ATP (a Mg^{2+} chelator) and Ca^{2+}/calmodulin. The outcome of this study argues against, but does not definitely rule out a role for intramicrovillar phosphoproteins or Ca^{2+} as local regulators of Na^+/H^+ and Cl^-/anion exchangers in the brush-border membrane of rat enterocytes.

INTRODUCTION

Studies of stimulus-secretion coupling in intestinal epithelium have revealed a crucial role for the second messengers cAMP, cGMP, Ca^{2+} and diacylglycerol as modulators of transepithelial Na^+ and Cl^- transport [1-3]. The major ion transport systems affected by these second messengers are (i) an electroneutral Na^+-Cl^- cotransport system localized in the brush-border membrane of the mature villus cell [1], presumably composed of a Na^+/H^+ and a $Cl^-HCO_3^-$ exchanger coupled by circular proton movements [4,5] and (ii) an electrogenic Cl^- channel apparently enriched in the apical membrane of the intestinal crypt cell [1]. The molecular nature of the transporters and of the signal transduction mechanisms involved is largely unknown. Studies of ion transport regulation at the level of isolated brush-border membranes, avoiding the complexity of the intact cell, may potentially allow a more detailed analysis of local regulatory factors within the intestinal microvilli capable of modulating Na^+ and Cl^- transport across the apical membrane. Phosphorylation studies with isolated brush-borders and brush-border membrane vesicles from intestinal villus cells have revealed an enrichment of the type II isoenzyme of cAMP-dependent protein kinase and a specific isoenzyme of cGMP-dependent protein kinase, and the additional presence of a Ca^{2+}/calmodulin- and a Ca^{2+}/phospholipid-dependent protein kinase in the microvillus membrane [6-9], each acting on one or several endogenous substrates. A 25 kDa proteolipid, which is co-phosphorylated by both cAMP- and cGMP-dependent protein kinases may fulfill a key role in the regulation of NaCl transport by both cyclic nucleotides [7,8]. Transport studies in small intestinal brush-border membrane vesicles have provided evidence for the existence of Na^+/H^+ and Cl^-/anion exchangers in the apical membrane which can operate in tandem as a coupled Na^+-Cl^- cotransport system [4,5], and for the presence of non-saturable electrogenic pathways for Na^+ and Cl^-, which could be reversibly stimulated by micromolar Ca^{2+} in the absence of ATP [10]. Micromolar Ca^{2+} in combination with calmodulin but in the absence of ATP was reported to inhibit coupled Na^+-Cl^- transport in brush-border membrane vesicles from rabbit ileum [11].

In the present study we have explored a possible ATP-dependent modulation of Na^+/H^+ and Cl^-/anion exchangers in rat intestinal brush-border membrane

vesicles by cyclic nucleotides and Ca^{2+}. We found no evidence for a significant change in Na^+/H^+ and Cl^-/anion exchange activities in response to an alteration in phosphorylation state of intravesicular proteins brought about by ATP- and cyclic nucleotide- loading during or following vesiculation. Also in situ exposure of intestinal epithelium to cholera toxin had no significant influence on the activity of the Na^+ and Cl^- transporters at the level of brush-border membrane vesicles. These results argue against, but do not definitely rule out, a regulation of one or both exchangers through Ca^{2+}- or cyclic nucleotide-provoked phosphorylation of the transport protein itself or of a regulatory protein tightly associated with the exchangers.

MATERIALS AND METHODS

Materials.

$^{22}NaCl$ (11 Ci/mmol), $Na^{125}I$ (2000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from Amersham International PLC, creatine kinase (18U/mg), cyclic AMP, cyclic GMP, the Ca^{2+}-ionophore A23187 and valinomycin were obtained from Boehringer, trifluoperazine (TFP) came from Rohm Pharma and 4β phorbol 12-myristate 13-acetate (PMA), 4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonic acid (SITS), cholera toxin and cAMP-dependent protein kinase (crude enzyme from bovine heart,1900 U/mg) from Sigma. Amiloride was a gift from Merck Sharp and Dohme and alamethicin was donated by Dr. J.E. Grady, Upjohn, Kalamazoo, MI. Calmodulin was purified from bovine brain as described in Ref. 12. All other chemicals were analytical grade.

Methods

Preparation of brush-borders and brush-border membrane vesicles

Each batch of brush-border membrane vesicles originated from jejunal-ileal segments of small intestine freshly obtained from 2-3 adult male Wistar rats weighing 300-350 g. Intact brush-border caps were isolated by vibration of everted gut segments in hypotonic (2.5 mM) EDTA as described in Ref. 13. Brush-border membrane vesicles hereafter referred to as KSCN-vesicles were prepared by exposing the brush-border caps to 0.52 M KSCN followed by a 7-fold dilution in buffer A (300 mM mannitol, 20 mM Hepes/-Tris pH 7.2) or buffer B (300 mM mannitol, 40 mM Hepes/Tris pH 7.2) and

differential centrifugation in principle as described in Ref. 14. Vesicles prepared by this method are virtually depleted of cytoskeletal proteins and calmodulin [10,14]. Brush-border membrane vesicles hereafter referred to as Mg-vesicles were prepared from isolated enterocytes by freeze-thawing and differential Mg^{2+} precipitation as described earlier in full detail [15].

Phosphorylation of brush-borders and brush-border membrane vesicles

Isolated brush-border caps (2-4 mg protein/ml) were prephosphorylated for 15 min at 30°C by incubation in the presence of 20 mM Hepes/Tris (pH 7.2), 25 mM β-glycerophosphate, 5 mM $MgSO_4$, 5 mM β-mercaptoethanol, 0.2% trypsin inhibitor, 20 mM creatine phosphate, 0.1 mg/ml creatine kinase, 2 mM ATP and other additions specified in the legends. To maintain phosphorylation conditions following vesiculation, the KSCN-exposed brush-borders were diluted 7-fold with the phosphorylation mixture (minus β-glycerophosphate, β-mercaptoethanol and trypsin inhibitor) in buffer A or buffer B prior to differential centrifugation (cf. Refs. 10,14). The quantitative entrapment of ATP at the interior of the KSCN-vesicles was confirmed by a luciferase assay [15]. Phosphorylation of brush-borders and brush-border membrane vesicles with radioactive ATP was performed in principle according to Ref. 9, as specified in the legends of the figures. Phosphoproteins were analysed by SDS-PAGE followed by autoradiography [9]. Mg-brush-border membrane vesicles were converted to a phosphorylated state by entrapment of ATP or ATP S (2mM), creatine phosphate (20 mM) and other additions specified in the Figure and Table legends by a freeze-thaw procedure described in full detail in Ref. 15. KSCN- or Mg-vesicles were finally suspended in buffer A or buffer B.

In situ exposure of rat intestinal epithelium (jejunum and ileum) to cholera toxin was carried out by intraluminal injection of 10 μg cholera toxin in 5 ml saline (or 5 ml saline alone; control experiment) into prerinsed intestinal segments (≈ 40 cm) using a surgical procedure described earlier in detail [16]. Three hours later the intestinal loops were quickly frozen by means of a Wollenberg clamp, precooled in liquid nitrogen. The frozen tissue was pulverized in a porcelain mortar, kept cold with liquid nitrogen, suspended in buffer B and thawed slowly in icewater. Thereafter brush-border membrane vesicles were rapidly isolated by differential Mg^{2+} precipitation as described earlier [15]. Alterna-

tively, the intestinal loops were rinsed in situ with ice-cold saline, excised and vibrated in hypotonic EDTA in order to obtain brush-border caps and KSCN-vesicles as described in the foregoing. In all cases the cholera toxin-treated rats showed a profound secretion of water into the intestinal lumen.

Transport assays

Na^+-driven H^+ uptake was determined by a method utilizing the pH-sensitive fluorescent dye acridine orange in principle as described by Reenstra et al. [17]. 20 µl of a vesicle suspension (15-20 mg protein/ml) preincubated for 1 h at 0°C with 100 mM NaCl, 25 mM KCl and 10 µM valinomycin in buffer A was injected into the cuvette of a Perkin Elmer PKF-3 fluorimeter kept at 20°C and containing 1.5 ml of buffer A, 100 mM choline-Cl, 25 mM KCl and 10 µM acridine orange . Maximal quenching of the fluorescence (excitation 490 nm, emission 540 nm) as reached within 30-60 s following vesicle injection, showed a linear correlation with the initital slope of the quenching, and was taken as a measure of the Na^+/H^+ exchange activity.

$^{22}Na^+$ uptake was measured by mixing 70-250 µl of brush-border membrane vesicles (4 mg/ml) in buffer B with 10 µl of buffer B containing $^{22}NaCl$ to reach a final concentration of 1 mM $^{22}Na^+$ (5 µCi/ml). At various time intervals 50 µl aliquots were transfered to small columns (0.6 ml packed resin) of Dowex 50W X8 cation exchanger (50-100 mesh, Tris form) and vesicles were quickly eluted with 1 ml of ice-cold buffer B as described previously [10].

Cl^-/anion exchange activity was determined by measuring the overshooting uptake of trace amounts of $^{125}I^-$ into the brush-border membrane vesicles driven by an outwardly directed Cl^- gradient as discussed previously [18]. Briefly, 100 µl of brushborder membrane vesicles (2-4 mg protein/ml) preloaded for 1 h at 0°C with 100 mM KCl in buffer A were loaded on Dowex-1 X8 anion exchange columns (50-100 mesh, gluconate form) and eluted with 300 µl of buffer A in order to replace the extravesicular Cl^- by gluconate. After addition of 1 nM $Na^{125}I$ to the freshly eluted vesicles 50 µl aliquots were transfered at various time points to similar anion exchange columns to separate the vesicles from the extravesicular $^{125}I^-$. $^{22}Na^+$ and $^{125}I^-$ were determined by γ-emission spectroscopy.

Initial rates of Na^+-driven [^{3}H]glucose uptake into brush-border membrane vesicles were determined by a Millipore filtration method as described in Ref. 19.
Protein was determined by the method of Lowry et al. using bovine serum albumin as a standard [20]. Alkaline phosphatase activity was determined according to Ref. 21, and sucrase activity measured in 0.1% Triton X-100 was determined by the method of Forstner et al. [22].

RESULTS

Phosphorylation studies

As shown in Fig. 1, cGMP was found to stimulate the incorporation of ^{32}P from [γ-^{32}P]ATP into two brush-border proteins, i.e. a 86 kDa substrate, identified as the cGMP-dependent protein kinase itself, and a 25 kDa proteolipid (cf. Refs. 7,8). The latter serves also as a substrate for endogenous cAMP-dependent protein kinase [7,8]. Fig. IA additionally shows that both phosphoproteins, when prelabeled with ^{32}P in the intact brush-borders, are able to maintain their phosphorylated state during the preparation of KSCN-brush-border membrane vesicles and actually copurify with the brush-border membranes. This indicates that the cGMP-dependent protein kinase and its 25 kDa substrate are tightly associated with the apical membrane and, considering their localization, are likely candidates to play a role in the regulation of ion transport systems in the brush-border membrane. To estimate the extent of phosphate incorporation into the two substrate proteins brought about by prephosphorylation of the brush-border caps for 15 min at 30 C with 2 mM ATP, brush-borders were washed to remove the non-radioactive ATP, and residual free acceptor sites for phosphate on the substrates were back-titrated with 10 μM [γ-^{32}P]ATP. As shown in Fig. 1B, brush-borders preincubated with ATP and cGMP displayed a very low level of ^{32}P incorporation into the 25 kDa protein as compared to brush-borders preexposed to GTP, whereas preincubation with ATP alone led to an intermediate value of 25 kDa protein labeling (60% of the GTP lane as determined by densitometric scanning). In contrast, ^{32}P labeling of the cGMP-dependent protein kinase itself (86 kDa band) was less completely inhibited by preincubation with ATP plus cGMP (25% of the incorporation seen in the GTP lane) but again partially inhibited by

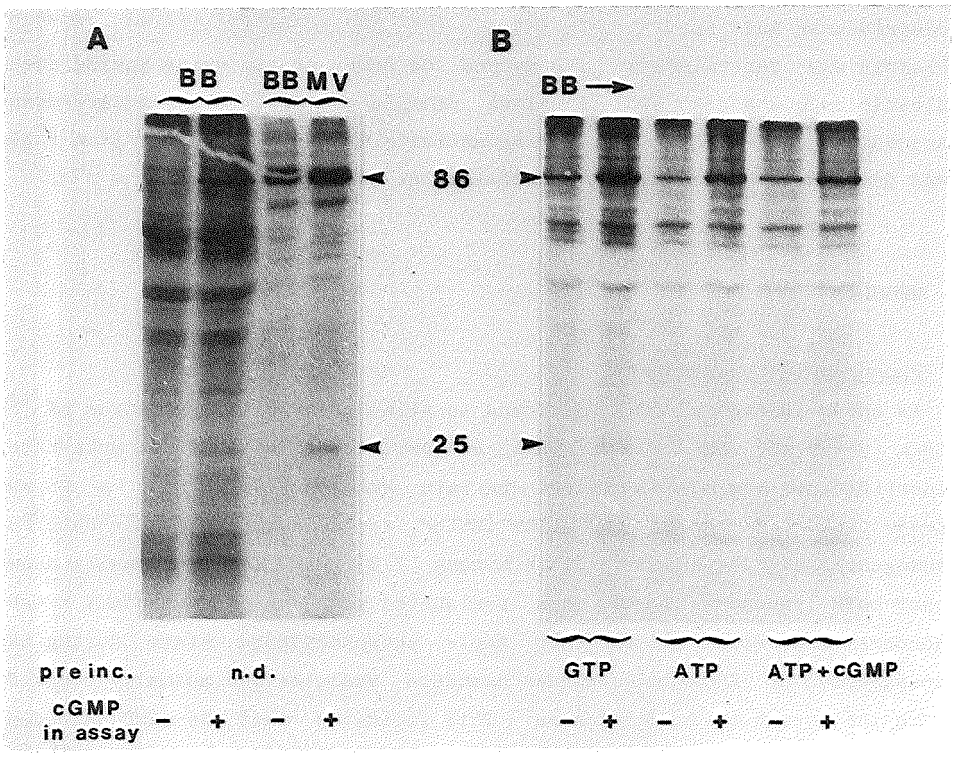

Fig. 1.

Autoradiographs showing basal and cGMP-dependent ^{32}P-labeling of proteins in isolated intestinal brush-border caps (BB) and KSCN-brush-border membrane vesicles (BBMV) (panel A) and the effect of prephosporylation with non-radioactive ATP on the ^{32}P-labeling profile of brush-borders (panel B).

Brush-border caps (2 mg/ml) were phosphorylated for 30 sec at 30°C in a 10 mM Tris/HCl buffer (pH 7.1) containing 25 mM β-glycerophosphate, 5 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP (10 Ci/mmol) and, where indicated, 10 μM cGMP. Phosphorylation was terminated directly (BB) or following KSCN treatment and vesicle isolation (BBMV) by addition of a SDS stopping solution and boiling [9]. Proteins were separated on a 12.5% SDS-PAGE gel and ^{32}P incorporation was visualized by autoradiography as described previously [9]. Molecular weights are indicated in kDa.

A: ^{32}P-labeled brush-borders were analyzed directly (lanes 1 and 2) or following vesiculation with KSCN (lanes 3 and 4).

B: Brush-border caps (2-4 mg protein/ml) were preincubated for 15 min at 30°C in a phosphorylation mixture containing 20 mM Hepes/Tris (pH 7.2), 5 mM β-mercaptoethanol, 25 mM β-glycerophosphate, 5 mM $MgSO_4$, 0.2% trypsin inhibitor, 20 mM creatine phosphate, 0.1 mg/ml creatine kinase (18 U/mg), 2 mM GTP (lanes 1 and 2), 2 mM ATP (lanes 3 and 4) or 2 mM ATP plus 10 μM cGMP (lanes 5 and 6). Brush-border pellets obtained by centrifugation (10 min, 3000 g) were washed twice with 20 ml 10 mM Tris/HCl buffer (pH 7.1) and were then phoshorylated with [γ-^{32}P]ATP as described above.

exposure to ATP alone (45% of the labeling in the GTP lane). These results indicate that the prephosphorylation procedure in the presence of ATP plus cGMP resulted in almost stoichiometric phosphorylation of the 25 kDa substrate, whereas the cGMP-dependent protein kinase was less completely phosphorylated (75% of the maximum) or slightly dephosphorylated during the washing procedure. Because preincubation of brush-borders with ATP alone also altered the phosphorylated state of the 25 kDa and the 86 kDa protein, indicating some basal phosphorylation of both proteins in the absence of cGMP, it appeared essential to test possible effects of second messengers on ion transport activities under at least three conditions, i.e. in ATP-free, ATP-loaded and ATP plus second messenger-loaded vesicles.

Back-titration of the phosphorylation sites on the 86 kDa cGMP-dependent protein kinase in Mg-brush-border membrane vesicles revealed that preloading with cGMP and ATP or ATPγS, in contrast to ATP alone, led to an almost stoichiometric prephosphorylation of the kinase as judged by the low extent of ^{32}P incorporation compared to ATP- and cGMP-free brush-border membrane vesicles (Fig. 2, compare lanes with cGMP in assay). Entrapment of ATPγS, which may produce a more stable thiophosphorylation due to the resistance of the sulphur-phosphate bond to phosphatases, was also effective in provoking a phosphorylated state of the 86 kDa protein (Fig. 2). Unfortunately, the inability of the endogenous cGMP-dependent protein kinase to phosphorylate the 25 kDa proteolipid in vesiculated microvillus membranes as noticed in earlier phosphorylation studies [7] prevented us to verify its phosphorylated state in isolated KSCN- or Mg-vesicles by means of the back-titration technique.

Effect of phosphorylation on Na^+/H^+ exchange

The activity of the Na^+/H^+ exchanger was measured first by fluorescence quenching of the pH-sensitive dye acridine orange. A steep Na^+ gradient across the vesicle membrane ($Na^+_{in} > Na^+_{out}$), in the presence of a Na^+/H^+ exchange mechanism, is converted into a pH gradient ($pH_{in} < pH_{out}$) resulting in a rapid uptake and quenching of the fluorescent dye (cf. Ref. 17). A possible contribution of Na^+ and H^+ conductance pathways to Na^+/H^+ exchange (electrogenic coupling) was virtually nullified by short-circuiting the membrane potential with K^+ and valinomycin. As shown in Table 1, the additional entrapment of 10 μM cGMP or 10 μM cAMP into ATP-

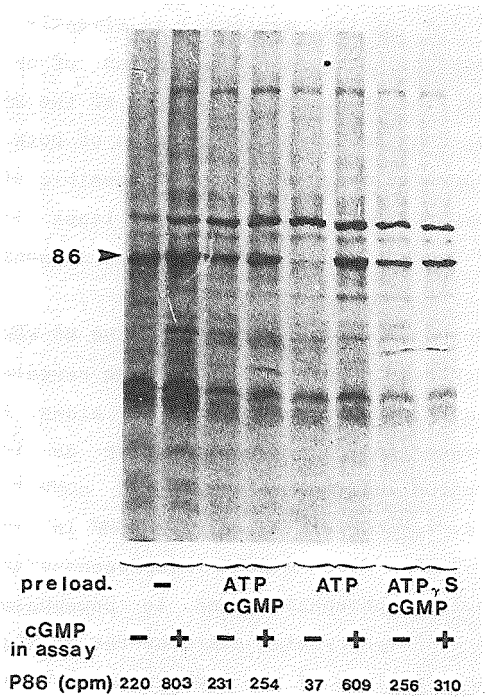

Fig. 2.
Autoradiograph showing basal and cGMP-dependent ^{32}P-labeling of proteins in Mg-brush-border membrane vesicles. Effect of preloading with ATP and cGMP.
Mg-brush-border membrane vesicles were preloaded with buffer alone (lanes 1 and 2), 20 mM creatine phosphate plus 2 mM MgATP (lanes 5 and 6), 2 mM MgATP plus 10 µM cGMP (lanes 3 and 4) or 2 mM MgATP S plus 10 µM cGMP (lanes 7 and 8) as described in Materials and Methods. The Mg-brush-border membrane vesicles were phosphorylated with 0.5 mM [γ-^{32}P]ATP for 1 min at 30°C in the absence or presence of 10 µM cGMP and other additions as specified in the legend of Fig. 1. Alamethicin (0.3 mg/ml) was added as a membrane permeabilizing agent. Phosphorylations were terminated with SDS and boiled samples were analyzed by SDS-PAGE (7.5% acrylamide gel) and autoradiography [9]. The 86 kDa phosphoprotein band was dissected from the gel and ^{32}P-incorporation was quantitated by liquid scintillation counting.

loaded Mg-brush-border membrane vesicles had no significant effect on the activity of the Na^+/H^+ exchanger as measured by the acridine orange technique. Prephosphorylation of brush-border proteins in the presence of 10 μM cGMP or 10 μM cAMP likewise did not result in a change in Na^+/H^+ exchange activity in KSCN-membrane vesicles prepared from the brush-borders as compared to incubation with ATP alone or in the absence of ATP (Table 1). However the conditions used for measuring Na^+/H^+ exchange (0.1 M Na^+ and acid intravesicular pH) may have been suboptimal for monitoring potential effects of cAMP- and cGMP-dependent phosphorylation on the Na^+ or H^+ affinities of the exchanger, e.g. through phosphorylation of its so-called H^+ modifier site identified by Aronson in renal brush-border membrane vesicles [23]. A shift in the affinity of the intracellular binding site for protons is presumably also the underlying mechanism in the stimulation of Na^+/H^+ exchange in fibroblasts by growth factors and protein kinase C [24,25]. We therefore repeated our measurements of Na^+/H^+ exchange activity at subsaturating concentrations of Na^+ (1 mM) and a physiologic intravesicular pH (7.2) by monitoring initial rates of

TABLE I

QUENCHING OF ACRIDINE ORANGE FLUORESCENCE IN BRUSH-BORDER MEMBRANE VESICLES BY AN OUTWARDLY DIRECTED Na^+ GRADIENT. EFFECTS OF PREINCUBATION WITH CYCLIC NUCLEOTIDES AND ATP.

Mg- and KSCN-brush-border membrane vesicles preloaded with 0.1 M NaCl and 25 mM KCl in buffer A in the presence of 10 μM valinomycin were injected into buffer A containing 0.1 M choline-Cl, 25 mM KCl and 10 μM acridine orange and quenching of fluorescence was monitored as described in Materials and Methods. Preloading of Mg- and KSCN-vesicles with the various compounds was also carried out according to Materials and Methods.

Preloading conditions	Quenching of fluorescence (percentage of control ± SE;n=4)	
	Mg-vesicles	KCSN-vesicles
Control (2 mM ATP, 20 mM creatine phosphate, 1 mM EGTA)	100±10	100±12
+ 10 μM cAMP*	90±10	103±10
+ 10 μM cGMP	89±10	87±11
no ATP, no creatine phosphate	93±9	95±10

*To the KSCN-vesicle crude cAMP-dependent protein kinase from bovine heart (0.1 mg/ml) was added in addition to cAMP to compensate for the washout of endogenous kinase.

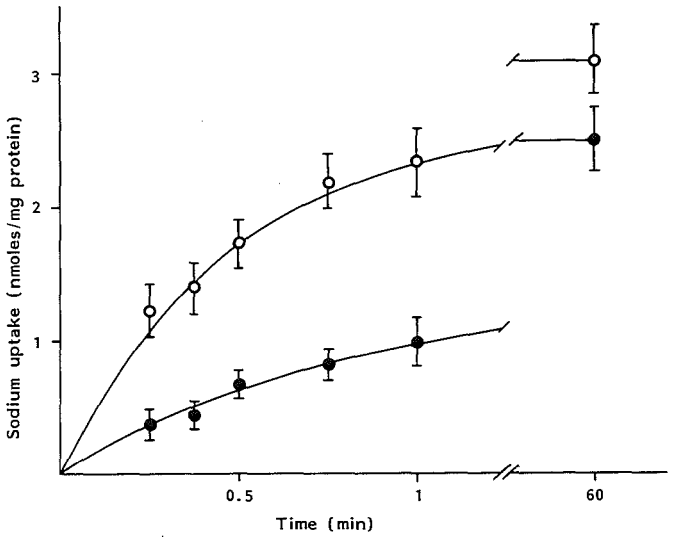

Fig. 3.
Uptake of $^{22}Na^{+}$ in KSCN-brush-border membrane vesicles.
Time-dependent uptake of $^{22}Na^{+}$ into KSCN-brush-border membrane vesicles was measured at 25°C following the addition of 1 mM $^{22}NaCl$ to the extravesicular medium in the absence (o) or presence (•) of 1 mM amiloride as described under Materials and Methods. Vertical bars indicate S.E.; n=3.

amiloride-sensitive $^{22}Na^{+}$ influx into the vesicles. As shown in Fig. 3, a large part of the $^{22}Na^{+}$ uptake (50-75%) is inhibitable by 1 mM amiloride which is characteristic for Na^{+}/H^{+} exchange. Table 2 shows that prephosphorylation in the presence of cAMP and cGMP did not effect the amiloride-sensitive $^{22}Na^{+}$ uptake neither in Mg-brush-border membrane vesicles nor in KSCN-vesicles. Omitting of ATP and creatine phosphate however resulted in a 75% inhibition of the amiloride-sensitive $^{22}Na^{+}$ uptake, whereas the amiloride-insensitive component remained unaffected. Interestingly, the additional omittance of Mg^{2+} from the phosphorylation mixture fully restored the amiloride-sensitive $^{22}Na^{+}$ uptake into the KSCN-brush-border

TABLE II

AMILORIDE-SENSITIVE $^{22}Na^+$ UPTAKE IN BRUSH-BORDER MEMBRANE VESICLES. EFFECT OF PREINCUBATION WITH CYCLIC NUCLEOTIDES AND Ca^{2+}.

Mg- and KSCN-brush-border membrane vesicles in buffer B were preincubated for 1 min at 25°C in the absence or presence of 1 mM amiloride and exposed for 20 s or 1 h to 1 mM $^{22}Na^+$ (5 μCi/ml). Intravesicular uptake of $^{22}Na^+$ was determined as described in Materials and Methods. Initial uptake of $^{22}Na^+$ measured at 20 sec was expressed as a percentage of the equilibrium uptake at 1 h and corrected for the amiloride-insensitive component of $^{22}Na^+$ influx. Mg- and KSCN-brush-border membrane vesicles were preloaded with the various compounds as described in Materials and Methods. n.d., not determined.

Preloading conditions	Amiloride-sensitive $^{22}Na^+$ uptake (percentage of control ± SE;n=5)	
	Mg-vesicles	KSCN-vesicles
Control (2 mM ATP, 20 mM creatine phosphate, 1 mM EGTA, 5 mM Mg^{2+})	100±11	100±10
no ATP, no creatine phosphate	20*±8	25*±10
no ATP, no creatine phosphate, no Mg^{2+}	n.d.	108±12
1 mM EGTA/Ca (pCa 5)	92±15	95±15
+ 10 μM cAMP** + 10 μM cGMP	95±12	92±12
+ 10 μM cAMP** + 10 μM cGMP, 1 mM EGTA/Ca (pCa5)	90±10	n.d.
+ 1 μM phorbol myristate acetate, 1 mM EGTA/Ca (pCa5)	91±10	n.d.
+ 20 μM calmodulin, 1 mM EGTA/Ca (pCa5)	n.d.	108±10
+ 50 μM trifluoperazine	28*±8	25*±10

*P 0.01 compared to control.

**to the KSCN-vesicles crude cAMP-dependent protein kinase from bovine heart (0.1 mg/ml) was added to compensate for the washout of the endogenous kinase.

membrane vesicles, suggesting that intravesicular Mg^{2+} (5 mM) inhibits the Na^+/H^+ exchanger but that this inhibition is masked by the Mg^{2+}-chelators ATP and creatine phosphate. Our finding that incubation of Mg-vesicles in the presence of 2 mM EDTA and 20 μM Ca^{2+}/Mg^{2+}-ionophore A23187 led to a 2-fold activation of the amiloride-sensitive $^{22}Na^+$ uptake (not shown) coroborates this suggestion.

Intracellular Ca^{2+}, which is also thought to modulate NaCl transport in small intestine, may directly interact with apical transport proteins or exert its action by stimulating Ca^{2+}/calmodulin or Ca^{2+}/phospholipid-dependent protein kinases in the brush-border region. We were therefore

also interested to study the effect of intravesicular Ca^{2+} on the Na^+/H^+ exchanger in ATP-loaded vesicles. As shown in Table 2, a comparison between Ca^{2+}-loaded (pCa 5) versus EGTA-loaded vesicles in the absence or presence of cyclic nucleotides did not reveal significant effects of Ca^{2+} or Ca^{2+}-stimulated endogenous phosphorylation on amiloride-sensitive $^{22}Na^+$ uptake in Mg- or KSCN-brush-border membrane vesicles. The Na^+/H^+ exchanger appeared also insensitive to the additional entrapment of phorbolester (PMA), an activator of protein kinase C, in Mg-vesicles or to the addition of calmodulin to KSCN-brush-border membrane vesicles, which are depleted of endogenous calmodulin [10]. These results also argue against the possibility that cyclic nucleotides and Ca^{2+} exert a synergistic effect on Na^+/H^+ exchange. In order to unmask a potential basal effect of endogenous calmodulin on Na^+/H^+ exchange in Mg-brushborder membrane vesicles which might manifest itself even in the presence of EGTA, vesicles were also preloaded with trifluoperazine (TFP), a calmodulin antagonist. Surprisingly, the encapsulation of 50 μM TFP inhibited the amiloride-sensitive $^{22}Na^+$ uptake for 70-75% in both Mg- and (calmodulin-depleted) KSCN-brush-border membrane vesicles. It is therefore likely that this inhibition reflects a direct interaction of TFP with the Na^+/H^+ exchanger itself or its lipid environment, instead of a specific inhibition of calmodulin action. However, this finding seems to compromize studies in which TFP is used to evaluate the role of calmodulin as a potential regulator of Na^+/H^+ exchange.

Effect of phosphorylation on Cl^-/anion exchange

As shown in Table 3, cyclic nucleotide- and Ca^{2+}-dependent phosphorylation was also unable to change the rate of Cl^--driven $^{125}I^-$ uptake in Mg- and KSCN-brush-border membrane vesicles. This exchange process was previously shown to consist of two components a Cl^-/HCO_3^- exchange enriched in rat ileum and a less specific Cl^-/anion exchange possessing a low sensitivity for HCO_3^- [18]. Similar experiments carried out with ileal Mg-brush-border membrane vesicles in the presence of 0.1 mM SITS which almost completely blocks the HCO_3^--insensitive Cl^-/I^- exchange but has only a minor effect on the Cl^-/HCO_3^- exchange, were likewise unable to demonstrate effects of Ca^{2+} or cyclic nucleotides on anion exchange activity in the vesicle membrane (not shown).

TABLE III

Cl^--DRIVEN $^{125}I^-$ UPTAKE IN BRUSH-BORDER MEMBRANE VESICLES. EFFECT OF PREINCUBATION WITH CYCLIC NUCLEOTIDES AND Ca^{2+}.

Mg- and KSCN-brush-border membrane vesicles were preloaded for 1 h at 0°C with 0.1 M KCl and passed through a Dowex anion exchange column (gluconate form) to remove extravesicular Cl^-. Subsequently Cl^--driven $^{125}I^-$ uptake was determined at 60 s at 25°C as described in Materials and Methods. The Mg- and KSCN-brush-border membrane vesicles were preloaded with the various compounds also as described in Materials and Methods. n.d, not determined

Preloading conditions	Cl^--driven $^{125}I^-$ uptake (percentage of control±SE;n=5)	
	Mg-vesicles	KSCN-vesicles
Control (2 mM ATP, 20 mM creatine phosphate 1 mM EGTA, 5 mM Mg^{2+})	100±11	100±12
no ATP, no creatine phosphate	97±10	93±11
+ 10 µM cAMP*, + 10 µM cGMP	91±10	88±10
1 mM EGTA/Ca, (pCa5)	95±11	96±9
+ 20 µM calmodulin, 1 mM EGTA/Ca (pCa5)	n.d.	110±10

*To the KSCN-vesicles crude cAMP-dependent kinase from bovine heart (0.1 mg/ml) was added in addition to cAMP to compensate for the washout of endogenous kinase.

Effects of in vivo treatment with cholera toxin on Na^+-dependent glucose transport, Na^+/H^+ exchange and Cl^-/anion exchange

Mg-brush-border membrane vesicles prepared from rat intestinal segments which were preexposed for 3 h to intraluminal cholera toxin in vivo showed a significantly higher rate of Na^+-driven [^{3}H]glucose uptake per mg protein as compared to Mg-brush-border membrane vesicles from control segments. However when the [^{3}H]glucose uptake rate was expressed on the basis of vesicular alkaline phosphatase or sucrase activity, known marker enzymes of the brush-border membrane, no significant difference was observed (Table 4). This suggests that the difference in glucose uptake was caused by a higher enrichment factor of apical membranes isolated from cholera toxin pretreated rats rather than by a specific stimulating effect

TABLE IV

EFFECT OF CHOLERA TOXIN-PRETREATMENT OF INTESTINAL EPITHELIUM IN SITU ON Na^+-DEPENDENT [^{3}H]GLUCOSE UPTAKE, AMILORIDE-SENSITIVE $^{22}Na^+$ UPTAKE AND CL^-- DRIVEN $^{125}I^-$ UPTAKE IN BRUSH-BORDER MEMBRANE VESICLES.

All transport measurements were performed in buffer B at 25°C as described in Materials and Methods. Na^+-dependent [^{3}H]glucose uptake was measured at 6 s in the presence of an inwardly directed 0.1 M NaCl gradient, the amiloride-sensitive $^{22}Na^+$ uptake was measured at 20 s following exposure to 1 mM $^{22}Na^+$, and Cl^--driven $^{125}I^-$ uptake was monitored at 60 s in the presence of an outwardly directed 0.1 M Cl^- gradient. Jejunal-ileal segments of rat intestine were exposed in situ to 5 ml saline (control) or 5 ml saline plus 10 µg cholera toxin. Three hours later the intestine was freeze-clamped or excised and Mg- or KSCN-brush-border membrane vesicles were isolated as described in Materials and Methods. n.d., not determined.

Transport functions	Ratio (±SE) of transport activity in vesicles from cholera-treated and sham-operated rats (n=5)	
	Mg-vesicles	KSCN-vesicles
Na^+-dependent [^{3}H]glucose uptake/mg protein	1.50*±0.12	0.90±0.10
Na^+-dependent [^{3}H]glucose uptake/unit alkaline phosphatase	1.03 ±0.10	0.92±0.10
Na^+-dependent [^{3}H]glucose uptake/unit sucrase	0.99 ±0.10	n.d.
Amiloride-sensitive $^{22}Na^+$ uptake/mg protein	n.d.	1.03±0.12
Cl^--driven $^{125}I^-$ uptake/mg protein	n.d.	0.92±0.10

*P 0.01 compared to control.

on the Na^+-glucose cotransporter itself. To avoid this complication, we prefered to use KSCN-brush-border membrane vesicle preparations, which showed a similar Na^+-dependent [^{3}H]glucose uptake per mg protein or unit of alkaline phosphatase whether derived from cholera toxin-treated or control rats, to investigate a potential effect of cholera toxin on Na^+/H^+ and Cl^-/anion exchange. However, as shown in Table 4, intraluminal injection of cholera toxin had no significant effect on amiloride-sensitive $^{22}Na^+$ uptake or Cl^--driven $^{125}I^-$ uptake. Back-titration of empty phosphorylation sites in brush-borders from cholera toxin-treated and control rats with [γ-^{32}P]ATP (cf. Fig. 1B) showed no significant difference in phosphorylation state of the 25 kDA protein between brush border preparations (not shown). When we assume that the 25 kDa protein became also phosphorylated by endogenous cAMP-dependent protein kinase in the

intact enterocyte, it is therefore likely that the rather time-consuming isolation procedure (2-3 h at 0°C) has led to a dephosphorylation of this 25 kDa protein and presumably also of other proteins.

DISCUSSION

As shown in the present study, our rather elaborate attempts to reconstitute and analyze a potential second messenger regulation of Na^+/H^+ and Cl^-/anion exchangers at the level of rat intestinal brush-border membrane vesicles were unsuccessful sofar. All three methods designed to ensure a cAMP-, cGMP-, Ca^{2+}- or diacylglycerol-dependent phosphorylation of intravesicular proteins, i.e. entrapment of the second messengers and ATP in Mg- or KSCN-vesicles; exposure to cholera toxin in vivo, followed by a isolation of Mg- or KSCN-vesicles, failed to demonstrate a significant change in Na^+/H^+ or Cl^-/anion exchange activity in the vesicle membrane. If we assume that an electroneutral Na^+-Cl^-cotransport composed of a separate Na^+/H^+ exchanger and a Cl^-/anion exchanger is indeed the main target for second messengers in the apical membrane of rat villus enterocytes (as postulated mainly on the basis of transport studies performed in rabbit and chicken intestine [1,2,26,27]), some of the following considerations may explain why this modulation was not observed at the level of the isolated brush-border membrane:

i The regulatory protein responsible for the modulation of Na^+/H^+ or Cl^-/anion exchange may not have been phosphorylated to a stoichiometric level in the ATP- and second messenger-loaded vesicles or may occur in a partially or completely prephosphorylated state in the control vesicles as the result of an imbalance of phosphorylation and dephosphorylation reactions under the in vitro conditions, e.g. following the loss or inactivation of protein phosphatases. Although in vitro phosphorylation experiments have provided evidence for a cAMP, cGMP and Ca^{2+} stimulation of ^{32}P incorporation into a number of substrate proteins in both brush-border caps and brush-border membrane vesicles [7,8], the percentage of occupied phosphorylation sites in each phosphoprotein prior to the ^{32}P incorporation assay is largely unknown [7,8]. In spite of clear evidence for a highly efficient prephosphorylation of the cGMP-

dependent protein kinase (86 kDa) itself and of its 25 kDa substrate protein in ATP plus cGMP-loaded vesicles obtained in the present study, it cannot be completely excluded that the (unknown) modulator is less efficiently phosphorylated under the in vitro conditions. In similar reconstitution experiments recently reported for brush-border membrane vesicles from rabbit proximal tubules [28,29], protein dephosphorylation was prevented by acidification of the intravesicular space (pH 5.5, i.e. far below the physiological range). Even under these acidic conditions, the observed inhibition of Na^+/H^+ exchange by cAMP and ATP [28], and its stimulation by PMA and ATP [29] were very small (i.e. 10-15%) and exclude a more detailed analysis of the molecular mechanisms involved in ion transport regulation.

ii The coupling between a modulator which is phosphorylated or directly activated by the second messengers, and the ion transporter is lost upon the isolation of the brush-border membrane vesicles. This would implicate that the Na^+/H^+ exchanger or the Cl^-/anion exchanger are not tightly connected to their regulatory subunits. Such an assumption seems to conflict with present models for Ca^{2+} and cyclic nucleotide regulation of other ion transport systems, e.g. a Ca^{2+}-activated K^+ channel in neurons [30], and a Cl^- channel in the apical membrane of the trachea [31], which could be both regulated by cAMP-dependent phosphorylation even after reconstitution in model membranes, and the conductance pathways for Na^+ and Cl^- in intestinal brush-border membrane vesicles which, in the presence of low Mg^{2+} levels, were directly activated by Ca^{2+} [10]. Moreover, some evidence for the possible redistribution/uncoupling of protein complexes provoked by the vesiculation process may be derived from our observation that endogenous cGMP-dependent protein kinase was no longer capable of phosphorylating its major substate, the 25 kDa proteolipid, upon vesiculation of the brush-border membrane despite a copurification of both proteins with the vesicle fraction.

iii The modulation of the Na^+/H^+ exchanger or Cl^-/anion exchanger by the cyclic nucleotides or Ca^{2+} is a more complex multi step process, e.g. involving cytosolic or cytoskeletal factors which are either not present or not functioning in the brush-border membrane

vesicles. Interactions of the second messengers with the cytoskeleton leading to a change in Na^+/H^+ exchange as observed by Friedman et al. [32] may not be detectable in brush-border membrane vesicles in which the cytoskeleton is possibly distorted (Mg-brush-border membrane vesicles) or absent (KSCN-brush-border membrane vesicles).

iv Regulation of transport in the apical membrane by insertion of ion transporters or transport modulators from intracellular stores, as has been shown for the Na^+ channel in the rabbit urinary bladder [33] or the H^+/K^+ pump in gastric mucosa [34], would remain undetected in studies in which the putative regulatory factors are entrapped during or following vesiculation of the membrane. This mechanism however is expected to become detectable if the intact epithelium is first preexposed to the secretagogues in vivo prior to vesiculation. Therefore the present observation that pretreatment of a rat intestinal segment with cholera toxin in vivo did not result in an irreversible change in Na^+/H^+ or Cl^-/anion exchange activity in brush-border membrane vesicles isolated from this segment argues strongly against a carrier recruitment model.

Our results do not confirm a direct modulation of Na^+-Cl^- cotransport by intravesicular Ca^{2+} as observed by Fan and Powell in rabbit ileal brush-border membrane vesicles [11], which apparently occurred even in the absence of ATP. The differences in vesicle origin and preparation method however precludes a direct comparison. The calmodulin antagonist TFP, which was used in their study and other reports (see Ref. 2) to demonstrate the involvement of calmodulin in the regulation of Na^+-Cl^- cotransport in ileum, was found to inhibit Na^+/H^+ exchange in brush-border membrane vesicles in our study, compromizing the use of TFP as a tool to investigate the role of calmodulin in Na^+ transport regulation in the intestine. The inhibitory effect of millimolar concentrations of Mg^{2+} on the Na^+/H^+ exchange also described by LaBelle [35] which could be masked by ATP or creatine phosphate should also be taken into account in studies on Na^+ transport regulation at the level of brush-border membranes routinely isolated in most laboratories by the differential precipitation technique employing 10 mM Mg^{2+}. This observation might be interpreted erroneously as evidence for an activating effect of ATP-provoked phosphorylation on the Na^+/H^+ exchanger instead of de-inhibition by Mg^{2+} complexation. Interestingly, the inhibitory effect of Mg^{2+} was not seen in

Na^+/H^+ exchange measurements by means of the acridine orange technique carrried out in the presence of 100 mM internal Na^+ instead of 1 mM external Na^+. The physiological significance of the inhibition of the Na^+/H^+ exchange by Mg^{2+} is therefore less clear, considering the much higher Na^+ concentration at the exterior of the intestinal cell in vivo and the unknown levels of free Mg^{2+} inside the intestinal microvilli in situ. Unfortunately, studies of Na^+/H^+ and Cl^-/anion exchange regulation in Mg^{2+}-depleted brush-border membrane vesicles may be complicated by the Ca^{2+}-provoked changes in cation and anion conductances observed earlier in Mg^{2+}-free conditions [10] which could indirectly influence electroneutral ion transport processes by triggering the collapse of Na^+ or Cl^- gradients across the vesicle membrane.

Our attempts to affect Na^+/H^+ and Cl^-/anion exchange activities in the vesicle membrane under more physiological conditions by preexposing the intestine in vivo to cholera toxin, were also unsuccessful, indicating that cholera toxin, in contrast to its irreversible activation of adenylate cyclase in villus cells [16], did not cause an irreversible inhibition of the Na^+/H^+ or Cl^- anion exchanger in rat intestine. Reversible effects mediated by phosphorylation may however be lost in the time-consuming isolation procedure of the KSCN-brush-border membrane vesicles, as suggested by back-titration of the phosphorylation sites on the 25 kDa protein. A more rapid isolation procedure, involving in situ freeze-clamping of the intestine followed by isolation of Mg-vesicles, did indeed result in a significant increase of Na^+-dependent glucose uptake in cholera toxin-treated compared to control rats in agreement with earlier observations by Murer et al. [36]. The difference however was likely to result from a slightly higher enrichment of apical membranes in the vesicle preparation obtained from cholera toxin-treated rats, since a similar increase (on a protein basis) was observed in the activity of the brush-border marker enzymes alkaline phosphatase and sucrase. In renal brush-border membrane vesicles isolated from kidney slices which were preincubated with parathyroid hormone or dibutyryl cAMP the inhibition of Na^+/H^+ exchange was preserved [37], suggesting a difference with respect to (expression of) the regulation of Na^+/H^+ exchange at the level of the brush-border membrane vesicle between rabbit kidney and rat intestine. The finding that PMA stimulates the Na^+/H^+ exchange in brush-border membrane vesicles from the kidney [29] is also in contrast with the putative effect

of protein kinase C activation on Na^+/H^+ exchange in the brush-border membrane from intestine, where PMA has been shown to act as an inhibitor of Na^+/H^+ exchange or NaCl absorption in rat colon [38], rabbit proximal colon [26], and chicken enterocytes [39].

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CHAPTER VI

GENERAL DISCUSSION

In the studies described in this thesis isolated intestinal brush border membranes were used as a tool to investigate the regulation of ion transport at the apical membrane of the enterocyte. Brush border caps and brush border membrane vesicles were shown to be a useful *in vitro* model for the characterization of the phosphatidylinositol metabolism in the apical membrane of the enterocyte, including the activation of a PIP- and PIP_2-specific phospholipase C by Ca^{2+} and nonhydrolyzable GTP-analogues. The role of the PI-cycle in the brush border membrane in the regulation of ion transport however is not clear considering the possible lack of receptors for hormonal activators of phospholipase C in this membrane as discussed in Chapter II. The BBMV could also be advantageously used to characterize Cl^-/anion exchange activities in the apical membrane of jejunal and ileal intestinal villus cells. The presence of an anion exchanger with a high affinity for HCO_3^- in ileal but not in jejunal BBMV is in agreement with the observation in vivo, that only the ileum contains a Cl^-/HCO_3^- exchange process [1,2].

In contrast, attempts to reconstitute the action of secretagogues on ion transport in the apical membrane by exposing the inside of the BBMV to the putative second messengers in the presence or absence of a phosphorylating system led to results which were more difficult to interprete. The increase in the Na^+, K^+ and Cl^- permeabilities observed in BBMV in response to micromolar levels of intravesicular Ca^{2+} is unlikely to reflect a physiological action of intracellular Ca^{2+} in the intact enterocyte upon its release by intestinal secretagogues, for the following reasons:

(1) Ca^{2+}-activated K^+ channels identified in intact enterocytes have been localized in the basolateral membrane, but not in the apical membrane [3,4] ; moreover, these channels are inhibited by Ba^{2+} or quinidine, in contrast to the Ca^{2+}-activated K^+ permeability in BBMV [3].

(2) An increase in the Na^+ permeability of the apical membrane is expected to promote rather than inhibit absorption of Na^+ *in vivo* ; however intracellular Ca^{2+} has been clearly identified as an inhibitor of NaCl absorption across the brush border membrane in intact enterocytes [5].

(3) The Ca^{2+} provoked increase of the Cl^- permeability of BBMV could in principle reflect the activity of a Ca^{2+}-activated Cl^- channel, recently demonstrated by patch clamp studies in the apical membrane of most Cl^- secreting epithelia [6]; however the Ca^{2+} sensitivity of this physiological Cl^- channel (submicromolar range) contrasts with the micromolar levels of intravesicular Ca^{2+} needed to activate Cl^- transport in intestinal BBMV ; moreover, a recent study of secretagogue-induced Cl^- channel opening in cultured colonic cells failed to demonstrate a modulation of apical Cl^- channels by intracellular Ca^{2+} [7].

(4) The (partial) inhibition of the Ca^{2+} effect on vesicular ion permeabilities by physiological concentrations of Mg^{2+} (2 mM) also renders the physiological significance of the Ca^{2+} action in BBMV more questionable.

Nevertheless, as discussed in Chapter III, the finding of Ca^{2+}-activatable and Mg^{2+}-inhibitable conductance pathways for cations and anions in intestinal BBMV may have important implications for the experimental design and interpretation of transport studies at the level of brush border membrane vesicles.

Subsequent attempts to reconstitute and analyze a possible second messenger regulation of Na^+/H^+ and/or Cl^-/anion exchangers at the level of rat small intestinal BBMV were not successful sofar. As described in Chapter V all three methods designed to ensure a second messenger (e.g. cAMP, cGMP, Ca^{2+} or DAG)-dependent phosphorylation of intravesicular proteins in BBMV i.e. entrapment of the second messengers and ATP in Mg-BBMV and KSCN-BBMV ; exposure to cholera toxin *in vivo*, followed by rapid isolation of BBMV, failed to demonstrate a significant change in Na^+/H^+ or Cl^-/anion exchange activities in the brush border membrane. The negative outcome of this study may find its explanation in one or several of the following considerations:

(1) The *in vitro* conditions may have been suboptimal for the reconstitution of transport regulation (cf. Discussion in Chapter V). A major difficulty in reconstitution experiments is the maintenance of a proper balance between phosphorylation and dephosphorylation reactions reflecting the intact cell conditions, i.e. to ensure a stoichiometric phosphorylation of intravesicular regulatory proteins in ATP-loaded vesicles solely in the presence but not in the absence of regulatory factors (e.g. cyclic nucleotides, Ca^{2+}) and to prevent protein dephosphorylation prior

to or during the transport measurements. In similar reconstitution studies recently carried out with BBMV from rabbit proximal tubules [8-10] protein dephosphorylation could be prevented by acidification of the intravesicular space in the ATP-loaded vesicles (pH 5.5, i.e. far below the physiological range; a condition deliberately avoided in our experiments, because, among other reasons, it would mask a possible second messenger-induced shift in the H^+ sensitivity of the Na^+/H^+ exchanger observed in intact cells at physiological pH, cf. Ref. [11]). Even under these acidic conditions, the observed inhibition of Na^+/H^+ exchange by cAMP and ATP [8,10] and its stimulation by PMA and ATP [9] were very small (i.e. 10-15%), and exclude a more detailed analysis of the molecular mechanisms involved in ion transport regulation. Moreover several other studies reporting a modulation of Na^+ and Cl^- transport systems in BBMV by intravesicular factors were found difficult to reproduce in the same or other laboratories. Examples are the small inhibition of Na^+-phosphate cotransport in renal BBMV by cAMP and ATP described by Hammerman [12], which could not be reproduced in Murers lab using cAMP- or Ca^{2+}- and ATP-loaded vesicles [13,14], the inhibition of Na^+-Cl^--cotransport in rabbit ileal BBMV by Ca^{2+} and calmodulin [15], and the activation of a Cl^- conductance by cAMP and ATP in rat intestinal BBMV [16] (Powell, personal communication; Vaandrager, unpublished results). As mentioned earlier, further complications in reconstitution studies may arise from the in vitro effect of Ca^{2+} and Mg^{2+} on ion permeabilities observed in the intestinal BBMV (Chapter III) and from the effect of Mg^{2+} on the amiloride-sensitive uptake of $^{22}Na^+$ which was masked by ATP (Chapter V).

(2) The cyclic nucleotide-, Ca^{2+}-or phorbolester-sensitive part of NaCl absorption by rat intestinal villus cells is not mediated by a double-exchanger but by a different Na^+-Cl^--cotransport system (see Chapter I) whose activity is lost or at least difficult to demonstrate under in in vitro conditions in isolated vesicles.

(3) The inhibition of intestinal Na^+/H^+ exchange and Na^+-Cl^--cotransport by second messengers observed in other epithelia (e.g. phorbolesters in rabbit proximal colon [17],cAMP and Ca^{2+} in chicken enterocytes [18], Ca^{2+}/calmodulin in rabbit ileum [5]) may not occur in rat intestinal epithelium as a consequence of species differences in salt transport regulation. A study of Hardcastle [19] showing an enhancement rather than

an inhibition of net NaCl absorption in rat mid-intestine by cAMP provides some evidence into this direction.

Unfortunately, a more detailed analysis of the regulatory mechanisms for salt transport in intact enterocytes of rat small intestine is hindered by a number of obstacles, e.g.:

- In comparison with rabbit ileum and colon, it is technically extremely difficult to remove both muscle layers from excised pieces of rat small intestine without damaging the epithelial layer. Complete stripping is however required to prevent ischemia in subsequent Ussing chamber experiments . Moreover the presence of paracrinic and neuronal cells in stripped mucosa which may also respond to Ca^{2+}-ionophore, phorbolester or cyclic nucleotides severely complicate the interpretation of salt transport regulation studies in intact mucosa (Chapter I).
- Techniques allowing the stable insertion of microelectrodes into epithelial cells in situ (cf. recent studies in colon [20] and gall bladder [21]) have not yet been worked out for small intestinal epithelium; such techniques may in principle allow the separate registration of changes in membrane potential or intracellular concentrations of ions in the absorptive villus and the secretory crypt cell.
- The "gigaohm" sealing of patch clamp pipettes to the luminal membrane of intestinal epithelial cells in situ is hampered by the microvillar structure of the brush border membrane and the mucus layer covering the luminal surface of the enterocyte in intact epithelium. Moreover the patch clamp technique is highly suitable for measuring single channel currents across epithelial membranes and for studying the kinetic and regulatory properties of such channels (e.g. the apical Cl^- channel in the crypt), but cannot be used for analysis of electrically neutral transport systems (e.g. Na^+-Cl^--cotransport in the villus).
- Preliminary attempts to show secretagogue-induced changes in Na^+ or Cl^- transport in rat ileal and jejunal enterocytes isolated by a vibration technique [22] or by hyaluronidase treatment [23] have been unsuccessful sofar (Vaandrager, unpublished results). Fluorescent measurements of cytosolic free Ca^{2+} in suspensions of rat enterocytes preloaded with the Ca^{2+}-probe quin-2 revealed the

existence of abnormally high basal Ca^{2+} levels (> 0.8 uM) in the absence of exogenously added secretagogues (De Jonge, unpublished results). Apparently the isolated intestinal cells were incapable of maintaining a physiological level of intracellular Ca^{2+} and are therefore unsuitable for the studies of stimulus-secretion coupling in which intracellular Ca^{2+} levels fullfil an important role (Chapter I). Similar experiments carried out on chicken enterocytes have been more successful: Hyun [24] was able to detect an inhibition of a Na^+ influx pathway by cholera toxin and 8-Br cAMP, whereas Semrad demonstrated in the same preparation an inhibition of Na^+/H^+ exchange by cAMP, apparently mediated by a rise in intracellular Ca^{2+} [18]. It is however questionable whether such results obtained in avian enterocytes may also apply to the mammalian villus cell.

An alternative, highly attractive model for studies of transport regulation is offered by (sub)clones of established epithelial cell lines. The human colonic cell line T84 for instance was successfully applied by Dharmsathaphorn et al. to elucidate the mechanism of Cl^- secretion by a crypt cell (see Chapter I). Using the HT-29 human colonic carcinoma cell line as a model system De Jonge et al. could show a transient increase of cytosolic free Ca^{2+} in response to the secretagogues carbachol, neurotensin, α_1-adrenergic agonists and bradykinin but not to forskolin or 8-Br-cAMP [25]. Another human colon carcinoma cell line, CaCo-2 shows symptoms of spontaneous in vitro differentiation including the development of apical microvilli and brush border specific hydrolases [26], and might offer a suitable model for the study of both Cl^- secretion (Grasset [26], De Jonge, unpublished results) and inhibition of NaCl absorption. In preliminary experiments it was found that PMA as well as forskolin but not Ca^{2+}-ionophore could inhibit $^{22}Na^+$ influx into CaCo-2 monolayers when they were fully differentiated (three weeks after reaching confluence). This inhibition was only partially mimicked by amiloride. These results indicate that the influx of Na^+ in CaCo-2 cells (presumably across the apical membrane) can be modulated by cAMP and protein kinase-C through inhibition of a Na^+ transport system different from the Na^+/H^+ exchanger and that this regulation does not involve a rise in intracellular Ca^{2+} (Vaandrager et al., manuscript in preparation).

All colonic cell lines studied sofar, however, are tumor cells implying that at least part of their intracellular regulatory mechanisms (i.e. involved in growth control) must be changed in the transformation process. Therefore it seems again rather questionable to what extent results of transport regulation studies obtained in cultured colonocytes can be extrapolated to normal colonocytes or villus and crypt enterocytes.

Considering the potential difficulties of intact cell studies summarized above, the reconstitution of ion transport regulation in membrane vesicles isolated from mammalian enterocytes may still be a valuable objective in future studies of the molecular mechanism of ion transport in the epithelium. In principle the isolated membranes lend theirselves easier for a study of the interactions between the various second messengers affecting ion transport in the enterocyte, because the multiple regulatory pathways may be more easily dissected than in the whole cell. For example, the question whether a rise in intracellular Ca^{2+}, induced by exposure of chicken enterocytes to 8-Br-cAMP, is causally linked to cAMP-inhibition of NaCl transport or reflects just a side effect of cAMP in addition to its more direct action on ion transport, is more difficult to elucidate in whole cell studies than in experiments with isolated membrane preparations, in which the effects of cAMP may be studied in the presence of carefully controlled levels of Ca^{2+}. The chances for a successful reconstitution of ion transport regulation at the level of isolated intestinal BBMV however may be increased by a more detailed information about the transport systems involved and their main regulators; studies exploiting the colon carcinoma cell lines may fill up at least part of the gaps in our present knowledge of stimulus-secretion coupling in the intestine.

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SUMMARY

The transport of water across the intestinal barrier, classified as a "leaky" epithelium, is secondary to the net movements of electrolytes, mainly Na^+ and Cl^-. Transepithelial transport of salt is controlled by a variety of hormones and neurotransmitters acting through receptors at the basolateral membrane of the enterocyte. The action of endogenous secretagogues (e.g. acetylcholine, vasoactive intestinal polypeptide) is mimicked by a number of enterotoxins (e.g. choleratoxin, heat-labile and heat-stable Escherichia coli toxin) secreted into the lumen upon bacterial colonization of the intestinal tract and adhering to specific receptors in the apical membrane of the epithelial cells. Active net secretion of salt and water in response to these toxins ("secretory diarrhoea") may lead to severe dehydration and could become lethal if not compensated by intravenous or oral rehydration therapy.

Both endogenous and exogenous secretagogues have been shown to modulate the levels of one or more intracellular signal molecules. The major targets for secretagogue action have been lozalized in the apical membrane of the enterocyte and include (1) an electroneutral Na^+-Cl^- cotransport system in the absorptive villus cell inhibited by secretagogues, and (2) a Cl^- channel enriched in intestinal crypt cells and activated by secretagogues.

Sofar the molecular nature of the secretagogue-sensitive ion transporters in the apical membrane and their regulatory properties are not known in any detail. In the present study isolated brush border membrane vesicles were used as a tool to analyze the possible interactions of second messengers (e.g. cyclic nucleotides, calcium, diacylglycerol) and protein kinases activated by such messengers with Na^+ and Cl^- transport systems in the apical border. Advantages and drawbacks of the use of isolated membrane vesicles as a model system for studying ion transport regulation are discussed extensively in Chapter I and VI.

Our study also provides the first evidence for the existence of a polyphosphoinositide cycle in the luminal membrane of an epithelial cell, which is activated by calcium and a GTP-binding protein. A detailed analysis of phosphatidylinositol (PI) metabolism in isolated intestinal brush borders and brush border membrane vesicles is given in Chapter II. Despite extensive washings in hypotonic EDTA, isolated brush borders

appeared to be enriched in the major enzymes of the polyphosphoinositide cycle, i.e. DAG-, PI- and PIP kinases, PIP, PIP_2, IP_3, and IP_2 phosphomonoesterases (other than alkaline phosphatase) and a specific PIP- and PIP_2-phosphodiesterase (phospholipase C). The latter enzyme could be activated by 10^{-4} M Ca^{2+}, but also by nonhydrolyzable GTP analogues in the presence of 10^{-7} - 10^{-6} M Ca^{2+}, which is diagnostic for the presence of a G protein associated with phospholipase C in the brush border membrane. G proteins have been shown to function as transducing elements between hormone receptors and second messenger generating processes like the breakdown of PIP_2 in mammalian plasma membranes. It is therefore remarkable that a G protein is coupled to phospholipase C in a membrane which presumably does not contain receptors for hormonal activators of this enzyme. The role of PI metabolism in the brush border in the regulation of ion transport systems is therefore not clear; plausibly the local generation of second messengers (IP_3, DAG) at the apical border could initiate a local release of Ca^{2+} from submicrovillar stores and activate a brush border pool of DAG-activated protein kinase C. Moreover, phosphorylation of PI in the microenvironment of an ion channel or carrier may influence the activity of the transporter.

Chapter III deals with the determination of ion permeabilities in brush border membrane vesicles (BBMV) by three independent methods; (i) measurement of Na^+-dependent ^3H-glucose uptake in the presence of various anions; (ii) determination of initial influx rates of $^{22}Na^+$, $^{36}Cl^-$ or $^{86}Rb^+$, and (iii) stopped-flow spectrophotometry of salt-induced osmotic swelling. The permeabilities (P) for the various ions were found to decrease in the following order: $P_{SCN^-} > P_{NO^-} > P_{Na^+} > P_{K^+} > P_{Cl^-} > P_{gluconate}$. Incubation of BBMV prepared by KSCN treatment (but not by differential Mg^{2+}-precipitation) with 10^{-5} M Ca^{2+} in the presence of the Ca^{2+}-ionophore A23187, resulted in a more than twofold increase in the permeabilities for Na^+,K^+ and Cl^- as compared to Ca^{2+}-free conditions. The effect of Ca^{2+} on the ion permeabilities was half-maximal at 10^{-6} M, fully reversible and dependent on internal Ca^{2+}, suggesting a localization of Ca^{2+} sensor sites at the inner surface of the microvillus membrane. The influx of small uncharged molecules as glucose and mannitol was not affected by Ca^{2+}, showing that micromolar Ca^{2+} did not cause a general leakiness of the vesicle membrane. The effect of Ca^{2+} on the ion permeabilities could be blocked by Ba^{2+} (10^{-3} M) and Mg^{2+} (10^{-2} M), which

explains the lack of effect of Ca^{2+} on BBMV isolated by a Mg^{2+}-precipitation. In contrast amiloride (10^{-3} M), an inhibitor of Na^+ channels, apamin (2.10^{-7} M) and quinine (5.10^{-4} M) both inhibitors of K^+ channels, and trifluoperazine (10^{-4} M), a calmodulin antagonist, were all unable to affect vesicular ion transport. It is at present unclear as to whether Ca^{2+} activates a non-selective cation and anion channel or multiple selective channels in the BBMV. Considering the antagonistic effect of Mg^{2+} ("Mg^{2+}-brake") on the Ca^{2+} activation of vesicular ion permeabilities, the physiological significance of the effect of Ca^{2+} is likewise questionable.

In addition to electrogenic channel-type pathways for ion transport, intestinal brush borders also contain electroneutral and saturable cation (Na^+/H^+) and anion (e.g. Cl^-/HCO_3^-) exchange activities which may be coupled by circular proton movements. A sensitive method for the demonstration of Cl^-/anion exchange, which under certain conditions (isotope equilibrium, 25 mM KCl) may account for as much as 75% of the $^{36}Cl^-$ influx across the vesicle membrane, is described in Chapter IV. The overshooting uptake of tracer amounts of $^{125}I^-$ into BBMV in the presence of a large Cl^- gradient (in >> out) was interpreted as evidence for the operation of an electroneutral Cl^-/anion exchange system rather than an anion channel considering the insensitivity of the overshoot to voltage clamping by K^+ and valinomycin and its inhibition by SITS, an anion exchange inhibitor, and a number of other anions such as NO_3^-, SCN^-, Cl^- and formate. The Cl^-/I^- exchange process in ileal BBMV showed a clear heterogeneity in respect to the inhibitory effects of SITS, HCO_3^- and SO_4^{2-}. Approximately 30% of the Cl^-/I^- exchange activity showed a relatively low sensitivity for SO_4^{2-} (IC_{50} > 20 mM) and SITS (IC_{50} = 1 mM) but could be inhibited for 80% by 2 mM HCO_3^-. This exchange presumably reflects the activity of the classical Cl^-/HCO_3^- exchanger. The residual 70% of the exchange activity showed a relatively high sensitivity for SO_4^{2-} (IC_{50} ≈ 0.5 mM) and SITS (IC_{50} ≈ 2.5 μM) but was less sensitive for HCO_3^-. An anion exchanger with similar properties as this last one was also identified in BBMV isolated from rat jejunal segments. Both exchangers may play a role in net Cl^- transport by catalyzing the exchange of Cl^- for HCO_3^- or formate or by performing Cl^-/SO_4^{2-}-exchange, followed by a SO_4^{2-}/OH^- exchange. Some of these anions i.e. HCO_3^-, formate and OH^- may recycle across the membrane following their protonation facilitated by the Na^+/H^+ exchanger.

Both the Na^+/H^+ exchanger and Cl^- anion exchangers may therefore function as targets for secretagogues which inhibit Na^+ and Cl^- absorption across the luminal membrane of the intestinal villus cells. In Chapter V the possibility was explored that second messengers of secretagogues, i.e. cAMP, cGMP, Ca^{2+} or DAG, could inhibit Na^+/H^+ or anion exchange either by direct allosteric interaction or through the activation of brush border bound protein kinases and subsequent phosphorylation of the exchange protein itself or a regulatory protein. For that purpose BBMV were preloaded with ATP and the second messengers either by a freeze-thaw procedure or alternatively, by provoking the vesiculation of isolated brush border caps by KSCN treatment in the presence of these agents. In both vesicle preparations a change in phosphorylation state of cGMP-regulated phosphoproteins following entrapment of cGMP and ATP was confirmed by back-titration with [γ-^{32}P]ATP. However, neither the Na^+/H^+ exchange activity as measured by quenching of the pH sensitive dye acridine orange or as amiloride sensitive influx of $^{22}Na^+$, nor the anion exchange activity as measured by Cl^--driven $^{125}I^-$ uptake was significantly altered by the encapsulation of ATP alone or ATP plus cyclic nucleotides, Ca^{2+} or phorbol ester (PMA). The latter compound acts as a specific activator of a DAG-dependent protein kinase (PK-C) in the presence of Ca^{2+}. Surprisingly, the amiloride sensitive influx of $^{22}Na^+$ appeared strongly inhibited by encapsulation of 5 mM Mg^{2+} or 50 μM TFP. The physiological significance of such an internal Mg^{2+}-modifier site on the Na^+/H^+ exchange is unclear. Moreover, TFP is unlikely to act as a specific inhibitor of calmodulin or PK-C because a similar inhibition occurred in the absence of Ca^{2+} or ATP.

In an alternative approach jejunal-ileal segments of rat intestine were pre-exposed <u>in situ</u> to cholera toxin in order to provoke maximal rates of NaCl and water secretion. BBMV were rapidly isolated from this tissue and used for measurements of Na^+/H^+ or anion exchange activities. However, no significant differences were found in transport properties of BBMV from saline- or cholera toxin-injected intestinal segments.

Our failure to demonstrate modulation of Na^+/H^+ or anion exchange activities in BBMV by internal Ca^{2+} or by Ca^{2+}-, phorbolester-, or cyclic nucleotide-dependent phosphorylation of intravesicular substrate proteins argues strongly against a direct regulatory role of Ca^{2+}/calmodulin (in the absence of phosphorylation) or of Ca^{2+}- and cyclic nucleotide-

dependent phosphorylation reactions with respect to these exchangers. Instead the second messengers may possibly act through a more complex mechanism involving critical steps which are either located outside the brush border region, or irreproducible at the level of isolated BBMV. Alternatively, the inhibition of coupled NaCl absorption by cyclic nucleotides and Ca^{2+} observed in intact epithelium might not reflect an inhibition of Na^+/H^+ or Cl^-/HCO_3^- exchangers, but could instead result from the blockade of a different Na^+-Cl^- cotransport system coexisting in the same membrane (as demonstrated earlier in other absorptive epithelia) whose activity could be lost following vesicle isolation.

As discussed in Chapter VI, a better characterization of the secretagogue-sensitive ion transport systems and their regulation by second messengers in the intact epithelium is needed to improve the chances for a successful reconstitution of ion transport regulation at the level of isolated membrane vesicles. Filter-grown monolayers of human colon carcinoma cell lines expressing hormone- and bacterial toxin-sensitive Cl^- channels (e.g. T84 and CaCo-2) and Na^+ influx pathways (CaCo-2) may prove to be highly useful model systems for such studies.

SAMENVATTING

Het transport van water door de darmbarrière, een zogenaamd "lek-epitheel", is afhankelijk van de netto verplaatsingen van electrolyten, voornamelijk Na^+ en Cl^-. Het transepitheliaaltransport van zout wordt gecontroleerd door een groot aantal hormonen en neurotransmitters, die werken via receptoren aan de basolaterale membraan van de enterocyt. De werking van endogene secretagogen wordt nagebootst door een aantal toxines (b.v. choleratoxine, hitte-stabiele en hitte-labiele Escherichia coli toxine) die gesecreteerd worden door pathogene bacteriën in het darmlumen. Deze toxines binden aan specifieke receptoren in de apicale membraan van de epitheelcellen. De actieve netto secretie van water en zout, veroorzaakt door deze toxines ("secretoire diarree") kan leiden tot een ernstige dehydratie, en zelfs tot de dood, indien ze niet gecompenseerd wordt door intraveneuze of orale rehydratietherapie.

Van zowel de endogene als de exogene secretagogen is aangetoond dat ze de niveaus van een of meer intracellulaire boodschappermoleculen beïnvloeden. De belangrijkste aangrijpingspunten van de secretagogen liggen in de apicale membraan van de enterocyten en bestaan uit (1) een electroneutraal Na^+-Cl^--cotransportsysteem in de absorberende villuscellen, dat geremd wordt door de secretagogen en (2) een Cl^--kanaal, dat voor het grootste deel gelegen is in de cryptcellen en dat gestimuleerd wordt door de secretagogen.

Over de moleculaire aard van de secretagoog-gevoelige ionentransporteurs in de apicale membraan en hun regulatie bestaat tot nu toe weinig exacte kennis. In het hier beschreven onderzoek werden geïsoleerde borstelzoommembraan-vesicles gebruikt als modelsysteem om de mogelijke interacties te bestuderen van de intracellulaire boodschappermoleculen, de zogenaamde second messengers (b.v. cyclische nucleotiden, calcium en diacylglycerol), met de Na^+- en Cl^--transporterende systemen in de apicale membraan. Bij deze interactie kunnen eiwit-kinasen, die geactiveerd worden door zulke second messengers, een rol spelen. Voor- en nadelen van het gebruik van geïsoleerde membraan-vesicles als model voor de bestudering van ionentransport-regulatie worden besproken in hoofdstuk I en VI.

Deze studie leverde het eerste bewijs voor de aanwezigheid van een "polyfosfoinositide-cyclus" in de luminale membraan van een epitheelcel, die geactiveerd kan worden door Ca^{2+} en een GTP-bindend eiwit. Een

gedetailleerde beschrijving van het fosfatidylinositol (PI)-metabolisme in geïsoleerde darmborstelzomen en borstelzoommembraan-vesicles wordt gegeven in hoofdstuk II. Geïsoleerde borstelzomen bleken, zelfs na een uitgebreide wasprocedure in hypotone EDTA de belangrijkste enzymen van de polyfosfoinositide-cyclus te bevatten d.i. DAG-, PI- en PIP-kinasen, PIP-, PIP_2-, IP_3- en IP_2-fosfomonoesterasen (anders dan alkalische fosfatase) en een specifiek PIP- en PIP_2-fosfodiesterase (fosfolipase C). Het laatst genoemde enzym kon geactiveerd worden door 10^{-4} M Ca^{2+}, maar ook door niet-hydrolyseerbare GTP-analogen in de aanwezigheid van 10^{-7}-10^{-6} M Ca^{2+}, wat duidt op de koppeling van een G-eiwit met fosfolipase-C in de borstelzoommembraan. Het is aangetoond dat G-eiwitten functioneren als transducers in de koppeling tussen hormoonreceptoren en systemen, die second messengers genereren in plasmamembranen van zoogdiercellen. Het is daarom opmerkelijk dat een G-eiwit gekoppeld is aan fosfolipase-C in een membraan dat vermoedelijk geen receptoren bevat, die dit enzym activeren. De functie van het PI-metabolisme in de borstelzoom is dan ook niet duidelijk; vermoedelijk kan het second messengers (IP_3, DAG) bij de apicale membraan genereren. Deze kunnen zowel locaal Ca^{2+} vrijmaken uit reservoirs in of vlak onder de microvillus als een DAG-geactiveerd eiwit kinase-C in de borstelzoom activeren. Verder zou de fosforylering van PI rondom een ionentransporteur de activiteit van die transporteur kunnen beïnvloeden.

Hoofdstuk III behandelt de bepaling van de permeabiliteit voor ionen van de borstelzoommembraan-vesicles (BZMV) door middel van drie onafhankelijke methoden: (1) bepaling van Na^+-afhankelijke [^{3}H]glucose opname in aanwezigheid van verschillende anionen; (2) meting van de initiële instroomsnelheden van $^{22}Na^+$, $^{36}Cl^-$ en $^{86}Rb^+$ en (iii) stopped-flow spectrofotometrie van de door zout-geïnduceerde osmotische zwelling. De permeabiliteiten (P) voor de verschillende ionen bleken af te nemen in de volgende volgorde: $P_{SCN^-} > P_{NO^-} > P_{Na^+} > P_{K^+} > P_{Cl^-} > P_{gluconaat}$. Incubatie van de BZMV die gemaakt waren door middel van een thiocyanaatbehandeling (en niet door een differentiële Mg^{2+}-precipitatie) met 10^{-5} M Ca^{2+}, in aanwezigheid van de Ca^{2+}-ionofoor A23187, leidde tot een ruim tweevoudige toename in de permeabiliteit voor Na^+, K^+ en Cl^-, in verhouding tot een incubatie in de afwezigheid van Ca^{2+}. Het effect van Ca^{2+} was (i) half-maximaal bij 10^{-6} M, (ii) volledig omkeerbaar en (iii) trad alleen op als Ca^{2+} aan de binnenkant van de vesicles aanwezig was, wat suggereert dat de Ca^{2+}-

gevoelige plaatsen gelegen zijn op het binnenoppervlak van de microvillusmembraan. De opname van kleine ongeladen moleculen zoals glucose en mannitol werd niet beïnvloed door Ca^{2+}, wat erop duidt dat micromolaire hoeveelheden Ca^{2+} geen aspecifieke lekkage van de vesiclemembraan veroorzaakten. Het effect van Ca^{2+} op de ionenpermeabiliteit kon geremd worden door Ba^{2+} (10^{-3} M) en Mg^{2+} (10^{-2} M), wat het ontbreken van het Ca^{2+}-effect in BZMV, die gemaakt waren met behulp van een Mg^{2+} precipitatie verklaart. Amiloride (10^{-3} M), een remmer van Na^{+}-kanalen, apamine (2.10^{-7} M) en kinine (5.10^{-4} M), beide remmers van K^{+}-kanalen, en trifluoperazine (10^{-4} M), een calmoduline antagonist, waren echter niet in staat het ionentransport in de BZMV te beïnvloeden. Het is nog niet duidelijk of Ca^{2+} een weinig selectief kationen en anionen kanaal of meerdere specifieke kanalen in de BZMV activeert. Het is ook de vraag of het effect van Ca^{2+} op de permeabiliteiten voor ionen in de BZMV enige fysiologische betekenis heeft, gezien de remmende werking van Mg^{2+} ("Mg-brake") op het Ca^{2+}-effect.

Naast de electrogene op kanalen gelijkende routes voor ionen bevatten darmborstelzomen ook verzadigbare electroneutrale kationen- (Na^{+}/H^{+}) en anionen (zoals Cl^{-}/HCO_3^{-})-uitwisselaars die gekoppeld kunnen worden door circulerende protonbewegingen. Een gevoelige methode voor het aantonen van Cl^{-}/anionenuitwisseling, die onder bepaalde omstandigheden (isotoop-equilibrium, 25 mM KCl) tot 75% van de $^{36}Cl^{-}$-opname in de BZMV kan verzorgen, is beschreven in hoofdstuk IV. De opname van kleine hoeveelheden $^{125}I^{-}$ in BZMV onder invloed van een steile Cl^{-} gradiënt (inwendig >> uitwendig) bleek ver boven de evenwichtswaarde uit te komen. Dit werd geïnterpreteerd als een bewijs voor een anionenuitwisselingsproces, en niet voor een anionenkanaal omdat het accumuleren van $^{125}I^{-}$ niet beïnvloed kon worden door het constant houden van de membraanpotentiaal met K^{+} en valinomycine; daarentegen kon dit proces wel geremd worden door SITS, een remmer van anionenuitwisseling en andere anionen zoals NO_3^{-}, SCN^{-}, Cl^{-} en formaat. Het Cl^{-}/I^{-}-uitwisselingsproces in BZMV, verkregen uit het ileum, vertoonde een duidelijke heterogeniteit met betrekking tot de remmende werking van SITS, SO_4^{2-} en HCO_3^{-}. Ongeveer 30% van de Cl^{-}/I^{-}-uitwisseling had een relatief lage gevoeligheid voor SO_4^{2-} (IC_{50} > 20 mM) en SITS (IC_{50} = 1 mM) maar kon al voor 80% geremd worden door 2 mM HCO_3^{-}. Deze uitwisseling geeft vermoedelijk de activiteit van de klassieke Cl^{-}/ HCO_3^{-}-uitwisselaar weer. De overige 70% van de Cl^{-}/I^{-}-uitwisseling vertoonde een

relatief hoge gevoeligheid voor SO_4^{2-} ($IC_{50} \approx 0.5$ mM) en SITS ($IC_{50} \approx 2.5$ μM), maar was ongevoeliger voor HCO_3^-. Een anionenuitwisselaar met vergelijkbare eigenschappen als deze laatstgenoemde werd ook in BZMV geïsoleerd uit jejunum aangetroffen. Beide uitwisselaars zouden een rol kunnen spelen in het netto transport van Cl^- door het katalyseren van een uitwisseling van Cl^- tegen hetzij HCO_3^-, hetzij formaat, of door het uitwisselen van Cl^- tegen SO_4^{2-}, gevolgd door de uitwisseling van SO_4^{2-} tegen OH^-. Deze anionen d.i. HCO_3^-, formaat en OH^-kunnen recirculeren door de membraan nadat ze geprotoneerd zijn met behulp van de Na^+/H^+-uitwisselaar. Zowel de Na^+/H^+ uitwisselaar als de Cl^-/anionenuitwisselaars kunnen daarom als aangrijpingspunten voor de secretagogen dienen, die de absorptie van Na^+ en Cl^+ door de luminale membraan van de villuscellen in de darm remmen.

Zoals beschreven in hoofdstuk V werd de mogelijkheid onderzocht dat second messengers, die door de secretagogen gegenereerd worden d.i. cAMP, cGMP, Ca^{2+} en DAG, de Na^+/H^+ of de anionenuitwisselaar direct, door een allosterische interactie of indirect door fosforylering van de uitwisselaars zelf of van een regulerend eiwit, kunnen remmen. Daarvoor werden BZMV opgeladen met ATP en de second messengers met behulp van een vriesdooimethode of door het maken van vesicles uit borstelzomen met een thiocyanaatbehandeling in aanwezigheid van deze stoffen. Met behulp van een terugtitratie met [γ-^{32}P]ATP werd een verandering in de fosforyleringsgraad van de door cGMP-gereguleerde fosfoëiwitten na insluiting van cGMP en ATP bevestigd. De activiteit van zowel de Na^+/H^+-uitwisselaar, die gemeten werd met behulp van de pH-gevoelige stof acridine orange en door middel van amiloride-gevoelige opname van $^{22}Na^+$ als de anionenuitwisselaar, die gemeten werd als Cl^--gedreven $^{125}I^-$-opname waren echter niet significant veranderd door de insluiting van ATP alleen of van ATP samen met cyclische nucleotiden, Ca^{2+} of phorbolester (PMA). Laatstgenoemde stof is een specifieke stimulator van de DAG-afhankelijke eiwitkinase (PK-C) in de aanwezigheid van Ca^{2+}. De amiloride-gevoelige opname van $^{22}Na^+$ bleek echter wel aanzienlijk geremd na insluiting van 5 mg Mg^{2+} of 50 μM TFP. De fysiologische betekenis van de Mg^{2+}-remming is onduidelijk. TFP werkt hier vermoedelijk niet als een remmer van calmoduline of van PK-C, aangezien een dergelijke remming ook gezien werd in afwezigheid van Ca^{2+} of ATP.

In een andere benadering werden gecombineerde segmenten van ileum en jejunum in situ blootgesteld aan choleratoxine om een maximale secretie

van water en zout te induceren. Uit deze darmen werden BZMV geïsoleerd waarin zowel de Na^+/H^+- als de anionenuitwisselingsactiviteit bepaald werden. Er werden echter geen significante verschillen in ionentransport gevonden tussen de BZMV, die geïsoleerd waren uit de met fysiologisch zout behandelde en de met choleratoxine behandelde segmenten.

Het feit dat we geen verandering van Na^+/H^+- of anionenuitwisseling in BZMV konden aantonen met behulp van Ca^{2+}, of met cyclische nucleotiden, Ca^{2+} of phorbolester-afhankelijke fosforylering van intravesiculaire substraateiwitten pleit tegen een directe rol van Ca^{2+}-calmoduline (in de afwezigheid van fosforylering) of van Ca^{2+} en cyclische nucleotiden-afhankelijke fosforyleringsreacties in de regulatie van de uitwisselaars in de apicale membraan. In plaats daarvan zouden de second messengers misschien via een meer complex mechanisme kunnen werken, waarvan een deel of buiten de borstelzoom plaatsvindt of niet meer gereproduceerd kan worden in een geïsoleerde borstelzoommembraan-vesicle. Het zou echter ook kunnen dat de waargenomen remming van NaCl-absorptie door cyclische nucleotiden en Ca^{2+} in een intact epitheel geen remming van de Na^+/H^+- of de Cl^-/HCO_3^--uitwisselaars weerspiegelen. In dat geval zou een ander type NaCl-cotransporteur in dezelfde membraan (zoals aangetoond in andere absorberende epithelia), waarvan de activiteit verloren gaat tijdens de isolatie van de vesicles, door secretagogen geremd kunnen worden.

Zoals in hoofdstuk VI besproken wordt kan een betere karakterisering van de secretagoog-gevoelige transportsystemen en hun regulatie door second messengers in een intact epitheel de kansen op een succesvolle reconstitutie van de regulatie van het ionentransport in geïsoleerde membraanvesicles vergroten. Humane colonepitheelcellijnen, die zowel hormoongevoelige Cl^--kanalen (T84, CaCo-2) als Na^+-opnameroutes (CaCo-2) tot expressie brengen, zouden als modelsystemen kunnen dienen voor dergelijke studies.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd op 21 oktober 1958 geboren in Utrecht. In 1977 behaalde hij het diploma Gymnasium β aan het Coornhert Gymnasium te Gouda. In datzelfde jaar begon hij met de studie Scheikunde aan de Rijksuniversiteit Utrecht. Het kandidaatsexamen S_2 werd in september 1980 en het doctoraal examen in mei 1983 behaald met als bijvak immunologie en als specialisatie biochemie. Vanaf juni 1983 tot december 1986 werd in dienst van FUNGO/ZWO aan de afdeling Biochemie I van de Erasmus Universiteit Rotterdam en de Gastrointestinal Division of Columbia University, New York (november en december 1985) het onderzoek verricht dat tot dit proefschrift geleid heeft.
Vanaf juni 1987 werkt hij op de afdeling Biochemie I van de Erasmus Universiteit Rotterdam aan een project van de Nederlandse Hart-stichting.