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Effects of diclofenac sodium and disodium ethylenediaminetetraacetate on electrical parameters of the mucosal membrane and their relation to the permeability enhancing effects in the rat jejunum

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The effects of diclofenac sodium and disodium ethylenediaminetetraacetate (EDTA) on electrical parameters of rat jejunal membrane were investigated, together with measurement of the mucosal-to-serosal flux of sulphanilic acid or L-phenylalanine. Both adjuvants increased the flux rate of sulphanilic acid to a similar extent when added to the mucosal solution at 10 mM, but there were apparent differences in their effects on the electrical parameters. The addition of EDTA induced the gradual reduction in the membrane resistance (Rm) by 6–8 ohm cm^{-2}, while the effect of diclofenac on Rm was complicated and concentration-dependent. The short circuit current (Isc) was reduced rapidly to the level of 30–40 $\mu A\ cm^{-2}$ by the addition of diclofenac, but was less affected by EDTA. The flux rate of L-phenylalanine was decreased extensively by diclofenac or the 10 mM concentration of EDTA, suggesting an inhibition of carrier-mediated transport systems in the membrane. Together with our preceding communication (Yamashita et al 1985, J. Pharm. Pharmacol. 37: 512–513), it became obvious that the sites of action of diclofenac and EDTA were different, the former directly interacting with the epithelial cell to alter the permeability and functions of the cell membane, while the primary effect of EDTA could be at the intercellular junctions.

To develop effective and safe promoters for drug absorption from the gastrointestinal tract, it is necessary to estimate their effects quantitatively and also to characterize the mechanisms of action. Various information about promoters has been reported (Murakami et al 1982; Sithigorngul et al 1983; Nakanishi et al 1984b; Nishihata et al 1985; Shiga et al 1985; Yata et al 1985).

In an earlier report (Yamashita et al 1985), we clarified the site of action of two promoters, diclofenac sodium and disodium ethylenediaminetetraacetate (EDTA), using electrophysiological techniques. The ability of diclofenac and EDTA to enhance drug absorption from the gastrointestinal tract has been observed in a variety of studies. Nakanishi et al (1984a, b) showed that a number of non-steroidal anti-inflammatory drugs such as diclofenac, indomethacin and phenylbutazone could promote the rectal absorption of sulphanilic acid and enhance the rectal clearance of creatinine in the rat. EDTA, with its high chelating activity, was thought to alter the structure of the epithelial cell layer by removing calcium from the cell junction (Cassidy & Tidball 1967). This action is expected to cause permeability enhancement of the intestinal membrane to drugs. However, the effect of diclofenac and EDTA on physiological functions of the membrane have not yet been clarified.

In this report, changes in electrophysiological parameters of rat jejunal mucosa caused by diclofenac or EDTA have been examined to clarify differences in their effects on membrane function. Also their enhancing effect on intestinal permeability to drugs has been discussed.

MATERIALS AND METHODS

Materials

Diclofenac sodium (Kodama Pharmaceutical Co., Ltd, Japan) was used as supplied. All other reagents were of reagent grade and were used without further purification.

Preparation of jejunal sheets

Jejunal sheets were prepared from Wistar strain male rats, 200–250 g. The sheets were mounted between two Lucite half chambers (Ussing-type) as described by Yamashita et al (1984).

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Preparation of drug solutions

The composition (mM) of the normal Ringer solution was: NaCl 125, KCl 5, $CaCl_2$ 1·4, NaH_2PO_4 1·2, $NaHCO_3$ 10 and 200 mg dL^{-1} of D-glucose. All solutions which contained sulphanilic acid, EDTA, and/or diclofenac were prepared to have the same osmolarity and sodium concentration as normal Ringer solution. If necessary, an appropriate amount of mannitol was added to maintain the osmolarity. Furthermore, $CaCl_2$ was replaced with the equivalent amount of mannitol for reasons described under Results. Only L-phenylalanine (L-Phe) was added to each Ringer solution directly without a further modification of the composition. In this case, osmolarity change was thought to be negligible, since the concentration of L-Phe was 1 mM. Before the experiment, all solutions were adjusted to pH 7·4 at 37 °C and oxygenated with 95% O_2–5% CO_2.

Measurements of electrical parameters

The transmural potential difference (PD) and the short-circuit current (Isc) of the membrane were measured at 5–10 min intervals. The membrane resistance (Rm) was calculated by Ohm's law taking into account the resistance of the bathing solution. The apparatus for these measurements were as described by Kimura et al (1982). In the present study, since the compositions of Ringer solution were slightly different between the mucosal and serosal sides of the membrane when sulphanilic acid and/or a promoter were present in the mucosal side, measured Isc would not be precisely consistent with the flux of actively transported ions as described by Ussing & Zerahn (1951). However, the concentration of sodium, which plays a main role in supporting the PD and Isc of intestinal membrane (Schultz & Zalusky 1964), was always the same as normal Ringer solution. Also, the main purpose of our experiments was to compare the differences in the variation of membrane functions between the control and promoter-treated membranes. Thus, we considered it justifiable to use the values of 'measured Isc' as a parameter of membrane function.

Measurements of mucosal-to-serosal fluxes

Mucosal-to-serosal flux rates of sulphanilic acid and L-Phe were measured in the absence and presence of the promoter in the mucosal solution. After the preincubation with normal Ringer solution for 25 min, a test solution was introduced to the mucosal side of the membrane. At the end of the preincubation, the PD and Rm were checked and only those membranes with the PD above 3 mV and Rm in the range of 30 to 60 ohm cm^2 were used. 1 mL samples were taken every 10 min from the serosal side for 1 h and volume of the bathing solution was kept constant by the addition of fresh Ringer solution. Flux rate was calculated from the rate of increase in the serosal concentration of sulphanilic acid or L-Phe as a function of time.

Analytical methods

Sulphanilic acid was estimated spectrophotometrically as described by Kimura et al (1981). L-Phe was determined using the high performance liquid chromatograph after *o*-phthaldialdehyde derivatization as described by Yamashita et al (1986).

RESULTS

Effect of calcium exclusion from mucosal solution on membrane functions

Diclofenac rapidly precipitated in the presence of calcium. In addition, since it has been suggested that chelate formation by EDTA with membrane calcium is involved with its ability to enhance drug absorption (Tidball 1964), the calcium in the bathing solution could mask the action of EDTA. Therefore, it was necessary to investigate the effect of the agents in calcium-free conditions. However, it has been reported that small amounts of calcium are necessary in the incubation medium to ensure the good survival of biomembranes (Barry & Diamond 1970). Fig. 1

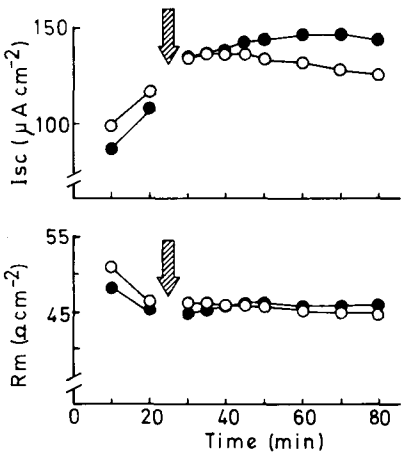

FIG. 1. The short-circuit current (Isc; upper) and the membrane resistance (Rm; lower) of rat jejunum as a function of time: effect of calcium exclusion from the mucosal solution. The normal ($CaCl_2$, 1·4 mM) (○) or calcium-free (●) Ringer solution was introduced to the mucosal side of the membrane after the preincubation with normal Ringer solution for 25 min (indicated by arrows). Each point represents the mean of at least 6 experiments.

shows the time-course of Isc and Rm when calcium-containing (1·4 mM) or calcium-free Ringer solution was introduced to the mucosal side of the membrane after preincubation with normal Ringer solution. The exclusion of calcium from the mucosal solution caused no change in the parameters. Isc in the absence of calcium was slightly greater but not significantly so. Also, the mucosal-to-serosal flux rate of sulphanilic acid was not affected by the calcium exclusion (Table 1). Therefore, it may be considered that the functions of the intestinal membrane were maintained normally, at least during this experimental period, regardless of the presence or absence of calcium in the mucosal solution. Subsequent experiments were carried out in calcium-free conditions.

Effects of diclofenac and EDTA on the flux rate of sulphanilic acid

Table 1 shows the mucosal-to-serosal flux rate of sulphanilic acid when the various concentrations of the promoters were added to the mucosal solution. Since the acid is completely ionized and highly polar at physiological pH ranges, it is poorly absorbable and its absorption from the intestinal tract is rate-determined by the penetration step across the intestinal epithelium. Thus, the increase in the flux rate of sulphanilic acid by diclofenac or EDTA observed in our in-vitro experiments would correspond to the promoting effect of these agents on the intestinal absorption of drugs, as reported in in-situ or in-vivo experiments.

The flux rate of sulphanilic acid increased as the concentration of the promoter increased and at 10 mM both promoters showed a similar effect. A low concentration (1 mM) of diclofenac caused only a 1·1–1·2 fold increase in the sulphanilic acid flux rate (not significantly different from the control), while 1 mM EDTA induced a marked effect that exceeded that of 5 mM diclofenac.

Table 1. Effect of diclofenac or EDTA on the mucosal-to-serosal flux rate of sulphanilic acid (SA).

Adjuvant	SA flux rate ($nmol\ cm^{-2}\ min^{-1}$)	Ratio
None		
Ca-1·4 mM	2·05 ± 0·07 (6)	
Ca-free	1·90 ± 0·07 (6)	1
Diclofenac		
1 mM	2·04 ± 0·15 (4)	1·1
2 mM	2·32 ± 0·08* (4)	1·2
5 mM	3·05 ± 0·21** (6)	1·6
10 mM	4·70 ± 0·17** (5)	2·5
EDTA		
1 mM	3·53 ± 0·02** (4)	1·9
5 mM	4·39 ± 0·39** (4)	2·3
10 mM	4·93 ± 0·18** (4)	2·6

Initial concentration of SA in the mucosal solution was 10 mM. SA flux rates are expressed as the mean ± s.e. with the number of experiments in the parentheses.

* $P < 0·01$, ** $P < 0·001$, significantly changed by the presence of adjuvant (by analysis of Fisher's t-test).

Effects of diclofenac and EDTA on electrical parameters of intestinal mucosa

The time course of Rm after the addition of diclofenac or EDTA to the mucosal solution is shown in Fig. 2. The results are expressed as change with reference to the values at 20 min (5 min before the addition of a promoter). While under control

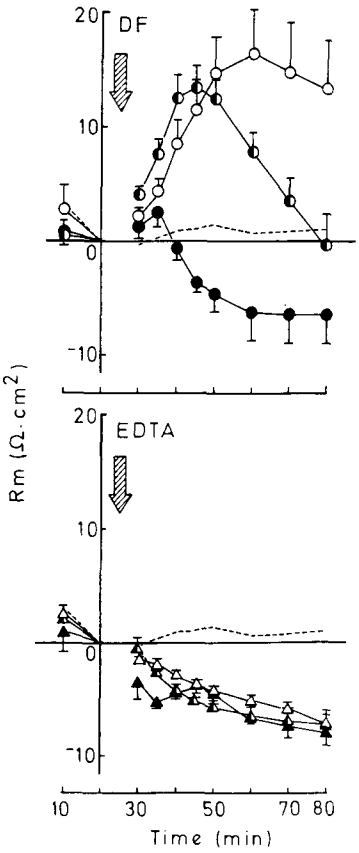

FIG. 2. Effects of diclofenac (DF) or EDTA on the membrane resistance (Rm) of rat jejunum. Results are expressed as the changed values of Rm with reference to those at 20 min. The Ringer solution containing the following adjuvants was introduced to the mucosal side of the membrane after preincubation with normal Ringer solution for 25 min (indicated by arrows): DF 1 mM (○), 5 mM (◐), 10 mM (●); EDTA 1 mM (△), 5 mM (▲), 10 mM (▲). Dotted lines represent the results of control experiments. Each point represents the mean of at least 4 experiments with s.e.

conditions, Rm was maintained nearly constant during the experimental period (Fig. 1); it decreased gradually by about 6–8 ohm cm^2 after the addition of 1 mM or 5 mM EDTA. When the concentration of EDTA was raised to 10 mM, a more rapid decrease with a temporary increase in Rm was observed. On the other hand, the addition of 1 mM or 5 mM diclofenac resulted in an immediate increase in Rm, the maximal increment being 13–16 ohm cm^2 (results at 2 mM were almost the same as at 1 mM, not shown). 10 mM diclofenac caused only a slight increase in Rm which thereafter decreased to a level similar to that with EDTA.

The effect of diclofenac or EDTA on Isc was also examined in the same manner as Rm. Fig. 3 shows the time-course of Isc when diclofenac or EDTA

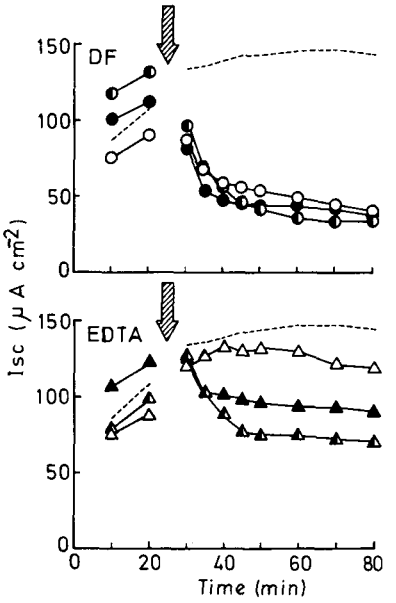

FIG. 3. Effects of diclofenac (DF) or EDTA on the short-circuit current (Isc) of rat jejunum. The Ringer solution containing the following adjuvants was introduced to the mucosal side of the membrane after preincubation with normal Ringer solution for 25 min (indicated by arrows): DF 1 mM (○), 5 mM (◐), 10 mM (●); EDTA 1 mM (△), 5 mM (▲), 10 mM (▲). Dotted lines represent the results of control experiments. Each point represents the mean of at least 4 experiments.

were in the mucosal solution. The addition of diclofenac resulted in a rapid decrease in Isc to 30–40 μA cm^{-2} regardless of its concentration. The effect of EDTA was much less over the concentration range used but was greater at 5 mM than at 10 mM (Isc values were 68·7 and 89·4 μA cm^{-2}, respectively).

Effects of diclofenac and EDTA on the flux rate of L-phenylalanine

To estimate the effect of promoters on the carrier-mediated transport system of the intestinal membrane, the mucosal-to-serosal flux rate of L-Phe was measured (Table 2). The transport activity for L-Phe was unaffected by the exclusion of calcium from the mucosal solution, but the addition of promoter at 10 mM decreased the flux rate of L-Phe. EDTA, at 1 mM, reduced the flux rate to 64% of the control, while the effect of 1 mM diclofenac was similar to that of 10 mM EDTA (Table 2).

Table 2. Effect of diclofenac or EDTA on the mucosal-to-serosal flux rate of L-Phe.

Adjuvant	L-Phe flux rate (nmol cm^{-2} min^{-1})	Ratio
None		
Ca-1·4 mM	4·41 ± 0·39 (7)	
Ca-free	5·01 ± 0·39 (5)	1
Diclofenac		
1 mM	0·674 ± 0·082** (3)	0·13
10 mM	0·252 ± 0·019** (4)	0·050
EDTA		
1 mM	3·22 ± 0·17* (3)	0·64
10 mM	0·608 ± 0·06** (3)	0·12

Initial concentration of L-Phe in the mucosal solution was 1 mM. L-Phe flux rates are expressed as the mean ± s.e. with the number of experiments in the parentheses.
*$P < 0·05$, **$P < 0·001$, significantly changed by the presence of adjuvant (by analysis of Fisher's t-test).

DISCUSSION

Electrophysiological techniques have been widely used to characterize membrane functions and the permeability to various substances ever since the conventional method of measuring the short-circuit current was developed by Ussing & Zerahn (1951). It has been shown that some bile salts, which were reported to enhance the intestinal absorption of water-soluble drugs (Kakemi et al 1970a, b), alter the ion transport properties of the intestinal membrane, thereby increasing the short-circuit current (Binder & Rawlins 1973; Volpe & Binder 1975; Freel et al 1983a). In addition, Freel et al (1983b) demonstrated a good correlation of bile salt-induced alteration in the membrane permeability and ion transport.

In our study, apparent differences have been demonstrated in the effect of diclofenac and EDTA on electrical parameters of the intestinal membrane. Table 3 summarizes the results of the voltage-clamp experiment reported earlier by Yamashita et al (1985), which evaluated the sulphanilic acid flux by dividing it into two individual components, transcel-

Table 3. Selective effects of diclofenac and EDTA on the paracellular and transcellular fluxes of sulphanilic acid (SA) at the short-circuited condition.

	SA flux rate (nmol cm^{-2} min^{-1})	
Adjuvant	Transcellular	Paracellular
None	0·75 ± 0·15	0·91 ± 0·12
Diclofenac 10 mM	2·80 ± 0·28*	1·67 ± 0·21*
EDTA 10 mM	0·95 ± 0·52	4·06 ± 0·40*

This result was derived from the voltage-clamp experiments reported by Yamashita et al (1985). Each value is shown with s.d. * Significantly changed by the presence of the adjuvant ($P < 0·01$ by analysis of variance).

lular and paracellular. It was revealed that diclofenac increased both the transcellular and paracellular fluxes of the acid significantly, while the effect of EDTA was located only in the paracellular pathway.

The interaction of EDTA with biomembranes such as an intestinal epithelium has been thought to be due to its high chelating activity with calcium thereby enhancing membrane permeability (Cassidy & Tidball 1967; Murakami et al 1982). Since the major ionic conductance pathway in leaky tissues, such as the small intestine or the gallbladder, is thought to be the paracellular route (Powell 1981), the decrease in Rm after the addition of EDTA (Fig. 2) might indicate a reduction in paracellular resistance. This observation and the results in Table 3 support the hypothesis that the removal of calcium from the intestinal cell junction by the formation of calcium-EDTA complexes loosens the structure of the tight junction and enhances paracellular permeability. On the other hand, Nishihata et al (1985) claimed that the site of EDTA action is the protein fraction of the membrane. However, the effects of 1 mM EDTA on both Isc and L-Phe flux rate were small, in contrast to the 1·9-fold increase in sulphanilic acid flux rate. It has been shown that the greater part of Isc across the intestinal membrane is attributed to the electrogenic active transport of Na^+, especially the co-transport with glucose (Barry et al 1965) and, in addition, L-Phe is transported from the mucosal to the serosal side of the intestinal membrane by a carrier-mediated transport system (Schultz & Frizzell 1975). At higher concentrations of EDTA, these transport systems were markedly damaged. This would correspond with solubilizing or otherwise releasing proteins from the membrane by EDTA, resulting in the irreversible change in the membrane function as reported by Nishihata et al (1985). Therefore it was suggested that the primary effect of EDTA in enhancing membrane permeability was located in the extracellular portion, probably the tight junction, of epithelial cells, and when the concentration of EDTA became higher, functional damage to the membrane would occur.

Previously (Nakanishi et al 1984b), it was shown that diclofenac is highly accumulated in the intestinal mucosa and that its binding with the membrane components (protein and lipids) plays an important role in its permeability-enhancing effect. So, it is likely that the rapid reduction in Isc after the addition of diclofenac was due to its binding with the carrier-protein of the Na^+-glucose co-transport system which is located in the apical membrane of epithelial cells. Active transport of L-Phe was also inhibited by diclofenac regardless of the concentration.

It is apparent that 10 mM diclofenac mainly enhanced the permeability of the transcellular pathway, as shown in Table 3. Although the detailed mechanisms of its action in enhancing the transcellular permeability have not yet been clarified, it seems likely that by binding to the protein or lipid the regular arrangement of lipid molecules which constitute the cell membrane is disordered. In such a state, the barrier functions of the apical membrane to prevent the permeation of drugs into the cell might be reduced. It is supposed that the effect of diclofenac on the transcellular permeability to sulphanilic acid would not correlate appreciably with change in Rm, although there is no adequate explanation for changes in Rm after the addition of diclofenac. Moreover, the question remains why the low concentration of diclofenac did not suppress the flux rate of sulphanilic acid in spite of the rapid increase in Rm. One possible explanation is that the low concentration of the drug had opposing effects, probably similar in magnitude, on the paracellular and transcellular pathways, i.e. it enhanced the transcellular while reducing the paracellular permeability. Since 1 mM diclofenac reduced Isc and L-Phe flux rate to a similar level as 10 mM diclofenac, it may be considered that the 1 mM concentration enhanced the permeability of the transcellular pathway. With the 10 mM concentration, Rm decreased to a level similar to that attained with EDTA and the sulphanilic acid flux rate through the paracellular pathway was also significantly enhanced. These results indicated that the high concentration of diclofenac has the ability to enhance the permeability not only of the transcellular but also of the paracellular pathway.

Apart from the permeability enhancing effects, the functional alterations of intestinal membranes

induced by these drugs suggests the need for caution in their clinical applications. It may be possible that inhibition of the active transport of Na^+ and other nutrients by diclofenac would result in the malabsorption of electrolytes and water to cause some types of diarrhoeal diseases. Although it has been reported that the enhancing effect of diclofenac was reversible (Nakanishi et al 1984a), this must be a practical problem to be considered. As for EDTA, this would be more difficult to use clinically because of the irreversible damage to the intestinal membrane.

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