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The inhibition of human duodenal adenylate cyclase activity by Ca^{2+} and the effects of EGTA

Julie A. Smith[a], Martin Griffin[b], Stewart E. Mireylees[b] and Richard G. Long[a]

[a]*Medical Research Centre, City Hospital, Nottingham, NG5 1PB, UK* and [b]*Department of Life Sciences, Nottingham Trent University, Nottingham, NG11 8NS, UK*

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This study demonstrates that the inhibition of adenylate cyclase activity by Ca^{2+} is enhanced in the presence of increasing [EGTA] (0, 0.3, 1, 2.5 mM) by 2 orders of magnitude. It has been established that this effect is not because of poor Ca^{2+} buffering by low [EGTA] or high Ca^{2+} binding by the membrane preparation. It is present irrespective of stimulus. We suggest the enhanced sensitivity of adenylate cyclase to Ca^{2+} induced by EGTA is caused by the Ca–EGTA complex being a more inhibitory species than Ca^{2+}. Thus consideration of the effects of the Ca–EGTA complex should be made when interpreting the results from experiments involving Ca^{2+} and EGTA.

Adenylate cyclase; Calcium; EGTA; Human duodenum

1. INTRODUCTION

The regulation of fluid and electrolyte flux by cyclic AMP and Ca^{2+} is the paradigm of second messenger function in the small intestine. The relationship between intracellular cyclic AMP and Ca^{2+}, however, remains obscure in this tissue. In other tissues (e.g. brain) Ca^{2+} and calmodulin (a Ca^{2+}-binding protein) are known to regulate adenylate cyclase activity [1]. A number of investigators have demonstrated that Ca^{2+} inhibits the activity of adenylate cyclase at concentrations higher than 1 μM while at submicromolar Ca^{2+} concentrations activation of the enzyme occurs [1–8]. It is thought that this activation of adenylate cyclase activity is mediated by calmodulin but the inhibition of the enzyme by Ca^{2+} is calmodulin-independent [9].

The use of the Ca^{2+}-chelating agent EGTA, is an established method for the in vitro control of free calcium concentrations in enzyme assay systems [10–14]. Contamination of buffered solutions by Ca^{2+} (in some cases up to 20 μM) necessitates the use of buffers containing EGTA to control and estimate free [Ca^{2+}] [11,14]. EGTA is also used to deplete membrane preparations of calcium and/or calmodulin [1,2,4,15–18]. The concentration at which EGTA is used varies considerably from micromolar [19–24] through to millimolar concentrations [2,8,13,16,25–27]. The inhibition of adenylate cyclase activity by EGTA has been taken as an indication that the enzyme has a calmodulin-sensitive component and thus a requirement for Ca^{2+}. The effective [EGTA] that inhibits adenylate cyclase activity is also subject to variability depending on the source of the tissue under study [10,19,21,28–33].

Previously we have reported the nature of human duodenal adenylate cyclase activity to be calmodulin-insensitive and inhibited by submicromolar free [Ca^{2+}] [34]. During that study it was observed that the concentration of free Ca^{2+} that inhibited adenylate cyclase activity decreased as controlling [EGTA] increased. This suggests that the presence of EGTA in the assay systems increases the sensitivity of the enzyme to Ca^{2+}. In this study we have examined this phenomenon and established that the Ca–EGTA complex is a more inhibitory species than Ca^{2+} to adenylate cyclase activity.

Correspondence address: J.A. Smith, Medical Research Centre, City Hospital, Nottingham, NG5 1PB, UK. Fax: (44) (602) 602 140.

Abbreviations: GMP-PNP, 5′-guanylyl-imidodiphosphate; Fura-2, 1(2(5-carboxyoxozol-2-yl)-6-ammobenzo-furan-5-oxy)-2-(2′-amino-5′-methylphenoxyl)-ethane-*N,N,N′,N′*-tetraacetic acid.

2. MATERIALS AND METHODS

Biopsies from the second part of the human duodenum were collected and prepared as described previously [34]. Adenylate cyclase activity was assayed in the membranes by the method of Salomon [35] as previously described [34]. Incubations were conducted in the presence of 30 μg membrane protein for 30 min at 37°C, pH 7 [36].

2.1. *The effects of calcium and EGTA on adenylate cyclase activity*

The Ca^{2+} concentration response of adenylate cyclase activity in biopsy particulate membranes was conducted with different controlling [EGTA]. For each calcium chloride addition, the free [Ca^{2+}] was computed using an iterative ligand–metal binding programme [34,37,38]. Stock [Ca^{2+}] were checked with a calcium-sensitive electrode and the pH of the buffers did not vary over the [Ca^{2+}] range studied [16].

To further investigate the effects of EGTA on Ca^{2+} inhibition of adenylate cyclase activity, the concentration dependence of adenylate cyclase activity to EGTA in the absence and in the presence of (1 μM)

free calcium was measured under basal and various stimulating conditions.

2.2. *Calcium contamination and calcium-binding capacity of membranes*

The concentrations of contaminating Ca^{2+} in the buffer solutions and the Ca^{2+}-binding capacity of the biopsy particulate membrane preparation were estimated using the Ca^{2+} fluorescence indicator Fura-2 [39,40]. The contaminating free [Ca^{2+}] was estimated in the Mg^{2+}/HEPES tissue buffer and in a bulk volume of the adenylate cyclase reaction mixture. Excitation scans of 2 ml of 5 mM Mg^{2+}/2 mM HEPES or adenylate cyclase reaction mixture [34] containing 5 μM Fura-2 were performed in 1 cm light path, 4 ml fluorimetric cuvettes in a Kontron SFM 25 spectrofluorimeter at 37°C. The emission wavelength was set at 505 nm. A standard curve of Ca^{2+} concentration against peak excitation wavelength was established using Mg^{2+}/HEPES tissue buffer containing 0.3 mM EGTA and $CaCl_2$ to give calculated free [Ca^{2+}] ranging from 0.00788 μM to 104.5 μM. From the curve the Ca^{2+} contamination in the Mg^{2+}/HEPES buffer was estimated to be 1.4 μM and in the adenylate cyclase reaction mixture to be 2 5 μM.

The Ca^{2+}-binding capacity of the biopsy particulate membrane preparations was estimated using this system and compared to that of EGTA. Six membrane preparations were each resuspended in 2 ml Mg^{2+}/HEPES buffer containing 5 μM Fura-2 (about 2 mg/ml membrane protein) and excitation-scanned as described above. For comparison 2 ml Mg^{2+}/HEPES buffer containing 0.3 mM EGTA was also excitation scanned.

2.3. *Ethical permission*

Ethical permission for the study was obtained from the Nottingham City Hospital Ethical Committee and patients gave informed written consent.

3. RESULTS AND DISCUSSION

3.1. *The effect of calcium and EGTA on adenylate cyclase activity*

The mean Ca^{2+} concentration at which adenylate cyclase activity was inhibited by 50% (IC_{50}) was deduced from three Ca^{2+}-response curves obtained in the presence of 0, 0.3, 1.0 and 2.5 mM EGTA (Table I). The results show that the concentration of free Ca^{2+} that inhibited adenylate cyclase activity in biopsy particulate membranes decreased as the [EGTA] was increased, i.e. EGTA shifts the adenylate cyclase concentration response to free Ca^{2+} to the left (left shift). The left shift was observed for both basal and NaF stimulated adenylate cyclase activity.

Table I

Inhibition (50%) of human duodenal adenylate cyclase activity by Ca^{2+} in the presence of increasing controlling EGTA concentrations

[EGTA] (mM)	IC_{50} for basal activity (μM)	IC_{50} for NaF (10 mM) stimulated activity (μM)
0.0	51.32 ± 15.77	62.82 ± 3 76
0.3	42.19 ± 10.59	31 05 ± 0 57
1.0	1.50 ± 0.14	2.99 ± 0.70
2 5	0.19 ± 0.06	0.19 ± 0 04

3.2. *Membrane calcium binding*

It has been postulated that high Ca^{2+} binding capacity of membrane preparations in the presence of low [EGTA] may account for the left shift, i.e. high [EGTA] are necessary to effectively buffer free Ca^{2+} [3,13]. This may have particular relevance to duodenal membranes which have a significant physiological role in the uptake of Ca^{2+} from the gut lumen. The hypothesis was tested in duodenal biopsy membrane preparations using the

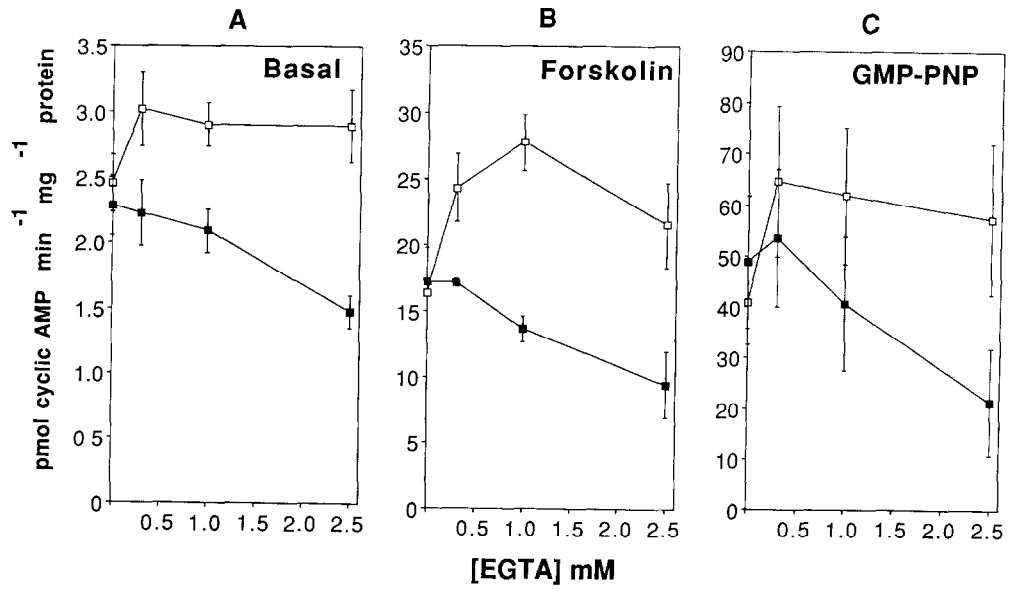

Fig. 1. The response of adenylate cyclase activity to increasing EGTA concentrations in the presence of no added $CaCl_2$ (□) and 1 μM free Ca^{2+} (■). Basal (A), 50 μM forskolin (B) and 1 μM GMP-PNP (C) stimulated activities are shown in particulate membranes from human duodenal biopsies Membranes were washed in buffer (4°C) containing 5 mM EGTA, 5 mM Mg^{2+} and 2 mM HEPES to remove any bound Ca^{2+} prior to adenylate cyclase assay. Data are the mean of at least 3 separate membrane preparations, assayed in triplicate.

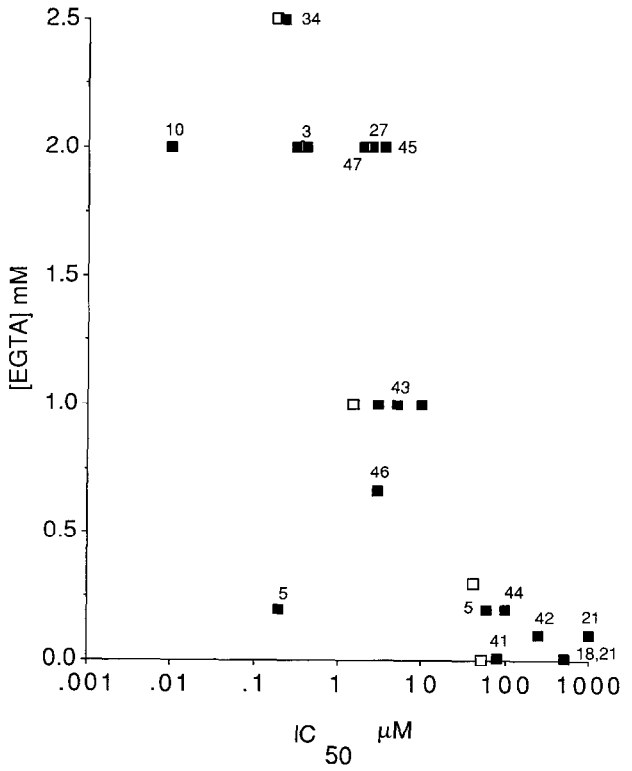

Fig. 2. Shows a range of IC_{50} values and EGTA concentrations obtained from published results (■). Each point is referenced. The IC_{50} data from Table I are superimposed for comparison (□). The different tissues used are as follows: 3, guinea-pig brain and heart ventricles; 5*, NCB-20 cells (biphasic response); 10, dog heart sarcolemma; 18*, guinea-pig enterocyte membranes; 21*, crayfish muscle; 25, luteal cells; 27, bovine aorta smooth muscle microsomes; 34, human duodenal membranes; 41* and 42, turkey erythrocyte membranes; 43, *Drosophila* wild-type heads and abdomens, rutabaga heads; 44*, rat cerebellum; 45, moth brain; 46, dog parathyroid; 47*, rabbit heart plasma membranes. * indicates IC_{50} values estimated from a graph or table.

fluorescent indicator Fura-2. Duodenal biopsy membranes added to Mg^{2+}/HEPES tissue buffer containing Fura-2 (in which the contaminating Ca^{2+} concentration was approximately 1.4 μM), caused a shift in the peak excitation spectra to the right (i.e. longer wavelength, mean 332.67 nm ± 0.81 S.D., $n = 6$) equivalent to a free Ca^{2+} concentration of 0.7 μM. EGTA (0.3 mM), on the other hand, caused a shift in the peak excitation spectra equivalent to a reduction in the free [Ca^{2+}] to 0.003 μM (excitation peak = 344 nm, $n = 3$). Therefore, the Ca^{2+}-binding capacity of the membranes was negligible compared to that of EGTA. The increased sensitivity of adenylate cyclase to Ca^{2+} in the presence of EGTA, cannot therefore be explained by inadequate buffering by low [EGTA].

3.3. *Ca–EGTA complex*

The left shift effect may be explained by considering the Ca–EGTA complex as a more active inhibitory species than Ca^{2+}. To test this hypothesis [EGTA] responses were conducted with and without a fixed concentration of free Ca^{2+} under differing stimulating conditions (Fig. 1A–C).

In the presence of a maintained free [Ca^{2+}] (1 μM), adenylate cyclase activity was inhibited by EGTA, whereas in the absence of a maintained [Ca^{2+}], EGTA stimulated enzyme activity. This supports the idea that, in these experiments, the Ca–EGTA complex was the more inhibitory species and not EGTA or Ca^{2+} per se. When the [Ca^{2+}] was maintained at 1 μM, $CaCl_2$ and EGTA were added at almost equimolar concentrations (Ca^{2+}/EGTA, respectively: 0.246 mM: 0.3 mM; 0.824 mM: 1 mM; 2.049 mM: 2.5 mM). Therefore the concentration of the Ca–EGTA complex increased as the [EGTA] increased. In the absence of added Ca^{2+} (i.e. when EGTA stimulated enzyme activity and the ratio of Ca^{2+} to EGTA was very small), EGTA was freely available to chelate the contaminating Ca^{2+} in solution (2.5 μM) and the [Ca–EGTA] remained low as the [EGTA] was increased. In the absence of added Ca^{2+}, EGTA did not inhibit adenylate cyclase activity at any [EGTA] tested. Under these conditions EGTA may have been available to chelate membrane-bound Ca^{2+} resulting in apparent enzyme stimulation. This suggests that human duodenal adenylate cyclase activity may be regulated by Ca^{2+} directly and not via calmodulin [5].

The effects of Ca–EGTA on adenylate cyclase activity were similar irrespective of which component of the adenylate cyclase system was stimulated (i.e. forskolin stimulated catalytic unit or GMP-PNP stimulated G proteins). It is therefore conceivable that the mechanism involves interaction between Ca–EGTA and the catalytic unit of the enzyme. Interaction of EGTA with a hydrophobic region of particulate adenylate cyclase from rat cerebral cortex has been previously demonstrated. However, it was unknown whether this hydrophobic region represents membrane associated calmodulin or the catalytic unit [29,30]. Our data, using duodenal particulate membranes, suggests Ca–EGTA interacts with the catalytic unit of adenylate cyclase.

Fig. 2 is a survey of published IC_{50} values for the inhibition of adenylate cyclase activity by Ca^{2+} and the corresponding [EGTA] used by other workers to control free Ca^{2+} in assay media. Despite the widely differing tissues and assay conditions employed, Fig. 2 highlights the left shift observed in this study. In the absence of EGTA, Ca^{2+} apparently does not inhibit adenylate cyclase activity at physiologically relevant intracellular concentrations (at resting < 1 μM, stimulated 1–2 μM [3,9]).

Finally both this data and that shown in Fig. 2 indicates that in some tissues it is difficult to draw firm conclusions about specific mechanisms for Ca^{2+} regulation of adenylate cyclase activity because of the interfering effects of the Ca–EGTA complex (effects that are obviously not present in vivo). Thus careful consideration of the effects of Ca–EGTA should be made when

interpreting the results from experiments involving Ca^{2+} and EGTA.

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