Elevated Intracellular Ca^{2+} Acts through Protein Kinase C to Regulate Rabbit Ileal NaCl Absorption

Evidence for Sequential Control by Ca^{2+}/Calmodulin and Protein Kinase C

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Abstract

Calcium/calmodulin is involved in the regulation of basal rabbit ileal active Na and Cl absorption, but the mechanism by which elevated intracellular Ca^{2+} affects Na and Cl transport is unknown. To investigate the roles of the Ca^{2+}/calmodulin and protein kinase C systems in ileal NaCl transport, two drugs, the isoquinolenesulfonamide, H-7, and the naphthalenesulfonamide, W_{13}, were used in concentrations that conferred specificity in the antagonism of protein kinase C (60 μM H-7) and Ca^{2+}/calmodulin (45 μM W_{13}), respectively, as determined using phosphorylation assays in ileal villus cells. W_{13} but not H-7 stimulated basal active NaCl absorption. H-7 inhibited changes in Na and Cl absorption caused by maximal concentrations of Ca^{2+} ionophore A23187 and carbachol and serotonin, secretagogues that act by increasing cytosol Ca^{2+}, while W_{13} had no effect. In contrast, neither H-7 nor W_{13} altered the change in NaCl transport caused by the cyclic nucleotides 8-Br-cAMP and 8-Br-cGMP. These data suggest that: (*a*) basal rabbit ileal NaCl absorption is regulated by the Ca^{2+}/calmodulin complex and not by protein kinase C; (*b*) the effect of elevating intracellular Ca^{2+} to decrease NaCl absorption is mediated via protein kinase C but not by Ca^{2+}/calmodulin; (*c*) the effects of protein kinase C are not overlapping or synergistic with those of Ca^{2+}/calmodulin on either basal absorption or on the effects of increased Ca^{2+}; and (*d*) neither Ca^{2+}/calmodulin nor protein kinase C are involved in the effects of cAMP and cGMP on ileal active NaCl transport.

Introduction

Basal active Na and Cl transport in rabbit ileum is regulated by Ca^{2+}/calmodulin (CaM)[1] (1–7). The naphthalenesulfonamide, W_{13}, a Ca^{2+}/CaM inhibitor, stimulates a specific Na-absorptive process, active linked NaCl absorption. This suggests that, under basal conditions, this Na-absorptive process is under continuous inhibition by Ca^{2+}/CaM (2, 3, 7). Elevating intracellular Ca^{2+} in ileal epithelial cells by neurohumoral agents or by Ca^{2+} ionophores further decreases the neutral linked NaCl-absorptive process and also stimulates electrogenic Cl secretion. Surprisingly, when Ca^{2+} was increased in ileal epithelial cells by the Ca^{2+} ionophore A23187, carbachol or serotonin, the Ca^{2+}-induced changes in Na and Cl transport were not reversed by Ca^{2+}/CaM inhibitors (7). Thus, the mechanism by which elevated intracellular Ca^{2+} affects ileal electrolyte transport is not known. The present study was undertaken in an effort to determine the intracellular mediator responsible for the effects of elevated cytosol free Ca^{2+} in regulating rabbit ileal Na and Cl transport. The results suggest that elevated Ca^{2+} acts on NaCl absorption through protein kinase C.

Methods

Animals. Male New Zealand albino rabbits weighing 2–2.5 kg were maintained on a standard rabbit laboratory diet with free access to water. The animals were anesthetized with pentobarbital sodium, and the distal ileum was removed. Epithelial sheets or villus cells were prepared for study as described below.

In vitro active electrolyte transport. The methods used to measure active ileal electrolyte transport have been described previously (4). In brief, stripped ileal mucosa was mounted as a flat sheet between two lucite modified Ussing chambers that had an area of 1.13 cm^2, oxygenated, and maintained at 37°C. Usually six to eight pieces of ileum from a single animal were studied simultaneously. Transmural potential difference (PD), short-circuit current (I_{sc}), and conductance (G) were determined. An automatic voltage clamp corrected for fluid resistance between the PD-sensing bridges and provided continuous short-circuiting of the tissue. Unidirectional fluxes of ^{22}Na and ^{36}Cl were determined on the same tissue matched to differ in G by not more than 25% and were measured while the tissues were short-circuited.

Unless specified, the bathing solutions consisted of Ringer's-HCO_3 composed of (in millimolar): NaCl, 115; $NaHCO_3$, 25; K_2HPO_4, 2.4; KH_2PO_4, 0.4; $CaCl_2$, 1.2; and $MgCl_2$, 1.2. Osmolality was 275 mosmol/kg and pH was 7.4 after gassing with 95% O_2-5% CO_2. 10 mM glucose was added to the serosal and 10 mM mannitol to the mucosal bathing fluid at the time of mounting the tissues.

Separate experiments were performed to determine the effects of the isoquinolenesulfonamide, H-7, and W_{13} on basal transport and on changes in transport caused by glucose-dependent stimulation of Na absorption and on the change in transport caused by secretagogues. In the latter, experiments evaluating the effects of H-7, W_{13}, or indomethacin on changes in transport caused by the Ca^{2+} ionophore A23187, serotonin, carbachol, theophylline, 8-Br-cAMP, and 8-Br-cGMP were performed. In these experiments, either the inhibitors or solvent controls were added 20 min after adding isotope and 40 min after mounting ileal mucosa in the Ussing chambers. 10 min later, two 20-min periods of ion flux measurements were made (designated period A in Table I and in figure legends). This was followed by addition

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1. *Abbreviations used in this paper:* CaM, calmodulin; G, conductance; I_{sc}, short circuit current; PD, potential difference; PS, phosphatidylserine.

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of secretagogue or a solvent control and, starting 10 min later, by two further 20-min flux periods (designated period B in Table I and figure legends). Ethanol was added to the time controls up to the same ethanol concentration as the simultaneously studied test substances and in all cases was less than 0.1%.

Protein kinase C assay. Villus cells were obtained by lightly scraping the ileal mucosa as previously described (8) and cytosol prepared at 4°C. The mucosa was homogenized in a buffer solution containing 5 mM EDTA, 0.5 mM EGTA, 50 mM DTT, 0.2 mM PMSF, 0.01% leupeptin, 1 ng/ml phosphoramidone, 20 mM Tris, pH 7.5, in a glass/teflon homogenizer. The homogenate was centrifuged at 40,000 *g* for 1 h. Glycerol was added to the supernatant to a concentration of 10% and stored at −80°C until use. The supernatant was diluted 1:5 before use and protein kinase C was assayed within one week of preparation.

Protein kinase C activity was assayed by the measurement of transfer of ^{32}P from ATP to histone III-S which occurred in the presence of phosphatidylserine (PS), phorbol dibutyrate, and Ca^{2+} compared with Ca^{2+} alone. The protein kinase C assay mixture (100 μl) contained 10 mM Mg acetate, 1.1 mM $CaCl_2$, 1 mM EGTA, 2 mM DTT, 0.01% leupeptin, 40 μg of histone III-S, 5 μM or 500 μM [γ-^{32}P]ATP (3.8 μCi), ~ 15 μg of cytosol protein, 2 mM Tris, pH 7.5. When PS and phorbol dibutyrate were used, they were at concentrations of 40 μg/ml and 10 μM, respectively. The isoquinolenesulfonamide H-7 or the naphthalenesulfonamides W_{13} and W_{12} were added over a range of concentrations. PS and phorbol dibutyrate were prepared by bath sonication (1-min sonication of PS in water at room temperature followed by addition of phorbol dibutyrate and an additional 1-min sonication at room temperature) and were always added just before starting the reaction by the addition of cytosol. Assays were performed over 10 min at 30°C and terminated by the addition of 1 ml of 10% TCA-0.2% Na pyrophosphate to each tube, followed by heating at 100°C for 10 min. The protein precipitates were collected on glass fiber filters (model GF/A, Whatman, Inc., Clifton, NJ), dried overnight, and assayed by liquid scintillation spectrometry. Results were expressed as picomoles phosphate per milligram protein. The concentration-dependent inhibition of protein kinase C by H-7 or W_{13} was determined by Woolf-Hanes analyses for each experiment (9) by setting total activity in the absence of inhibitor as 100% and determining the inhibitor concentration which reduced activity to 50%.

Ca^{2+}/CaM-dependent phosphorylation of ileal microvillus membranes. Microvillus membranes were prepared as open vesicles from rabbit ileal villus cells as previously described (8). Basal phosphorylation and the increase in phosphorylation caused by Ca^{2+}/CaM-dependent protein kinase(s) in the microvillus membranes were determined as previously reported using broken cell phosphorylation techniques with [γ-^{32}P]ATP, separating the phosphorylated peptides by one-dimensional SDS-PAGE (5–15% continuous acrylamide gradient), identifying the phosphorylated peptides by a comparison with simultaneously run molecular weight standards, and quantifying the extent of phosphorylation by scanning densitometry (10–12). Results are expressed in arbitrary densitometry units. Phosphorylation was carried out at 5 and 500 μM ATP and in the presence and absence of 60 μM H-7; when present, the free Ca^{2+} concentration was 0.3 μM and exogenous CaM was 5 μM.

Theophylline was purchased from Eastman Kodak Co., Rochester, NY; Tris, ATP, 8-Br-cAMP, 8-Br-cGMP, carbachol, serotonin creatinine sulfate, W_{12}, W_{13}, H-7, indomethacin, PS, phorbol dibutyrate, histone III-S, and CaM were from Sigma Chemical Co., St. Louis, MO.

Results are expressed as mean±SEM. Statistical analyses, unless otherwise specified, were by Student's paired and unpaired *t* tests.

Results

Initially, conditions were determined under which H-7 affected ileal protein kinase C activity without significantly affecting Ca^{2+}/CaM-dependent protein kinase activity, as indicated by an effect on specific Ca^{2+}/CaM-dependent protein phosphorylation in the same tissue in which transport was being studied.

Effect of H-7 and W_{13} on ileal villus cell cytosol protein kinase C activity. Protein kinase C activity was measured in ileal absorptive cell cytosol using histone III-S as a substrate, under conditions of maximal stimulation by Ca^{2+} (free Ca^{2+} ~ 100 μM), PS (40 μg/ml), and phorbol dibutyrate (10 μM). H-7 caused a concentration-dependent inhibition of protein kinase C activity, as shown in Fig. 1, when protein kinase C activity was assayed using two different concentrations of ATP. In Fig. 1 (*left*), the ATP concentration was 5 μM, which is the concentration used in most published protein kinase C assays (13), and H-7 inhibited protein kinase C activity with an IC_{50} of 22±6 μM. A concentration-dependent inhibition by H-7 of protein kinase C was also seen in the presence of 500 μM ATP (Fig. 1 [*right*]), but now with an IC_{50} of 89±11 μM.

In contrast to the effects of H-7, the naphthalenesulfonamide W_{13} (at 45 μM, the IC_{50} for stimulation of ileal NaCl absorption [7]) failed to cause a significant inhibition of cytosol protein kinase C activity. When W_{13} was studied in the presence of 5 μM ATP protein kinase C activity was −13±8% of the control value (n = 3); 45 μM W_{12}, a hydrophobic control for W_{13} (14), also failed to cause any significant change and the cytosol protein kinase C activity was +5±9% of the control value (n = 3).

Effect of H-7 on ileal villus cell microvillus membrane Ca^{2+}/CaM-dependent phosphorylation. We have previously demonstrated that Ca^{2+}/CaM increased the phosphorylation of six microvillus membrane peptides, identified by one-dimensional SDS-PAGE and autoradiography (10–12), and that W_{13} caused a concentration-dependent inhibition of these phosphorylations with an IC_{50} of ~ 45 μM (unpublished observations). In order to determine the specificity of the H-7

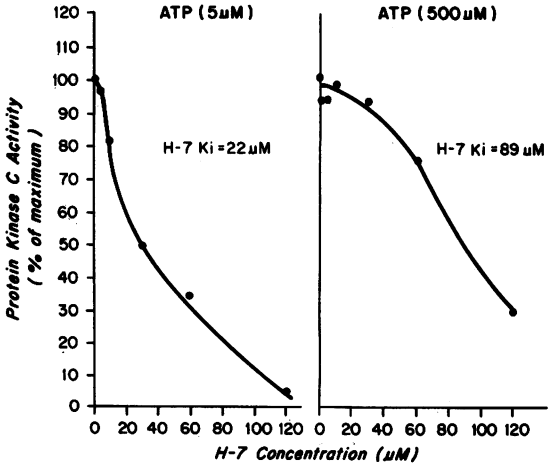

Figure 1. Concentration-dependent inhibition of ileal absorptive cell cytosol protein kinase C activity by H-7. Data shown are from three experiments and are expressed as a percent protein kinase C activity measured in the absence of H-7. Experiments shown in both panels were identical except that the total ATP concentration was either 5 or 500 μM. IC_{50} values were derived for each experiment by Woolf-Hanes linearization. IC_{50} for H-7 at 5 μM ATP was 22±6 μM and at 500 μM ATP the IC_{50} was 89±11 μM. In the absence of H-7, protein kinase C activity with 5 and 500 μM ATP was 322±112 and 475±205 pmol phosphate incorporated/mg protein per 10 min, respectively.

effect on protein kinase C, the effect of H-7 was determined on these Ca^{2+}/CaM-stimulated microvillus membrane phosphorylations studied in the presence of 5 and 500 μM ATP. The results were similar at both ATP concentrations. 60 μM H-7 caused a general decrease in basal microvillus membrane phosphorylation (Fig. 2 *A*, compare lanes *1* and *2*, and lanes *3* and *4*). In contrast to this general decrease in phosphorylation, the Ca^{2+}/CaM stimulation of phosphorylation of specific protein bands was not inhibited by H-7. As previously reported (10–12) and shown in Fig. 2 *A*, Ca^{2+}/calmodulin increased the phosphorylation of peptides with molecular weights of 137,000, 116,000, 77,000, 58,000, 53,000, and 50,000; these were all increased similarly whether in the presence or absence of H-7 (Fig. 2, *A* and *B*). Thus, under conditions of 60 μM H-7 exposure used for the intact tissue studies, H-7 did not significantly inhibit brush border Ca^{2+}/CaM-dependent phosphorylation although it did decrease basal phosphorylation.

Effects of H-7 on basal ileal electrolyte transport. The effects of 60 μM H-7, the inhibitor of protein kinase C, were determined in transport studies on intact tissue, and compared with the effects on transport caused by 45 μM W_{13}, the Ca^{2+}/CaM inhibitor. Shown in Table I are the effects of 60 μM H-7 and 45 μM W_{13} on basal ileal active electrolyte transport, compared with time controls. When values for two 20-min flux periods are compared to two 20-min flux periods from the same tissues before drug addition, 60 μM H-7 caused a slight increase in ileal I_{sc} and an increase in serosal-to-mucosal Cl flux, neither of which were significant when compared to time-related changes in control tissue. When two further 20-min flux periods were studied both in time control tissue and tissue to which 60 μM H-7 was added (i.e., total 50–90 min after H-7 addition), there were no significant changes in transport in either H-7 exposed or time control tissue. Of note, H-7 induced a rapid onset, small increase in I_{sc} which occurred maximally within 2 min of addition; the change in ion transport which explains this increase was not detected by flux measurements. 60 μM H-7 also did not alter the glucose-dependent increase in ileal I_{sc} (peak increase in I_{sc} which occurred within 10 min of addition of 10 mM glucose to the mucosal surface was 1.59±0.59 vs. 1.64±0.48 μeq/cm^2-h in H-7 exposed (n = 5) and untreated control tissue (n = 5), respectively).

Confirming our previous studies (7), W-13 caused a significant decrease in ileal I_{sc} and decrease in G (Table I). In addition, 45 μM W_{13} significantly increased mucosal-to-serosal Na and Cl unidirectional fluxes which led to a significant increase in both net Na and Cl absorption, but did not alter glucose-dependent Na absorption. In separate experiments, the effect of 45 μM W_{13} was determined over the same time course in ileal mucosa initially exposed for 50 min to serosal addition of 1 μM Ca^{2+} ionophore A23187 plus 60 μM mucosal plus serosal H-7. These effects of W_{13} were similar to the effects of W_{13} on basal transport shown in Table I. The effects of W_{13} in tissue pretreated with Ca^{2+} ionophore plus H-7 were (compare with W_{13} effect in Table I): I_{sc}, −0.81±0.26 μeq/cm^2-h; J^{Na}_{ms}, 1.03±0.41 μeq/cm^2-h; J^{Na}_{sm}, −0.51±0.89; J^{Na}_{net}, 1.55±0.68; J^{Cl}_{ms}, 2.00±.71; J^{Cl}_{sm}, 0.81±0.99; J^{Cl}_{net}, 1.19±0.30; n = 5.

Effects of H-7 on Ca^{2+}-mediated changes in ileal active electrolyte transport. The effects of H-7 on changes in ileal active electrolyte transport caused by Ca^{2+} (both as elevated by

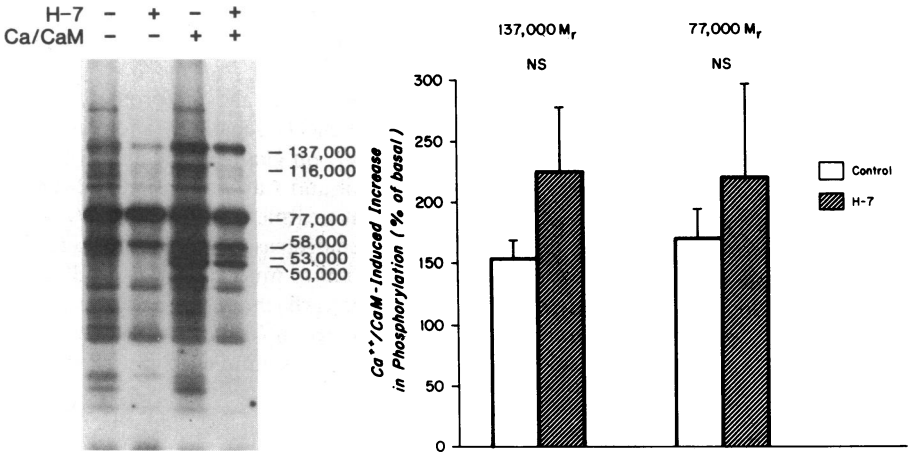

Figure 2. Effect of 60 μM H-7 on the Ca^{2+}/CaM-dependent increase in phosphorylation of ileal microvillus membranes. (*A*) Autoradiograms of phosphorylation of microvillus membranes with the incubation solution containing microvillus membranes and 500 μM [^{32}P]ATP, 3 mM EGTA, 5 mM $MgCl_2$, 10% (wt/vol) sucrose in lane *1* and, additionally, in lanes *3* and *4* from left, 0.3 μM free Ca^{2+} plus 5 μM exogenous CaM, and in lanes *2* and *4*, 60 μM H-7. Phosphorylation was performed at 0°C for 120 s and the reaction was stopped by addition of 50 μl of a solution of 0.1 M EDTA, 5% (wt/vol) SDS, 200 mM DTT, and 5 μg of pyronin Y/ml, followed immediately by immersion in boiling water for 2 min and incubation at 27°C for 20 min. Equal amounts of membrane protein (50 μg) were loaded on each lane of the polyacrylamide gel. Analysis was by scanning densitometry and the results are expressed in arbitrary densitometry units. H-7 decreased the basal phosphorylation (compare lanes *1* and *2*); however, Ca^{2+}/CaM increased phosphorylation comparably in six peptides in the presence and absence of H-7 (compare lanes *1/3* and *2/4*). The increase can be seen on the autoradiogram except for the 77,000-mol wt peptide which reached saturation on the photographic film. As previously reported, Ca^{2+}/CaM effects on phosphorylation of this peptide are best seen with 30 s of phosphorylation at 0°C and data from such phosphorylation studies are shown in *B*. To indicate the magnitude of the increase caused by Ca^{2+}/CaM in the presence and absence of H-7, the Ca^{2+}/CaM-dependent increase in phosphorylation of peptides with molecular weights of 137,000 and 77,000 caused in the presence and absence of H-7 is shown, with the data shown being the magnitude of the phosphorylation in the presence of Ca^{2+}/CaM expressed as a percent of basal phosphorylation. Note, Ca^{2+}/CaM caused a slightly, but not significantly greater increase in phosphorylation in the presence of H-7. Data are mean±SEM of three experiments. NS refers to comparison of the Ca^{2+}/CaM effect in the presence and absence of H-7. A similar conclusion, that the Ca^{2+}/CaM-induced increased phosphorylation of these peptides was not inhibited by 60 μM H-7, was reached when the magnitude of the Ca^{2+}/CaM-induced increase in phosphorylation of these six peptides was determined (in arbitrary densitometry units) in the presence and absence of H-7 (increase in phosphorylation of 137,000-mol wt peptide in the absence and presence of H-7, respectively, 0.22±0.04 vs. 0.16±0.04, n = 3, NS; increase in phosphorylation of 77,000-mol wt peptide in the absence and presence of H-7, respectively, 2.0±1.0 vs. 0.8±0.3, n = 3, NS).

*Table I. Effect of H-7 and W_{13} on Basal Ileal Active Electrolyte Transport Compared to Time Control**

Conditions	Period A									Period B				
	I_{sc}	PD	G	J^{Na}_{ms}	J^{Na}_{sm}	J^{Na}_{net}	J^{Cl}_{ms}	J^{Cl}_{sm}	J^{Cl}_{net}	I_{sc}	PD	G	J^{Na}_{ms}	J^{Na}_{sm}
0–0 (n = 8)	2.11	−2.8	20.7	10.46	9.18	1.28	5.95	6.15	−0.19	2.34	−3.1	20.9	10.52	9.76
	±0.31	±0.4	±1.0	±0.51	±0.84	±0.48	±0.47	±0.55	±0.49	±0.35	±0.5	±0.7	±0.63	±0.75
P^+														
O–H-7 (n = 10)	2.02	−3.2	15.8	7.79	7.87	−0.08	5.11	5.92	−0.80	2.69	−4.4	16.4	7.91	8.15
	±0.26	±0.4	±1.0	±0.71	±0.65	±0.53	±0.33	±0.36	±0.30	±0.20	±0.5	±1.4	±0.70	±0.74
P^+														
P^{++}														
O–W_{13} (n = 6)	2.13	−4.3	15.0	5.62	7.66	−2.04	6.28	6.32	−0.04	1.12	−3.7	13.9	7.20	8.36
	±0.20	±0.7	±2.3	±0.91	±0.81	±0.76	±1.12	±0.91	±0.99	±0.19	±0.5	±2.1	±0.72	±0.85
P^+														
P^{++}														

* Units for I_{sc} and fluxes are μeq/cm^2-h; PD, mV; G, mS/cm^2. n, number of animals studied. Period A: two 20-min flux periods starting 60 min after mounting tissue and 20 min after isotope addition. Period B: two 20-min flux periods starting 10 min after addition of H-7 or W_{13} and 70 min after isotope addition. P^+ refers to comparison of periods A and B in the same tissue (paired t test). P^{++} refers to comparison of period B − A vs. time control over the same period (unpaired t test).

the neurohumoral secretagogues carbachol and serotonin and by the Ca^{2+} ionophore A23187) and by the cyclic nucleotides, cAMP and cGMP, were studied. In these studies (Fig. 3), as has been previously demonstrated (15), 10^{-6} M carbachol added to the ileal serosal surface caused a significant increase in I_{sc} and decreased both net sodium and chloride fluxes due to a decrease in the mucosal-to-serosal Na and Cl fluxes as well as to a slight but not significant increase in the serosal-to-mucosal Cl flux. In the presence of 60 μM H-7, carbachol caused a significantly smaller increase in I_{sc} and failed to significantly decrease either net Na or net Cl fluxes. This was due to the inhibition by H-7 of the carbachol-induced decrease in the mucosal-to-serosal Na and Cl fluxes and increase in the serosal-to-mucosal Cl flux.

Similarly, as previously described (16, 17), serotonin increased ileal I_{sc} and decreased net Na and Cl fluxes, due to a decrease in mucosal-to-serosal Na and Cl fluxes (Fig. 4). Treatment with 60 μM H-7 inhibited the serotonin-induced increase in I_{sc} and prevented the serotonin-induced decreases in mucosal-to-serosal and net Na and Cl fluxes (Fig. 4).

As previously reported (7, 18), Ca^{2+} ionophore A23187 increased ileal I_{sc} and decreased net Na and net Cl fluxes with the effects being due to decreases in mucosal-to-serosal Na and Cl fluxes and an increase in the serosal-to-mucosal Cl flux (Fig. 5). Treatment with 60 μM H-7, similarly to the effects on the carbachol- and serotonin-induced changes in ileal transport, inhibited the calcium ionophore-induced increase in ileal I_{sc} and the decrease in net Na and net Cl fluxes with the effects being due to inhibition of the decreases in mucosal-to-serosal Na and Cl fluxes.

Because, in some cell types, Ca^{2+}/CaM and protein kinase C interact, we determined the effects, separately and together, of 60 μM H-7 and 45 μM W_{13} on changes in active ileal Cl transport caused by carbachol and Ca^{2+} ionophore A23187. The results in Fig. 6 show that treatment with 45 μM W_{13} did not alter the carbachol-induced decrease in mucosal-to-serosal Cl flux or the increase in serosal-to-mucosal Cl flux and consequently had no effect on the carbachol-induced decrease in net Cl transport. Thus the change in Cl fluxes caused by carbachol in the presence of W_{13} was similar to the effects of

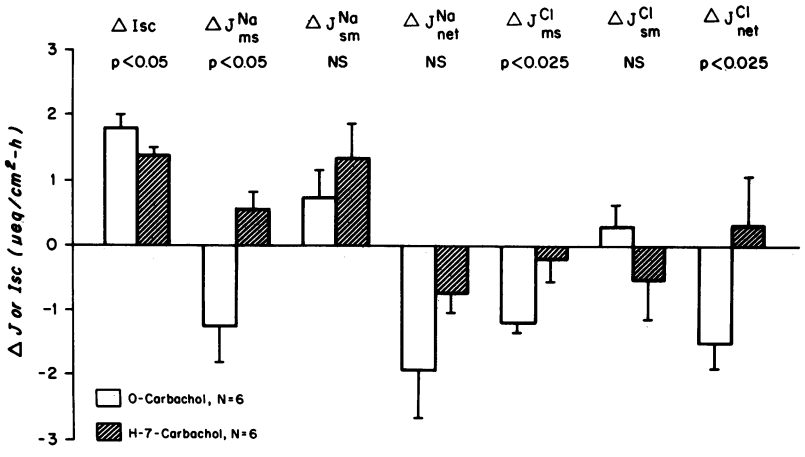

Figure 3. Effect of 60 μM H-7 on carbachol (10^{-6} M)-induced changes in ileal Na and Cl transport. During period A, tissue is either treated as control, with no addition, or exposed to 60 μM H-7 on the mucosal plus serosal surfaces and 10 min after H-7 addition, two 20-min flux periods were determined. Then, carbachol was added to the serosal surface and, again starting 10 min after addition, fluxes were measured over two further 20-min periods (period B). Data shown are the means±SEM of the difference between period B and period A. N refers to the number of animals studied. P values above the bars refer to comparison of the carbachol effect in the presence and in the absence of H-7 (paired t test).

				B − A									Glucose-dependent increase in I_{sc}
J^{Na}_{net}	J^{Cl}_{ms}	J^{Cl}_{sm}	J^{Cl}_{net}	I_{sc}	PD	G	J^{Na}_{ms}	J^{Na}_{sm}	J^{Na}_{net}	J^{Cl}_{ms}	J^{Cl}_{sm}	J^{Cl}_{net}	
0.76	5.81	6.52	−0.71	0.21	−0.3	0.2	0.06	0.58	−0.52	−0.14	0.37	−0.51	1.64
±0.65	±0.57	±0.51	±0.53	±0.12	±0.3	±0.6	±0.40	±0.28	±0.35	±0.11	±0.32	±0.30	±0.48
				NS	NS	NS	NS	NS	NS	NS	NS	NS	
−0.24	5.24	6.69	−1.45	0.67	−1.2	0.6	0.12	0.28	−0.17	0.13	0.78	−0.65	1.59
±0.58	±0.40	±0.30	±0.66	±0.24	±0.7	±0.6	±0.38	±0.38	±0.48	±0.41	±0.21	±0.48	±0.59
				<0.05	NS	NS	NS	NS	NS	NS	<0.05	NS	
				NS	NS	NS	NS	NS	NS	NS	NS	NS	
−1.16	7.64	6.38	1.26	−1.11	0.6	−1.1	1.59	0.70	0.89	1.36	0.06	1.30	1.90
±0.48	±0.78	±0.55	±0.61	±0.34	±0.5	±0.4	±0.47	±0.27	±0.33	±0.38	±0.18	±0.43	±0.56
				<0.05	NS	<0.05	<0.05	<0.05	<0.05	<0.05	NS	<0.025	
				<0.05	NS	NS	<0.05	NS	<0.05	<0.01	NS	<0.01	

carbachol alone. Furthermore, H-7 inhibited all aspects of Cl flux changes caused by carbachol, while the effects of H-7 in the presence of W_{13} were not different from the effects of H-7 alone. That is, the effects of carbachol on Cl fluxes were inhibited by H-7 with no evidence of synergism or additivity on the carbachol effects when H-7 and W_{13} were studied together.

As shown in Fig. 7, the effects of H-7 and W_{13} on the changes in Cl transport caused by calcium ionophore A23187 were similar to their effects on the changes in Cl transport caused by carbachol. W_{13} itself had no significant effect on the changes in Cl fluxes caused by Ca^{2+} ionophore A23187 and the combination of W_{13} plus H-7 was not different than the effects of H-7 by itself, which inhibited all Cl flux effects of the Ca^{2+} ionophore.

Effects of H-7 on cyclic nucleotide-induced changes in ileal active electrolyte transport. To study the specificity of the effect of H-7 in inhibiting Ca^{2+}-mediated changes in active ileal electrolyte transport, the effect of pretreatment with 60 μM H-7 was determined on the changes in active electrolyte transport produced by 8-Br-cAMP, 8-Br-cGMP, and 10 mM theophylline. As shown in Fig. 8, 100 μM 8-Br-cAMP increased ileal I_{sc} and decreased net Na and Cl fluxes with the effect on the net Cl flux exceeding the effect on the net Na flux. Pretreatment with 60 μM H-7 did not alter any aspect of active electrolyte transport affected by 8-Br-cAMP. Similarly, 100 μM 8-Br-cGMP altered active ileal electrolyte transport by increasing the I_{sc} and decreasing the net Na and Cl fluxes. Again, pretreatment with 60 μM H-7 did not alter the effects of 8-Br-cGMP on ileal I_{sc} or on net Na or net Cl fluxes.

Pretreatment with 60 μM H-7 did not alter the change in active ileal electrolyte transport caused by 10 mM theophylline added to the ileal serosal surface. The increase in ileal I_{sc} produced by theophylline was 61±12 vs. 60±7 μA/cm² in theophylline exposed tissue previously exposed to 60 μM H-7; the change in net Na flux was −0.84±0.38 vs. −0.71±0.24 μeq/cm²-h in theophylline-exposed tissue and in tissue exposed to theophylline after exposure to 60 μM H-7, respectively; the change in net Cl flux was −2.47±0.70 vs. −2.56±0.57 μeq/cm²-h in theophylline-exposed tissue and in tissue exposed to theophylline after exposure to 60 μM H-7, respectively.

Role of arachidonic acid metabolites via the cyclooxygenase pathway in the Ca^{2+}-induced changes in ileal electrolyte transport. In rat descending colon, the increase in electrogenic Cl secretion caused by phorbol dibutyrate (presumably acting by protein kinase C) is mediated by arachidonic acid metabolites via the cyclooxygenase pathway, whereas the decrease in

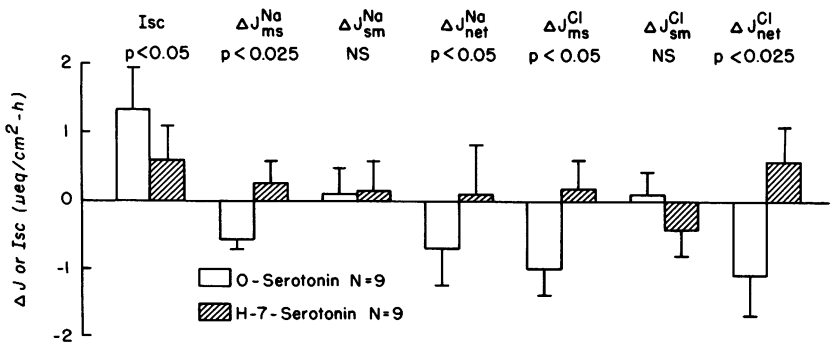

Figure 4. Effect of 60 μM H-7 on serotonin (2.6×10^{-4} M)-induced changes in ileal Na and Cl transport. These studies were performed as described in the legend to Fig. 3 and in Methods. The change in I_{sc} is the peak increase caused by serotonin, which occurred within 10 min of serotonin addition.

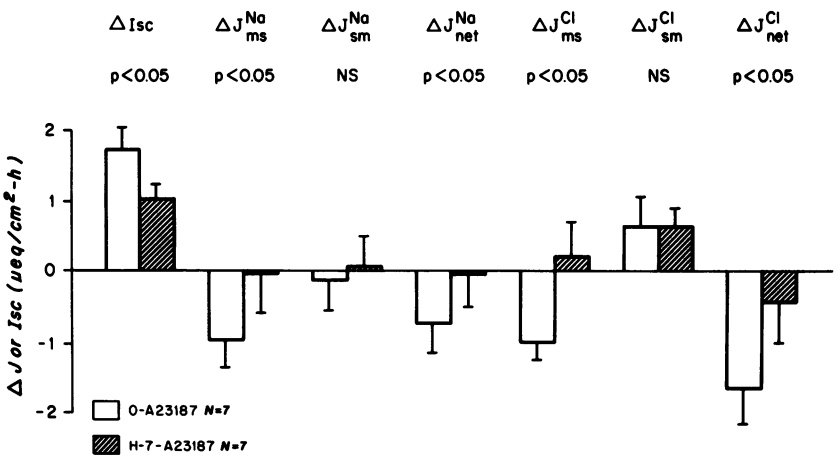

Figure 5. Effect of 60 μM H-7 on Ca^{2+} ionophore A23187 (10^{-6} M)-induced changes in ileal Na and Cl transport. The studies were performed as described in the legend to Fig. 3 and in Methods.

NaCl absorption, caused simultaneously, occurred independently of the cyclooxygenase pathway (19). Consequently, we determined whether the effects on active ileal electrolyte transport caused by Ca^{2+} ionophore A23187 or carbachol also were mediated by products of the cyclooxygenase pathway. Pretreatment of ileal mucosa with 10 μM indomethacin for 50 min did not alter the effects on active ileal electrolyte fluxes caused by Ca^{2+} ionophore A23187 (10^{-6} M) or carbachol (10^{-6} M) (Table II).

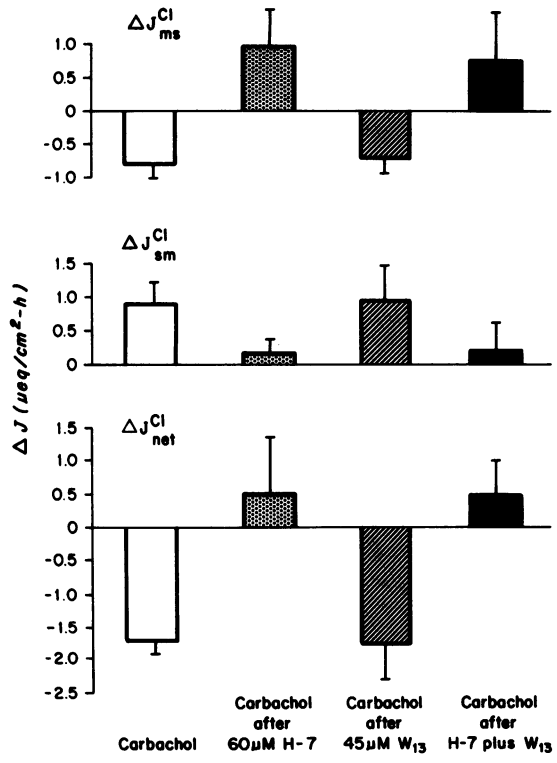

Figure 6. The effects of H-7 and/or W_{13} on carbachol (10^{-6} M)-induced changes in ileal Cl transport. Studies were performed as described in the legend to Fig. 3 with the change in Cl fluxes caused by 10^{-6} M carbachol determined for two 20-min flux periods starting 10 minutes after carbachol addition and compared to a basal period consisting of two 20-min flux periods either in the absence of additions or studied with 60 μM H-7, 45 μM W_{13}, or the combination of H-7 plus W_{13}. Data from six experiments are shown and expressed as means±SEM.

Discussion

In these studies, conditions were established in which H-7 was an effective inhibitor of protein kinase C activity in ileal villus epithelial cells, and its specificity, regarding protein kinases currently recognized as being involved in regulation of ileal electrolyte transport, was determined by a variety of protein kinase and transport studies. The conclusions reached in these studies are firmest for those cells in which the protein kinase activity was measured: i.e., the villus absorptive cells which contain the neutral NaCl absorptive process. Consequently, in the discussion we emphasize regulation of NaCl absorption and not Cl secretion. H-7 at 60 μM did not affect all ileal

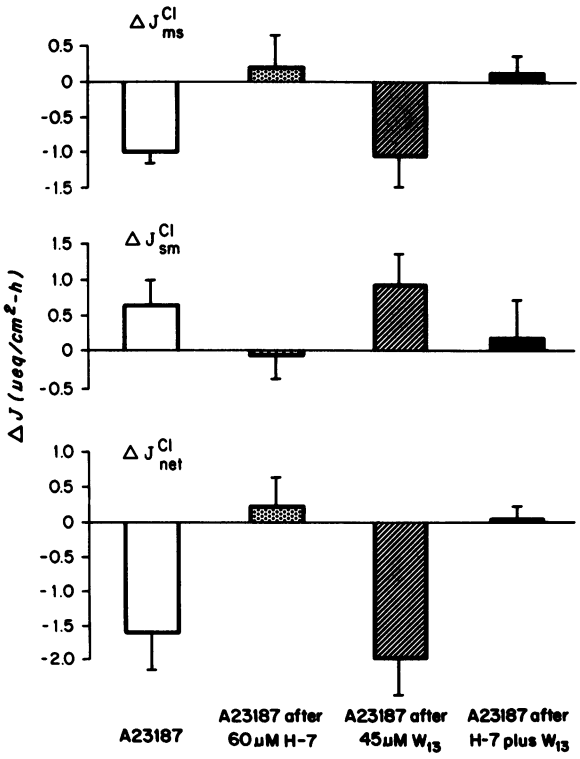

Figure 7. The effects of H-7 and W_{13} on Ca^{2+} ionophore A23187 (10^{-6} M)-induced changes in ileal Cl transport. Experiments were carried out as described in the legend to Fig. 6. Data from seven animals are shown and expressed as means±SEM.

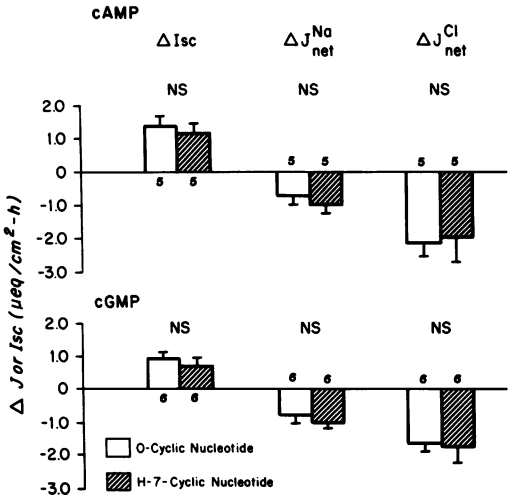

Figure 8. The effect of 60 μM H-7 on 8-Br-cAMP- and 8-Br-cGMP-induced changes in ileal Na and Cl transport. Data shown represent the effects of 100 μM 8-Br-cAMP (*top*) and 100 μM 8-Br-cGMP (*bottom*) on active ileal electrolyte transport determined during two 20-min flux periods starting 10 min after cyclic nucleotide addition (period B) compared to a control period with fluxes determined during two 20-min flux periods ending just before addition of the cyclic 8-Br-cAMP and 8-Br-cGMP (period A). For comparison are shown the effects of similar additions and concentrations of cyclic nucleotides performed in the presence of 60 μM H-7, which was present for ten minutes before initiating flux measurements during period A. Numbers below and above horizontal lines refer to the number of animals studied. Results are mean±SEM. *P* values above the bars represent comparison of the effects of cyclic nucleotides in the presence and absence of H-7 (paired *t* test).

protein kinases. Specifically, H-7 did not affect Ca^{2+}/CaM-dependent protein kinase activity in broken cell studies with the same ileal villus cells used for transport measurements and did not alter the cAMP- or cGMP-dependent protein kinases involved in regulation of ileal active Na and Cl transport. Further evidence that H-7 is not acting as an antagonist of Ca^{2+}/CaM-dependent protein kinases in these studies is summarized in Table III in which the effects of W_{13}, which is a Ca^{2+}/CaM antagonist, are compared with the effects of H-7. It is especially important to note, with respect to basal transport and the effects of agents which alter Na and Cl transport by altering cell Ca^{2+}, that W_{13} and H-7 had very few similar effects. That W_{13} had effects on basal transport not duplicated by H-7 and that W_{13}, not H-7, inhibited Ca^{2+}/CaM-dependent phosphorylation of specific peptides in ileal microvillus membranes (the location of the rate-limiting step for Na and Cl absorption) strongly suggests that in ileal villus cells, H-7 is not acting as a Ca^{2+}/CaM antagonist. Thus, in intact ileal villus cells, H-7 appears to be an inhibitor of protein kinase C and not an inhibitor of microvillus membrane Ca^{2+}/CaM-dependent protein kinase(s) or an inhibitor of cAMP- or cGMP-dependent protein kinases. H-7 did decrease basal ileal brush border phosphorylation (Fig. 2 *A*), performed in the absence of Ca^{2+}, indicating that it had effects not attributable to inhibition of protein kinase C; but this was not associated with a change in basal active ileal NaCl absorption. Thus this effect of H-7 is not involved in regulation of active NaCl absorption. This demonstrated specificity that 60 μM H-7 does not inhibit ileal Ca^{2+}/CaM-dependent protein kinase and does not affect the protein kinases through which cAMP and cGMP are presumed to alter ileal active Na and Cl transport is somewhat surprising. This is because, although H-7 is a poor inhibitor of the Ca^{2+}/CaM-dependent myosin light chain kinase activity, in some cells under broken cell conditions it is a better antagonist of cAMP-dependent protein kinase than it is a protein kinase C antagonist, and is an equally effective cGMP-dependent protein kinase antagonist (20–22). The discrepancies between the intact tissue studies presented here and that of bro-

Table II. Effect of Indomethacin (10 μM) on Carbachol (1 μM)- and Ca^{2+} Ionophore A23187 (1 μM)-induced Changes in Ileal Active Electrolyte Transport

Period	I_{sc}	PD	G	J^{Na}_{ms}	J^{Na}_{sm}	J^{Na}_{net}	J^{Cl}_{ms}	J^{Cl}_{sm}	J^{Cl}_{net}
A23187									
A. Indomethacin (10 μM)	1.23±0.22	−1.3±0.3	22.3±1.7	10.42±0.71	9.27±0.64	1.15±0.85	9.52±0.88	6.63±0.54	2.89±1.01
B. A23187 (1 μM)	1.75±0.19	−2.1±0.3	23.1±1.5	8.53±0.85	8.97±0.68	−0.44±0.78	8.33±0.58	8.56±0.58	−0.23±0.56
(n = 6) B − A	0.52±0.15	−0.8±0.3	0.8±0.4	−1.89±0.75	−0.30±0.70	−1.59±0.71	−1.19±0.69	1.93±0.43	−3.12±0.67
P^+	<0.05	<0.05	NS	<0.05	NS	<0.05	<0.05	<0.05	<0.025
A. O (ethanol)	1.72±0.33	−2.5±0.5	17.5±0.9	8.26±0.29	7.51±0.56	0.75±0.52	6.24±0.45	6.86±0.47	−0.62±0.34
B. A23187 (1 μM)	2.16±0.30	−3.3±0.5	17.5±0.9	7.40±0.24	7.95±0.53	−0.55±0.52	5.13±0.55	7.59±0.48	−2.46±0.26
(n = 6) B − A	0.44±0.07	−0.8±0.1	0.1±0.5	−0.86±0.23	0.44±0.29	−1.30±0.36	−1.11±0.23	0.73±0.35	−1.84±0.40
P^+	<0.05	<0.05	NS	<0.05	NS	<0.05	<0.05	<0.05	<0.025
P^{++}	NS	NS	NS	NS	NS	NS	NS	NS	NS
Carbachol									
A. Indomethacin	1.79±0.37	−2.6±1.2	19.9±1.6	11.68±0.96	9.06±0.59	2.59±0.81	8.31±0.74	7.08±0.66	1.23±0.88
B. Carbachol (1 μM)	2.05±0.30	−3.3±1.6	20.8±3.0	9.89±0.84	10.72±0.63	−0.83±0.79	7.14±0.63	8.70±0.80	−1.56±0.71
(n = 5) B − A	0.26±0.26	−0.7±0.6	0.9±0.8	−1.79±0.77	1.66±0.76	−3.43±0.81	−1.17±0.61	1.62±0.61	−2.79±0.69
P^+	NS	NS	NS	<0.05	<0.05	<0.025	<0.05	<0.025	<0.025
A. O (ethanol)	2.76±0.45	−4.1±0.5	18.2±1.3	8.63±0.71	8.65±0.55	−0.02±0.54	6.23±0.78	7.72±0.43	−1.49±0.62
B. Carbachol (1 μM)	2.94±0.23	−4.5±0.4	18.0±1.0	6.96±0.53	10.53±0.81	−3.57±0.64	4.84±0.66	8.95±0.89	−4.11±0.65
(n = 5) B − A	0.18±0.24	−0.4±0.3	−0.2±0.6	−1.67±0.59	1.88±1.06	−3.55±0.83	−1.39±0.61	1.23±0.65	−2.62±0.80
P^+	NS	NS	NS	<0.025	NS	<0.01	<0.05	<0.05	<0.01
P^{++}	NS	NS	NS	NS	NS	NS	NS	NS	NS

* Units as in Table I. *n*, number of animals studied. Period A: two 20-min flux periods starting 60 min after mounting the tissue, and 10 min after adding indomethacin or solvent control (0.1% ethanol). Period B: two 20-min flux periods starting 10 min after serosal addition of Ca^{2+} ionophore A23187 or carbachol. P^+ refers to comparison of periods A and B in the same tissue (paired *t* test). P^{++} refers to comparison of period B − A (A23187 or carbachol effect) in the absence vs. the presence of indomethacin (paired *t* test).

Table III. Different Effects on Basal and Stimulated Changes in Ileal Active Na and Cl Absorption Caused by a Ca^{2+}/CaM Antagonist (W_{13}) and a Protein Kinase C Antagonist (H-7)

	Protein kinase C antagonist H-7 (60 μM)	Ca^{2+}/CaM antagonist W_{13} (45 μM)
Basal		
NaCl absorption	–	↑
I_{sc}	↑ (slight)	↓
Glucose-dependent Na absorption	–	–
Ca^{2+}-mediated changes in Na and Cl absorption		
Carbachol: decreased NaCl absorption	↓	–
Serotonin: decreased NaCl absorption	↓	–
Ca^{2+} ionophore A23187: decreased NaCl absorption	↓	–
cAMP, cGMP-mediated changes in Na and Cl absorption		
Decreased NaCl absorption	–	–

–, no effect.

ken cell protein kinase studies are not understood. In this regard, it is of interest that the cAMP protein kinase inhibitor also appears far less effective on membrane bound than soluble cAMP-dependent protein kinases (23). This specificity may not pertain to all intact cells, and the specificity of drugs such as H-7 must be determined in each individual system in which they are used. This is why we limit our discussion to ileal villus Na-absorbing cells. That specificity cannot be assumed for any intact tissue studies was demonstrated recently in lymphocytes in which neither H-7 nor W_{13} demonstrated the specificity found in the current work (24).

Complicating the choice of conditions under which to study the effect of H-7 is the fact that H-7 competes with ATP in its action to inhibit protein kinases. Thus the concentration of ATP and the concentration achieved by H-7 at the site of interaction on a protein kinase becomes of great importance. In most cells ATP concentrations are thought to be in the range of 3–6 mM. With such high concentrations of ATP, it would not be expected that H-7 would be a very effective protein kinase inhibitor in intact tissue. In ileal villus cells the rate-limiting step for Na transport is at the brush border. The brush border ATP concentration has been estimated to be several millimolar (25); however this ATP appears to be found mostly in the cytoskeletal core, and it is possible that the ATP available to interact with the protein kinases in the plasma membrane is much lower.

The fact that inhibition of arachidonic acid metabolism by the cyclooxygenase pathway using indomethacin did not alter changes in active ileal Na and Cl fluxes caused by carbachol and Ca^{2+} ionophore A23187 indicates that cyclooxygenase pathway products are not involved in the transport effects which result from elevating Ca^{2+} in rabbit ileum. In addition to the experiments shown in Table II, we also determined the effect of indomethacin (10^{-5} M) added after addition of Ca^{2+} ionophore A23187 and demonstrated no difference in the Na and Cl flux changes caused by indomethacin in the two conditions (data not shown). Thus the effect of elevated Ca^{2+} in the neutral linked NaCl absorptive process does not appear to be mediated via the cyclooxygenase pathway. In addition, the decrease in I_{sc} caused by indomethacin (10^{-5} M) was only slightly but not significantly larger in Ca^{2+} ionophore-exposed tissue (maximum decrease after indomethacin in I_{sc} in control (n = 6) and A23187 (10^{-6} M) (n = 6) exposed tissue, respectively 32.8±7.4 vs. 46.7±8.5 μA/cm², NS).

Given the demonstrated specificity of H-7, the major findings of this work are that the effects of raised cytosol Ca^{2+} concentrations in ileal electrolyte absorption are mediated by protein kinase C, whereas Ca^{2+}/CaM, possibly acting via a Ca^{2+}/CaM-dependent protein kinase, regulates basal electrolyte transport. That is, the two systems exert sequential, nonoverlapping control over NaCl transport (see Fig. 9). The biochemical explanation for this nonoverlapping control has not yet been determined, but may have to do with the basal level of cytosol free Ca^{2+} being set higher in ileal villus cells than in other intestinal cells (1–3). For instance, basal cytosol free Ca^{2+} has been estimated in ileal villus cells using the Ca^{2+}-sensitive fluorescent dye fura-2 and a fluorescent microscope/imaging system. Cytosol free Ca^{2+} in rabbit ileal villus cells was estimated to be 133 nM vs 76 nM in the model Cl secretory line cell T_{84} measured under identical conditions (Reinlib, L., R. Mikkelsen, D. Zahniser, and M. Donowitz, unpublished observations).

Our studies support the view that Ca^{2+}/CaM, and not protein kinase C, is involved in regulation of basal active Na and Cl absorption in rabbit ileum. Whereas W_{13} stimulated neutral linked NaCl absorption, H-7 caused a slight increase in ileal I_{sc} without measurably affecting active Na and Cl transport. Further evidence of the independence of the two systems is that sequential addition of H-7 followed by W_{13} affected basal transport similarly to the effect of W_{13} addition by itself (unpublished observations) and that W_{13} caused similar effects in ileal mucosal Na and Cl transport under basal conditions and in tissue with intracellular Ca^{2+} increased by the Ca^{2+} ionophore A23187.

In regard to changes in transport caused by carbachol and the Ca^{2+} ionophore A23187, W_{13} had no inhibitory effect whatsoever whereas H-7 inhibited all aspects of the changes in Na and Cl transport. The combination of W_{13} plus H-7 was neither additive nor synergistic on the secretagogue-induced changes in Cl fluxes and thus the effect was strictly due to the inhibition caused by H-7 itself. Thus no hint of synergism or additivity of the Ca^{2+}/CaM and protein kinase C systems in regulating either basal or Ca^{2+}-stimulated changes in active Na and Cl absorption in ileum has been found. The results presented here do localize the protein kinase C effects at least partly, distal to the elevation of cytosol Ca^{2+} in the transport mechanism, since the Ca^{2+} ionophore effects were inhibited similarly to the effects of carbachol and serotonin. Thus, in addition to feedback effects on the plasma membrane hormone receptor—phosphatidylinositol turnover—diacylglycerol-IP_3 aspect of stimulus-secretion coupling which has been described (26, 27), our system represents an example in which protein kinase C is used to carry out the effect of elevated Ca^{2+}.

While many systems have shown multiple types of interactions of Ca^{2+}/CaM and protein kinase C (26–30), we postulate that rather than a synergistic or overlapping effect on the ileal

linked NaCl absorptive process, that Ca^{2+}/CaM and protein kinase C act in a sequential manner with Ca^{2+}/CaM regulating basal transport (as previously described [7]), and that when Ca^{2+} is elevated above basal levels, protein kinase C mediates the effect.

The concept that elevated cytosol Ca^{2+} is acting through protein kinase C in the regulation of ileal Na and Cl absorption and that Ca^{2+}/CaM is not involved is unusual. Most neurohumorally mediated systems that act through Ca^{2+} are believed to respond to neurohumoral secretagogues with an early increase in Ca^{2+}, which causes an effect via the Ca^{2+}/CaM complex, with a later, Ca^{2+}-dependent effect which occurs at a lower although still elevated Ca^{2+} concentration, being due to protein kinase C (28–30). What biochemically explains the sequential involvement of Ca^{2+}/CaM and protein kinase C is not yet known.

It is not known whether the effects of H-7 are exerted on epithelial cells directly, or on intermediate cells, and this issue has not been resolved by the current study. The fact that conditions have been selected in which H-7 inhibits ileal villus absorptive cell cytosol protein kinase C at the same concentration at which it regulates Ca^{2+}-dependent changes in active Na and Cl absorption that occur in the same cell type, suggests that the effects could be exerted directly on the epithelial cell but neither prove the point nor eliminate the possibility of H-7 affecting other cells indirectly involved in regulating Na and Cl absorption. Thus, these studies do not eliminate a role for protein kinase C at nerves, endocrine cells or inflammatory cells which are all present in the ileal preparation.

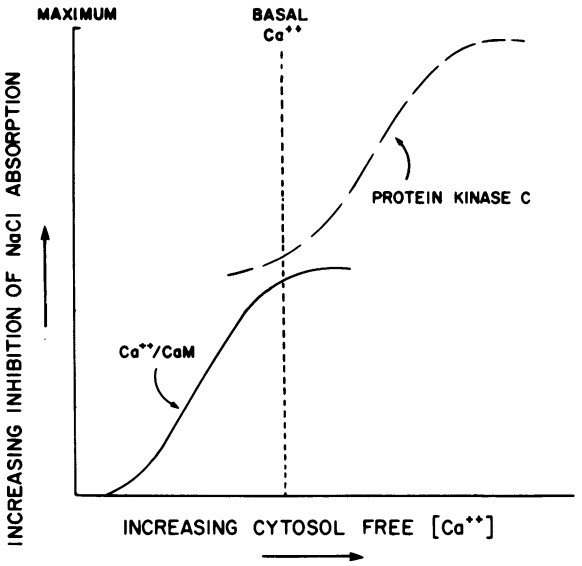

Figure 9. Hypothesis of sequential regulation of ileal villus cell-linked NaCl absorption by Ca^{2+}/CaM at basal levels of cytosol free Ca^{2+} and by protein kinase C at elevated levels of cytosol free Ca^{2+}. Shown is the effect of changes in cytosol free Ca^{2+} using the basal Ca^{2+} level as a reference (represented by the vertical dashed line) on ileal-linked NaCl absorption. The interaction of the two Ca^{2+} systems can be represented approximately as a single S-shaped curve made up of the two independent concentration response curves. Ca^{2+}/CaM inhibitors increase linked NaCl absorption indicating that under basal levels of Ca^{2+}, the Ca^{2+}/CaM complex is inhibiting this transport process. At basal Ca^{2+} this Ca^{2+}/CaM effect is at its maximum (as represented by the upper portion of the Ca^{2+}/CaM dose response curve to the right of the vertical) since W_{13} caused similar stimulation of NaCl absorption under basal Ca^{2+} conditions and when the intracellular Ca^{2+} was increased with a Ca^{2+} ionophore. When Ca^{2+} is elevated, protein kinase C causes further inhibition of linked NaCl absorption; however protein kinase C is not involved in regulation of NaCl absorption under basal conditions (as represented by the lower portion of the protein kinase C dose response curve to the left of the vertical) since H-7 had no effect on basal NaCl transport.

The fact that the effects of cAMP and cGMP on ileal Na and Cl transport were not altered by either H-7, as demonstrated in this study, or by W_{13}, as reported previously (7), suggests that neither Ca^{2+}/CaM nor protein kinase C are involved in the cyclic nucleotide-induced changes in ileal electrolyte absorption. It was demonstrated previously that agents that elevate cytosol free Ca^{2+} in ileal absorbing cells fail to alter the cyclic nucleotide levels (2, 3). Thus, the Ca^{2+} and cyclic nucleotide systems appear to act independently on ileal electrolyte absorption.

In summary, Ca^{2+}/CaM and protein kinase C appear to have separate effects in the regulation of active ileal NaCl absorption, as depicted in Fig. 9, with Ca^{2+}/CaM acting on basal transport to inhibit neutral NaCl absorption, while elevating cytosol free Ca^{2+} above basal levels further inhibits NaCl absorption. How the cell switches sequentially from the Ca^{2+}/CaM system to protein kinase C, based on the level of cytosol free Ca^{2+}, and why there does not appear to be overlap or synergism between these systems in ileum remain to be determined.

Acknowledgments

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