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Calcium-calmodulin-dependent Activation of Adenylate Cyclase in Prostaglandin-induced Electrically-monitored Intestinal Secretion in the Rat

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Abstract—The calcium-calmodulin antagonist 5-iodo-C_8-W7 inhibited the PGE_2-induced stimulation of cAMP production by isolated enterocytes from rat small intestine. It also reduced the secretory response of intestinal sheets to PGE_2, measured as a rise in short-circuit current. It did not however, inhibit the electrical responses to forskolin and dibutyryl cAMP, nor to acetylcholine, a secretagogue whose effect is not mediated by cAMP. It is concluded that the receptor-mediated activation of adenylate cyclase and the subsequent secretory response are dependent upon calcium-calmodulin.

Both calcium and cAMP are considered to act as intracellular messengers for the activation of intestinal secretion (Donowitz & Welsh 1986). In broken cell preparations of various tissues, adenylate cyclase activity is regulated by calcium-calmodulin (MacNeil et al 1985); this mechanism also appears to exist in the enterocyte, the transporting cell of the intestine. In membranes from rat enterocytes, calmodulin, in the presence of low calcium concentrations, increased adenylate cyclase activity (Amiranoff et al 1982; Pinkus et al 1983; Lazo et al 1984), while in isolated enterocytes the calmodulin antagonist trifluoperazine reduced prostaglandin E_1-induced accumulation of cAMP (Pinkus et al 1983). The relationship between these calcium-calmodulin-induced changes in adenylate cyclase activity and the secretory response of the intestine has not been examined and the aim of the present study was to determine whether the alterations in adenylate cyclase activity were accompanied by corresponding changes in secretion. The involvement of calcium-calmodulin in the activation of adenylate cyclase and the stimulation of secretion by prostaglandin E_2 (PGE_2) was investigated using the calmodulin antagonist W7, an *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide, and its derivative 5-iodo-C_8-W7 which has greater potency and specificity (MacNeil et al 1986). A preliminary report of the data has been presented to the Physiological Society (Ayton et al 1988).

Materials and Methods

Animals

Experiments were carried out on male Wistar rats, 230–250 g, obtained from the Sheffield Field Laboratories, UK, and allowed free access to food and water. They were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.).

Measurement of transintestinal electrical activity

The potential difference (PD), short-circuit current (SCC) and tissue resistance (R) were measured in paired sheets from the mid-region of the small intestine from which the muscle layers had been removed. These were mounted in Ussing chambers with an aperture of 1·925 cm^2 and incubated at 37°C in Krebs bicarbonate saline (Krebs & Henseleit 1932) gassed with 95% O_2–5% CO_2. The serosal solution contained 10 mM glucose and the mucosal solution 10 mM mannitol and each had a volume of 5 mL. The PD was measured using salt bridge electrodes connected via calomel half-cells to a differential input electrometer. Current was applied across the tissue using Ag/AgCl electrodes which made contact with mucosal and serosal solutions via wide-bore salt bridges. When short-circuiting the tissue a correction was made for the resistance of the medium as described by Field et al (1971). R was calculated from the PD and SCC using Ohm's law; values for each tissue pair did not differ by more than 25%.

After mounting, W7 or 5-iodo-C_8-W7 was added to the serosal solution of the test sheet while the control sheet received an equivalent volume of the vehicle (dimethylsulphoxide (DMSO) 1% v/v). The tissues were then allowed to stabilize for 10 min after which time readings were taken at 1 min intervals. After 5 min basal readings, PGE_2 (1·4 μM), forskolin (5 μM), dibutyryl cAMP (1 mM) or acetylcholine (ACh, 1 mM) was added to the serosal solution of both sheets and readings continued. The magnitude of the response was taken as the difference between the maximum SCC obtained in the presence of the secretagogue and the value immediately before its addition.

Estimation of cAMP levels in isolated enterocytes

The small intestine was removed from animals killed by a blow to the head and cervical dislocation. Enterocytes were isolated from the entire small intestine as described by Hardcastle et al (1980). cAMP levels were estimated using the competitive binding protein method of Brown et al (1971) and related to the protein content which was determined by the method of Lowry et al (1951).

Expression of results

Results are expressed as mean values ± s.e.m. of the number of observations indicated. A paired, two-tailed Student's *t*-

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test was used to assess the significance of 5-iodo-C_8-W7 and W7 action.

Chemicals

Prostaglandin E_2 was obtained from Upjohn Co, Kalamazoo, MI, USA; adenosine 3′,5′-cyclic phosphoric acid, acetylcholine chloride, dibutyryl cAMP and forskolin from Sigma Chemical Co, St Louis, MO, USA; D-glucose, mannitol and DMSO from BDH Chemicals Ltd, Poole, UK. Radioactive tracers were obtained from Amersham International, Amersham, UK. W7 and 5-iodo-C_8-W7 were synthesized by the Department of Chemistry, Sheffield University under the supervision of Dr G. M. Blackburn (MacNeil et al 1986).

Results

Effect of 5-iodo-C_8-W7 on the secretory response of intestinal sheets

The intestinal sheets generated a basal PD of $2{\cdot}7 \pm 0{\cdot}1$ mV, a SCC of $93{\cdot}0 \pm 6{\cdot}1$ μA cm^{-2} and a R of $35{\cdot}0 \pm 3{\cdot}7$ ohm cm^2 (n = 34). These values were unaffected by either W7 or 5-iodo-C_8-W7 at a concentration of 100 μM ($P > 0{\cdot}05$ in all cases). The stimulation of Cl^- secretion induced by PGE_2 (1·4 μM) was reflected in a rise in SCC and this response was reduced by 57% in the presence of 100 μM 5-iodo-C_8-W7 (Fig. 1). W7 (100 μM), however, failed to exhibit a significant inhibition (control: $79{\cdot}8 \pm 19{\cdot}1$ μA cm^{-2} (n = 7); +W7: $61{\cdot}6 \pm 10{\cdot}6$ μA cm^{-2} (n = 7), $P > 0{\cdot}05$). In contrast to its effects on PGE_2-induced secretion, 5-iodo-C_8-W7 (100 μM) did not reduce the response to 5 μM forskolin and slightly increased the response to 1 mM dibutyryl cAMP (Fig. 1). Neither W7 nor 5-iodo-C_8-W7 (100 μM) altered the response to 1 mM acetylcholine (control: $213{\cdot}8 \pm 19{\cdot}5$ μA cm^{-2} (n = 8), +W7: $191{\cdot}6 \pm 14{\cdot}1$ μA cm^{-2} (n = 8), $P > 0{\cdot}05$; control: $233{\cdot}8 \pm 28{\cdot}5$ μA cm^{-2} (n = 9), +5-iodo-C_8-W7: $207{\cdot}2 \pm 15{\cdot}7$ μA cm^{-2} (n = 9), $P > 0{\cdot}05$).

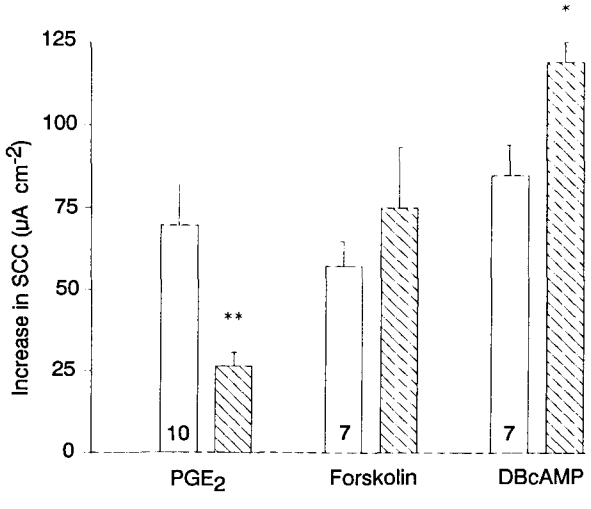

FIG. 1. 5-Iodo-C_8-W7 inhibited the rise in SCC generated by paired stripped sheets of rat mid-intestine in response to serosal application of PGE_2 (1·4 μM), but did not reduce the responses to forskolin (5 μM) or dibutyryl cAMP (1 mM). 5-Iodo-C_8-W7 (100 μM) was added to the serosal side of test sheets, while control sheets received an equivalent volume of vehicle (DMSO, 1% v/v). Each bar represents the mean ± s.e.m. of the number of observations indicated. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$. Control, □; 5-iodo-C_8-W7, ▨.

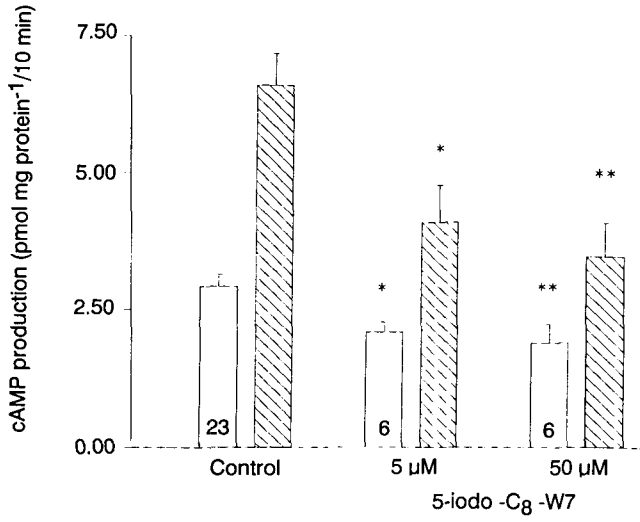

FIG. 2. 5-Iodo-C_8-W7 inhibited cAMP production by enterocytes isolated from rat small intestine under basal conditions and in the presence of PGE_2 (3·6 μM). 5-Iodo-C_8-W7 was present at the concentrations indicated, with control cells receiving an equivalent volume of vehicle (DMSO, 5% v/v). Each bar represents the mean ± s.e.m. of the number of experiments indicated, each carried out in triplicate. *$P < 0{\cdot}01$; **$P < 0{\cdot}001$. Basal, □; PGE_2, ▨.

Effect of 5-iodo-C_8-W7 on cAMP production by isolated enterocytes

PGE_2 (3·6 μM) increased cAMP production by isolated enterocytes ($P < 0{\cdot}001$). This response was reduced by 32 ± 4% (n = 6) in the presence of 5 μM 5-iodo-C_8-W7 and by 53 ± 4% (n = 6) with 50 μM (Fig. 2). These concentrations of 5-iodo-C_8-W7 also reduced basal cAMP production (Fig. 2), although the degrees of inhibition (16 ± 2% (n = 6) with 5 μM, 32 ± 4% (n = 6) with 50 μM) were less (both $P < 0{\cdot}05$) than with PGE_2-stimulated cAMP levels. W7 (100 μM) had no effect on either basal (control: $2{\cdot}93 \pm 0{\cdot}22$ pmol (mg protein)$^{-1}$/10 min (n = 23); +W7: $2{\cdot}97 \pm 0{\cdot}59$ pmol (mg protein)$^{-1}$/10 min (n = 5), $P > 0{\cdot}05$) or prostaglandin-stimulated cAMP levels (control: $6{\cdot}60 \pm 0{\cdot}58$ pmol (mg protein)$^{-1}$/10 min (n = 23); +W7: $6{\cdot}09 \pm 1{\cdot}23$ pmol (mg protein)$^{-1}$/10 min (n = 5), $P > 0{\cdot}05$).

Discussion

Most investigations designed to examine the possible involvement of calcium-calmodulin in the activation of intestinal Cl^- secretion have relied on the use of calmodulin antagonists. These inhibit the stimulation of secretion measured both as a rise in SCC and directly using isotopic flux determinations (Ilundain & Naftalin 1979; Smith & Field 1980; Hardcastle et al 1984). However, calmodulin antagonists are hydrophobic compounds and consequently exhibit non-specific interactions with lipid membranes and the hydrophobic regions of proteins (Johnson & Mills 1986). One protein affected by calmodulin antagonists is protein kinase C which has been implicated as a mediator of the intestinal secretory response (Chang et al 1985; Fondacaro & Henderson 1985; Weikel et al 1985; Sjoqvist et al 1986). Such lack of specificity suggests that the anti-secretory actions of these antagonists in the intestine may not necessarily result

from their ability to inhibit calmodulin. Evidence that this was the case came from the demonstration that the calmodulin antagonists W12 and W13 failed to inhibit secretagogue-induced changes in electrolyte transport by rabbit ileum, although both of these agents enhanced basal Na^+ and Cl^- absorption; W13, the more potent calmodulin antagonist, had a greater effect than W12, which has a similar hydrophobicity (Donowitz et al 1985). These findings suggest that calcium-calmodulin is not involved in the stimulation of secretion, although it may be implicated in the regulation of NaCl absorption, a view supported by the direct demonstration of calcium-calmodulin-induced inhibition of NaCl uptake into isolated brush-border membrane vesicles (Fan & Powell 1983).

Calcium-calmodulin is involved in the activation of adenylate cyclase (MacNeil et al 1985), and stimulates this enzyme in membrane preparations derived from enterocytes isolated from the small intestine (Amiranoff et al 1982; Pinkus et al 1983; Lazo et al 1984). Since both adenylate cyclase (Murer et al 1976) and calmodulin (Scully et al 1988) have been localized to the basolateral membrane of the enterocyte, such an interaction may occur in the intact cell. The present study has utilized 5-iodo-C_8-W7, a more potent and selective calmodulin antagonist with improved discrimination between the effects on calmodulin and protein kinase C (MacNeil et al 1986). In isolated enterocytes 5-iodo-C_8-W7 inhibited the stimulation of adenylate cyclase activity induced by PGE_2 (Fig. 2) and also reduced the rise in SCC, a reflection of increased Cl^- secretion (Fig. 1). The 53% inhibition of PGE_2-stimulated cAMP production in isolated enterocytes by 50 μM 5-iodo-C_8-W7 compares with a concentration of 3 μM that is required to inhibit calcium-calmodulin activity by 50% in a broken cell preparation (Barton et al 1987). Considering the different types of preparation used these values are in reasonable agreement. In the broken cell preparation W7 was one-tenth as effective in inhibiting calcium-calmodulin activity, 30 μM being required to cause a 50% reduction (Barton et al 1987). In the present study 100 μM W7 failed to influence PGE_2-stimulated cAMP production. In the intestinal sheet preparation 100 μM 5-iodo-C_8-W7 reduced the electrical response to PGE_2 by 57%. The requirement for a higher concentration to induce a similar inhibition might be due to the different preparations used; in the intestinal sheets, even with the muscle layers removed, the drug has to cross the considerable diffusion barrier of the subepithelial tissue before it reaches the enterocyte basolateral membrane, while it has direct access to isolated enterocytes. Not surprisingly, W7, at the same concentration, failed to demonstrate any inhibitory action.

The inhibitory effects of 5-iodo-C_8-W7 on prostaglandin-induced secretion do not appear to result from a non-specific action on the secretory process, as the rise in SCC in response to acetylcholine, which induces Cl^- secretion by a mechanism independent of cAMP (Isaacs et al 1976), was unaffected by the drug. Moreover, the electrical responses to forskolin, which activates adenylate cyclase directly (Seamon et al 1981), and dibutyryl cAMP, which mimics the actions of endogenous cAMP, were also unaffected by 5-iodo-C_8-W7. Thus the calmodulin antagonist does not appear to inhibit adenylate cyclase directly, nor to affect the subsequent secretory response to the cAMP produced. This points to the link between receptor occupation and adenylate cyclase activation as the site of calcium-calmodulin action. The calcium that activates calmodulin leading to the stimulation of adenylate cyclase may originate from the extracellular fluid, as prostaglandin-induced secretion is dependent on external calcium (Hardcastle et al 1984).

The present study indicates that although calcium-calmodulin does not now appear to be directly concerned with the mechanism for activating Cl^- secretion by the intestine, it is involved in the stimulation of adenylate cyclase activity by receptor-mediated secretagogues. Its action at this point is essential for the subsequent Cl^- secretion which is reduced when calcium-calmodulin activity is inhibited by 5-iodo-C_8-W7.

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