Measurement of tissue transglutaminase activity in a permeabilized cell system: its regulation by Ca^{2+} and nucleotides

Peter A. SMETHURST and Martin GRIFFIN*

Department of Life Sciences, Faculty of Science and Mathematics, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, U.K.

Electropermeabilized human endothelial cells (ECV-304) were used to study the regulation of tissue transglutaminase (tTGase) activity in the intracellular environment. An ELSA (enzyme-linked sorbent assay) plate assay was developed for intracellular tTGase activity, using the incorporation of a biotinylated primary amine, 5-{[(*N*-biotinoylamino)hexanoyl]amino}pentylamine (biotin-x-cadaverine; BTC), into endogenous protein substrates of tTGase. This incorporation process was inhibited by competitive inhibitors of tTGase, cystamine and monodansylcadaverine, in a dose-dependent manner. Over a 30 min period tTGase and its protein substrates did not leak out of the cell, and no incorporation of BTC occurred in unpermeabilized cells, indicating the reaction to be intracellular. In the presence of 10 nM or 10 μM Ca^{2+}, when nucleotides ATP and GTP were added at concentrations mimicking cytosolic levels, tTGase activity was decreased virtually to zero. Only at 100 μM Ca^{2+}, when nucleotides were low or absent was tTGase activity observed. Under these conditions a variety of proteins was labelled by the enzyme, with the major labelling found in a protein of molecular mass around 51 kDa when analysed by SDS/PAGE/Western blotting.

INTRODUCTION

Transglutaminases (EC 2.3.2.13) are a diverse family of Ca^{2+}-dependent enzymes with distinct genes, structures and biological functions [1] which catalyse an acyl-transfer reaction between peptidyl glutamine residues and primary amines. The primary amine may include the side chains of peptidyl lysine, resulting in protein cross-linking via ϵ-(γ-glutamyl)lysine bonds, or polyamines such as spermine and putrescine, resulting in peptide-bound γ-glutamyl polyamine conjugates [2]. Transglutaminase activity may be measured by the incorporation of suitably labelled primary amines into γ-glutamyl-containing protein substrates [3,4].

The tissue (tTGase, type 2, cytosolic) transglutaminase is the most widespread of the family which reside in mammalian tissues. However, the physiological function of the enzyme is still not fully understood. Suggested roles for tTGase have been proposed in stimulus–secretion coupling [5], receptor-mediated endocytosis [6], programmed cell death [7], cell differentiation [8] and tumour growth [9,10].

Although tTGase is essentially an intracellular enzyme, there is now increasing evidence to suggest that it has the ability to act at the cell surface, which may be important in both cell adhesion [11] and stabilization of the extracellular matrix [8,12].

In addition to its protein cross-linking activity, tTGase is a GTP-binding protein [13,14] with GTPase activity [15], which may also be important in transducing extracellular signals via the α_1-adrenergic receptor to phospholipase C [16]. Studies with purified tTGase indicate that GTP, and to a lesser extent ATP, are capable of inhibiting its Ca^{2+}-dependent cross-linking activity by altering the conformation of the enzyme and decreasing its affinity for Ca^{2+} [13,14]. It has been suggested that this regulation by GTP may be one way of modulating its cellular activity, such that the intracellular role of tTGase would be limited to that of a GTP-binding protein. Any cross-linking role for the enzyme would then be limited to cell-surface reactions, where nucleotides are absent and Ca^{2+} is plentiful, or in the dying cell, where ATP is lower and much larger intracellular Ca^{2+} concentrations are experienced [17]. However, these studies [13,14] were carried out with Ca^{2+} concentrations of 100 μM or above with purified constituents, which may behave differently in the intracellular environment, where the association of tTGase with other cellular factors may also exert an effect on activity. Examples of such interactions would be those of tTGase with a particulate-associated inhibitory factor in endothelial cells [18], or with calmodulin [19], which may affect its response to nucleotides and Ca^{2+}. In order to observe tTGase activity on native substrates under cytosolic conditions, we used electropermeabilized cells.

Electropermeabilization was the method of choice, because it allows the modulation of the cytosolic environment and the introduction of labelled substrates, but preserves much of the cellular integrity and the spatial arrangement of intracellular constituents [20,21]. This is a well-founded technique which has been applied to the study of kinase activity [22] and both Ca^{2+}- and GTP-stimulated exocytosis [22,23]. In an initial report, we used electropermeabilization to study tTGase function in permeabilized rat islets of Langerhans [24]. In the present paper, we have extended this principle to study the regulation of tTGase in an established human endothelial cell line [25]. Endothelial cells contain relatively high levels of tTGase activity [18] and are thus amenable to this type of study.

EXPERIMENTAL

All chemicals were of the highest available grade and were purchased from either Sigma (Poole, Dorset, U.K.) or BDH (Lutterworth, Leics., U.K.). Biotin-x-cadaverine (BTC) was purchased from Molecular Probes (Eugene, OR, U.S.A.) and was stored as an aqueous stock at −20 °C. Tissue-culture plastic and 96-well plates (code 25802) were purchased from Corning. All water was deionized (Elga, High Wycombe, Bucks., U.K.).

Treatment of cells

The ECV-304 human umbilical-vein cloned endothelial cell line [25] was obtained from the European Collection of Animal Cell Cultures (Porton Down, Wilts., U.K.). The cells were grown at

Abbreviations used: BTC, biotin-x-cadaverine; ELSA, enzyme-linked sorbent assay; tTGase, tissue transglutaminase.

* To whom all correspondence should be addressed.

37 °C with air/CO_2 (19:1) in growth medium, consisting of Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% foetal-bovine serum (Advanced Protein Products, Brierley Hill, W. Midlands, U.K.) and 1% penicillin/streptomycin (Sigma). The cells were trypsin-treated before each experiment by using 0.05% trypsin in DMEM; the trypsin was decanted after centrifugation at 2000 ***g*** for 2 min and the cells were resuspended in growth medium (containing serum) to give suspensions with clumps of 50 cells or less. The cells were then permeabilized as stated below.

Preparation of electropermeabilized cells

Batches of up to 10^7 cells were collected into a Microfuge tube and pelleted by centrifugation in a Microfuge (Jouan A40) at 2000 ***g*** for 2 min at room temperature. The supernatant was replaced with ice-cold 'poration buffer', containing 140 mM potassium glutamate, 7 mM $MgSO_4$, 1 mM EGTA, 0.5 mg/ml BSA (A-7030; Sigma), with $CaCl_2$ added to give a Ca^{2+} concentration of 10 nM. The cells were washed three times in this buffer, by resuspension and centrifugation, and then were exposed to five pulses (each 0.1–0.2 ms) of an electric field of 4.0 kV/cm from a capacitance of 3 μF, in a 0.4 cm-wide cuvette in a Gene Pulser apparatus (Bio-Rad, Hemel Hempstead, U.K.).

Labelling of intracellular proteins by transglutaminase in permeabilized cells

After electropermeabilization, the cells were washed once more in poration buffer and placed into microfuge tubes in batches of 2×10^5 cells. These constituted individual determinations in the assays. The cells were pelleted as above and placed on ice. To start the 5 min preincubation, the supernatant was replaced with buffer (50–100 μl) containing different concentrations of BTC, nucleotides and tTGase inhibitors. The cells were then transferred from ice to a water bath at 37 °C, together with the addition of $CaCl_2$ or water to give the various Ca^{2+} levels used. Reactions were stopped by addition of 500 μl of PBS containing 100 μM iodoacetamide and mixing. The cells were then centrifuged at 13000 ***g*** for 2 min at room temperature and the supernatants discarded. The pellets were then stored at -20 °C before assay or were treated for SDS/PAGE as described below. The Ca^{2+} concentrations were calculated by the method described by Yaseen et al. [26]. The Ca^{2+} concentrations were then checked by direct electrode measurement by the method of Baudet et al. [27].

SDS/PAGE/Western blotting/probing of cellular proteins after incorporation of BTC

Before SDS/PAGE, permeabilized cells were washed once in 0.25 M sucrose/5 mM Tris/2 mM EDTA, pH 7.4, before being resuspended in Laemmli sample buffer, consisting of 62.5 mM Tris, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.01% (w/v) Bromophenol Blue. The samples were then sonicated on ice with 3×5 s pulses, with 5 s gaps, at 5 μm peak width using a fine probe (Soniprep; M.S.E., Loughborough, U.K.), and then incubated at 90–100 °C for 5 min before loading on gels. SDS/PAGE was conducted by using a 3% stacking and a 10% resolving gel on a Mini-Protean system (Bio-Rad), by the method of Laemmli [28]. Western blotting was conducted with a semi-dry system (LKB Multiphor; Pharmacia–LKB, Uppsala, Sweden) with a continuous transfer buffer as described by Harlow and Lane ([29]; pp. 488–489). The blot was then incubated in 2% BSA in PBS, pH 7.4, overnight at 4 °C, followed by a wash in PBS containing 0.05% Tween-20, pH 7.4 (PBS-T), for 10 min. Incubation of the blot was then in PBS-T containing 1% BSA and ExtrAvidin peroxidase (1/2000, v/v) for 60 min, with shaking. The blot was then washed for 2×10 min in PBS-T, followed by a rinse in deionized water. The blot was then incubated in substrate buffer containing 4-chloro-1-naphthol, made up as described on p. 506 of [29], until labelled proteins were detected, whereupon the blot was transferred to water and subsequently photographed.

Measurement of incorporation of BTC into cellular proteins by enzyme-linked sorbent assay (ELSA)

The wells of an 96-well plate were pretreated with 100 μl of poly-L-lysine (Sigma; 1/100, v/v, in PBS) for 60 min at room temperature and washed three times with PBS. Cell pellets were thawed and resuspended in 0.67 ml of ice-cold PBS, pH 7.4. They were sonicated as described above, and then a further 1.0 ml of PBS was added and the samples were mixed and a 50 μl portion was placed in each well. This protein loading per well gave an optimal signal:background ratio (results not shown). The plates were then covered and left overnight at room temperature, with shaking. Then 50 μl of glutaraldehyde (0.1% in PBS) was added and the sample left for a further 60 min, with shaking. The wells were carefully evacuated and then the plates washed three times with PBS. The wells were then filled with 250 μl of 2% BSA in PBS, and then the plates were incubated at 37 °C for 120 min. The wells were washed three times with PBS-T, then 100 μl of ExtrAvidin peroxidase conjugate (Sigma; 1/40000 in PBS-T) was added to most of the wells and the plates were incubated at room temperature for 60 min, with shaking. To some wells, PBS was added instead of conjugate for plate blanks as stated below. After this, the wells were carefully evacuated and then the plates washed three times with PBS-T, three times with water and once with sodium acetate buffer (0.1 M, pH 6.0). The substrate solution was then added (100 μl per well), which consisted of 40 ml of sodium acetate buffer containing 300 ml of TMB solution (10 mg/ml 3,3,5,5-tetramethylbenzidine in DMSO) and 50 μl of H_2O_2 (aq. 3%). The reaction was terminated by addition of 2.5 M H_2SO_4 (50 μl). The plates were then read at 450 nm on an ELISA plate reader (ICN, Thame, Oxon, U.K.). Each plate was set to zero on wells containing cellular proteins which had not been probed with ExtrAvidin peroxidase and typically produced an absorbance of 0.08. Each experiment also had a value for the incubation at 100 μM Ca^{2+} + 1 mM iodoacetamide, which was probed with ExtrAvidin peroxidase and gave a typical absorbance value of 0.044 ± 0.013 ($n = 6$). Since iodoacetamide is an irreversible inhibitor of transglutaminases by carbamidomethylating the active-site thiol group [30], this was assumed to represent non-specific binding of the conjugate to cellular proteins and was subtracted from each value within an experiment.

Statistics and presentation

For the ELSA, absorbance values from three to five wells from one sonicated sample were used to obtain a mean, which constituted a single determination. Data were then expressed as a mean $\pm$ S.E.M. of at least three separate determinations (as in Figures 1 and 2 and in Table 2) or as a mean $\pm$ S.E.M. from a number of separate experiments (n) undertaken on separate days, each containing at least three determinations (as in Table 1). The differences between means were assessed by Student's t test, on the *Fig-P* graphics package (BioSoft, Cambridge, U.K.), which was used to produce the graphs.

RESULTS

Permeabilization of endothelial cells and incorporation of BTC into endogenous proteins of permeabilized ECV-304 cells by tTGase

Various field strengths ranging from 1 to 4 kV/cm using five pulses from a fixed capacitance of 3 μF were used to permeabilize the ECV-304 cells. By using Trypan Blue uptake as a measure of permeabilization, it was found that five pulses of 4 kV/cm led to $60.5 \pm 34\,\%$ ($n = 6$) of cells becoming permeabilized, as compared with 1 % before exposure. Incubation of permeabilized ECV-304 cells with increasing concentrations of BTC (0–3.4 mM) at 100 μM Ca^{2+} over a set time period of 5 min led to a substrate-dependent increase in the incorporation of this primary amine into cellular proteins (Figure 1). This incorporation was found to be Ca^{2+}-dependent, since incorporation was negligible at 10 nM Ca^{2+} (results not shown), indicating it to be a transglutaminase-mediated event. Incorporation of substrate was linear over a 5 min assay period (Figure 2). Treatment of the data, shown in Figure 1, by the method of Eadie [31] allowed the calculation of the K_m for BTC incorporation as 6.9 mM. For further assays, 2.26 mM (1 mg/ml) BTC was chosen as an appropriate concentration to use (as indicated by the broken line in Figure 1).

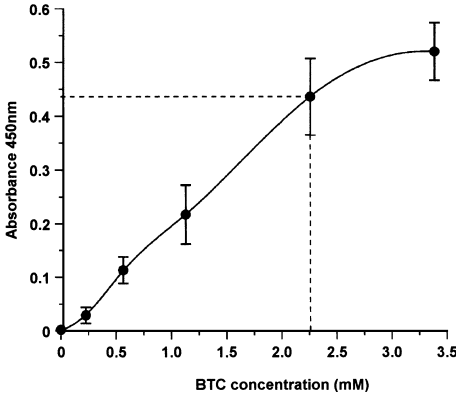

Figure 1 Effect of BTC concentration on rate of incorporation of BTC into cellular proteins

Incubations of permeabilized ECV 304 cells were conducted over 5 min at 37 °C, with 100 μM Ca^{2+}. Each point represents the mean ± S.E.M. of three separate determinations. Incorporation was assessed by the ELSA method as described in the Experimental section.

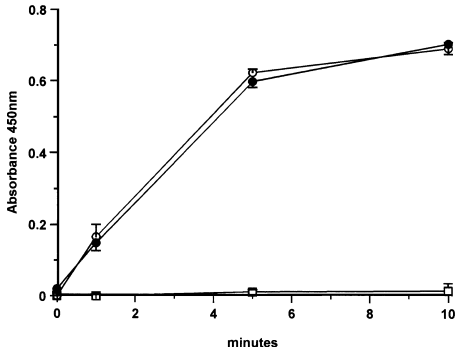

Figure 2 Time course of BTC incorporation into cellular proteins

Incubations of ECV 304 cells were conducted at 37 °C in the presence of 100 μM Ca^{2+} and 2.26 mM BTC. Before these incubations, cells were permeabilized immediately (●), or permeabilized and left for 20 min on ice and washed (○), or not permeabilized (□). Each point represents the mean ± S.E.M. of three separate determinations. In both cases, the incorporation was then assessed by the ELSA method as described in the Experimental section.

Containment of reactive cellular constituents within permeabilized cells

To check whether cellular constituents were being lost from the permeabilized cells over the time of incubation, the rate of substrate (BTC) incorporation was measured in cells which had been permeabilized immediately before assay, and in cells which had been permeabilized 20 min earlier. If the enzyme or its endogenous protein substrates leaked out over this time frame, then a decrease would be observed in the rate or the maximal absorbance value respectively. As shown in Figure 2, a reduction in neither parameter is observed, which indicates that neither the enzyme nor its substrates leak out over the time frame chosen for assay. The total time allowed for possible leakage out of the cells was therefore the 20 min delay plus the 10 min incubation time.

It can also be observed from Figure 2 that when the cells are not permeabilized, there is negligible substrate incorporation, indicating that under the conditions of this particular assay the tTGase is only active inside the cells.

Modulation of transglutaminase activity by nucleotides and Ca^{2+}

When conducted with an absence of nucleotides in the bathing medium, the intracellular activity of transglutaminase shows a dose-related increase in response to raised cytosolic Ca^{2+} (Table 1). In particular, a significant increase is observed when Ca^{2+} is raised from 10 nM to 10 μM, from $2.6 \pm 1.1\,\%$ to $24.3 \pm 1.8\,\%$ ($P < 0.0001$) when the activities are expressed as a percentage of the activity at 100 μM Ca^{2+}. However, this is an unphysiological condition generated by the dialysis of the cells' endogenous nucleotides and other low-molecular-mass solutes into the bathing medium, which occurs upon permeabilization [20]. The cytosol of living cells would normally contain levels of ATP of 8–11 mM [32], though some of this may be in bound form, and concentrations of GTP are in the range 50–300 μM [33,34], with an estimated free GTP concentration of around 100 μM [35]. A maximum response for exocytosis has been elicited by 500 μM GTP in one model [36]. Even if the cell was transiently stimulated, leading to the activation of G-proteins and kinases, the cytosol would still contain equivalent levels of the corresponding nucleoside diphosphates. Only in the rare event of anaerobiosis would the cytosol be depleted of both nucleoside tri- and di-phosphates [32]. Therefore, any study which wishes to assess the activity of transglutaminase in the cytosol must include these constituents. When these compounds are included in the bathing medium, these rapidly equilibrate with the cytosol of the cells. Bearing in mind the estimated cytosolic levels of ATP and GTP quoted above, we chose 5 mM ATP + 100 μM GTP and 0.5 mM ATP + 10 μM GTP to represent two extremes of the energy status of cells. To mimic a possible energy-depleted state, where nucleoside triphosphates become diphosphates, 5 mM ADP + 100 μM GDP was chosen. As shown in Table 1, 5 mM ATP + 100 μM GTP decreases the activity of the enzyme to levels approaching zero at Ca^{2+} levels of 10 nM–10 μM. A slight elevation of tTGase activity above basal (10 nM Ca^{2+}) is observed at 100 μM Ca^{2+} ($14.9 \pm 3.5\,\%$; $P < 0.02$). The same pattern is shown with 5 mM ADP + 100 μM GDP, indicating that even in an energy-depleted state tTGase is not activated. In the presence of low levels of nucleotides such as 0.5 mM ATP + 10 μM GTP, tTGase activity is extremely low and there is still no significant activation of tTGase at 10 μM Ca^{2+}, whereas 100 μM Ca^{2+} evokes a marked elevation of TG activity above background ($58.8 \pm 3.9\,\%$; $P < 0.001$). At the highest levels of nucleotides

Table 1 Activity of tTGase inside permeabilized cells at different levels of free Ca^{2+} and nucleotides in the bathing medium

Data, expressed as a percentage (mean ± S.E.M.) of the activity at 100 μM Ca^{2+} in the absence of nucleotides over 5 min, were obtained with permeabilized ECV-304 cells by the ELSA technique as described in the Experimental section; n.d., not detected. Numbers of separate experiments performed (within which there were three individual determinations) are given in parentheses. Activity at 100 μM Ca^{2+} = 0.497 ± 0.076 (12) A_{450} unit.

	Nucleotide levels				
Free [Ca^{2+}]	0 ATP 0 GTP	0.5 mM ATP 10 μM GTP	5 mM ATP 100 μM GTP	5 mM ADP 100 μM GDP	5 mM ATP 500 μM GTP
10 nM	3.0 ± 1.2 (5)	1.4 ± 1.7 (3)	0.6 ± 0.7 (3)	n.d. (3)	n.d. (4)
10 μM	24.3 ± 2.0 (5)	5.4 ± 3.4 (3)	0.7 ± 0.4 (3)	n.d. (3)	n.d. (4)
100 μM	(100)	58.8 ± 4.8 (3)	14.9 ± 4.3 (3)	9.7 ± 5.4 (3)	2.4 ± 1.7 (4)

Table 2 Effects of competitive inhibitors on tTGase activity

Data are expressed as a percentage (mean ± S.E.M.) of the activity at 100 μM Ca^{2+} in the absence of nucleotides over 5 min for three separate determinations, and were obtained with permeabilized ECV-304 cells by the ELSA technique as described in the Experimental section. Activity over a 5 min time period at 100 μM Ca^{2+} = 0.566 ± 0.052 A_{450} unit. Abbreviations: MDC, monodansylcadaverine; DMC, dimethyl monodansylcadaverine; n.s.d., no significant difference.

Compound	Concentration	Inhibition (%)
Cystamine	2.5 mM	43.7 ± 15.0 ($P < 0.05$)
	5.0 mM	73.7 ± 1.1 ($P < 0.001$)
MDC	100 μM	28.4 ± 8.0 ($P < 0.05$)
	200 μM	41.7 ± 11.5 ($P < 0.02$)
DMDC (control)	100 μM	9.2 ± 17.7 (n.s.d.)
	200 μM	26.7 ± 6.2 ($P = 0.05$)

used, 5 mM ATP + 500 μM GTP, tTGase activity is only 2.4% of that in the absence of nucleotides at 100 μM Ca^{2+}.

Inhibition of BTC incorporation into endogenous cellular proteins by competitive inhibitors of tTGase

Further confirmation that the BTC incorporation into proteins of permeabilized cells was transglutaminase-mediated came from the use of competitive inhibitors of the enzyme. Cystamine is both a competitive and irreversible inhibitor of the enzyme, thought to undergo disulphide exchange with the active-site cysteinyl residue [37]. Table 2 shows that a dose-dependent inhibition was observed with levels of 2.5 mM (giving 43.7 ± 15.0% inhibition; $P < 0.05$) and 5 mM (giving 73.7 ± 1.1% inhibition; $P < 0.001$) cystamine. Similarly, the primary amine competitor monodansylcadaverine [3] showed dose-dependent inhibition when used at 100 μM (28.4 ± 8.0%; $P < 0.05$) and 200 μM (41.7 ± 11.5%; $P < 0.02$). This was not matched by inhibition of the tertiary-amine control compound dimethylmonodansylcadaverine at 100 μM, which showed no significant difference from the uninhibited reaction, although at 200 μM a small amount of non-specific inhibition was observed (26.7 ± 6.2%; $P = 0.05$).

Elucidation of endogenous protein substrates labelled with BTC in permeabilized cells

In order to identify the endogenous proteins into which BTC is incorporated, and to confirm further the Ca^{2+}-dependency of this enzymic reaction, proteins of ECV-304 cells were fractionated by SDS/PAGE, Western-blotted and then probed with ExtrAvidin peroxidase conjugate (Sigma). In the lane corresponding to the incubation at 10 μM Ca^{2+}, the labelling of a protein with a calculated molecular mass of around 51 kDa could be discerned (Figure 3, lane 1). When the free Ca^{2+} concentration was increased to 100 μM, many more proteins were revealed (Figure 3, lane 2), which confirmed the results obtained by the ELSA. The most labelled protein had a molecular mass of 51 kDa, with other proteins of 151, 117, 89, 80, 67, 60, 48, 44, 38, 36, 34 and 31 kDa also labelled. In addition, there is some polymeric labelled material which is retarded at the top of the stacking gel (> 1 MDa) and resolving gel (> 250 kDa). The inclusion of protease inhibitors leupeptin, pepstatin A and PMSF at their effective concentrations ([29]; p. 677) led to a greater labelling of the 60

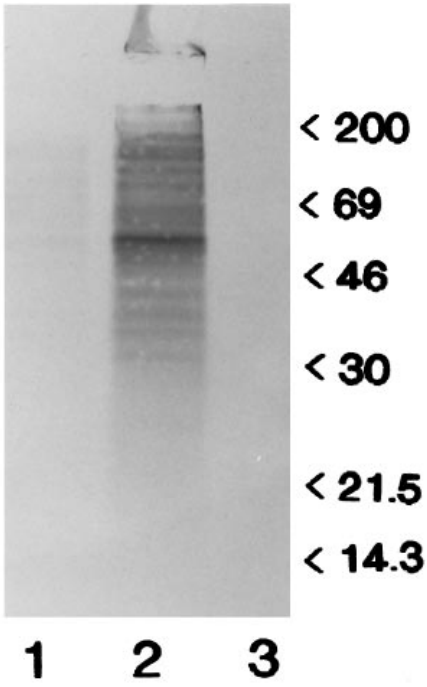

Figure 3 Western blot of proteins from permeabilized ECV 304 cells

Cells were incubated at 37 °C for 10 min in the presence of 2.26 mM BTC. A Western blot was produced after SDS/PAGE, and the proteins with BTC incorporated were revealed by using ExtrAvidin-peroxidase (Sigma) as described in the Experimental section. Protein loading was 80 mg per lane. Lane 1 is from cells incubated at 10 μM Ca^{2+}; lane 2 is from cells incubated at 100 μM Ca^{2+}; lane 3 is from cells incubated at 100 μM Ca^{2+} + 1 mM iodoacetamide. Molecular-mass standards with their values in kDa are displayed to the right.

and 67 kDa bands, but did not alter the prominence of the 51 kDa band (results not shown), making it unlikely that this is a proteolytic product generated by intracellular proteases. Lane 3 is the reaction conducted at 100 μM Ca^{2+} in the presence of the thiol inhibitor iodoacetamide (1 mM). Since no bands are revealed in this lane, this indicates that the reaction is not due to non-specific association of the conjugate with endogenous biotin-containing proteins, thus further validating the involvement of tTGase in this process.

DISCUSSION

The present study uses a novel assay system utilizing electropermeabilized cells to investigate the activation and regulation of tTGase activity in the intracellular environment. In addition, it allows the identification of endogenous intracellular γ-glutamyl-containing substrates under these conditions. Important to this study was the finding that neither enzyme nor protein substrates leak out of the permeabilized cells over a 30 min time frame. This concurs with the work of Knight and Scrutton ([20], and references therein), who found that only low-molecular-mass solutes are freely diffusible from electropermeabilized cells. This is markedly different from detergent permeabilization, where 'run-down' of cytosolic proteins is accepted and employed [38]. With cell permeabilization, the cytosolic solutes are dialysed into the surrounding medium and vice versa, allowing the levels of ATP, GTP and free Ca^{2+} to be fixed in the cytosol. In the cytoplasm of a resting energy-rich cell, the ATP levels may be as high as 8–11 mM [32] and GTP levels between 50 and 300 μM [33,34], with a proportion of each being bound to cytosolic proteins, giving lower free nucleotide concentrations. In the resting state the free Ca^{2+} is around 100–200 nM [39,40]. Under these conditions, our data suggest that tTGase activity in the cytosol would be switched off. This concurs with the conclusions of other workers from their studies with purified tTGase [13,14], and therefore indicates that no factors exist within the cytosol to support tTGase activity, in spite of the nucleotide levels. Even in the stimulated cell, where Ca^{2+} levels may reach 10 μM, tTGase activity is extremely low at physiological levels of nucleoside di- and tri-phosphates. Our data therefore suggest that, whatever the transient energy state of the cell, tTGase is unlikely to be activated in the cytosol without (i) falls in both nucleoside tri- and di-phosphate levels, (ii) a major influx of extracellular Ca^{2+}, and (iii) a post-translational modification of tTGase which removes the inhibitory activity of nucleotide and/or the need for Ca^{2+} binding in order for enzyme activation. An example of the latter is the pathological activation of synaptic TGase by tetanus toxin [41]. Given these possibilities, tTGase could possibly be activated during prolonged anaerobiosis, when nucleoside tri- and di-phosphate levels may be depleted [32]. This would probably lead to cell death via necrosis. tTGase could clearly be activated during the terminal stages of cell death, resulting from either necrosis or apoptosis, where intracellular Ca^{2+} concentrations increase and the cell becomes energy-depleted. This would fit in with the suggested role for tTGase in programmed cell death, where irreversible cross-linking of cellular proteins can only occur when the cell is destined to die [7]. Such proteins in endothelial cells may include those highlighted by this labelling procedure from Western blots, especially as this method tends to preserve the spatial integrity of the cell and thus prevents many non-specific reactions which occur in homogenates. In this respect, the labelling pattern may also reflect the degree of association of tTGase with other proteins within the cell. The molecular mass of 51 kDa may correspond to that of a tubulin subunit [42] or an intermediate-filament monomer [43], which have both been postulated as substrates of tTGase. Work is at present underway to characterize these key substrates.

In conclusion, our data obtained from electropermeabilized cells confirm and extend the hypothesis that both adenosine and guanosine tri- and di-phosphates are important intracellular regulators of the Ca^{2+}-activated cross-linking function of tTGase. If the enzyme has an intracellular role which is not linked with cell death, then this is likely to be as a GTP-binding protein, perhaps in such a way as that described by Nakaoka et al. [16], or as a potential regulator of cell-cycle progression, as recently proposed by Mian et al. [44], and not as an enzyme that catalyses the post-translational modification of proteins. There is now mounting evidence to suggest that tTGase can act at the cell surface, facilitating cell adhesion [11,12] and/or modification of the extracellular matrix [8]. Exposure to the extracellular environment, which lacks nucleotides and contains millimolar Ca^{2+}, would certainly activate the enzyme and may provide the switch between the two alternative functions of this protein.

We thank Ms. A. Cozzolino for her excellent technical assistance and Mr. G. L. Thomas for helpful discussions. This investigation was supported by the Polytechnics and Colleges Funding Council Research Initiative.

REFERENCES

1 Griffin, M. and Smethurst, P. A. (1994) Retinoids Today and Tomorrow **37**, 4–10
2 Folk, J. E. and Finlayson, J. S. (1977) Adv. Protein Chem. **31**, 1–131
3 Lorand, L., Parameswaran, K. N., Stenberg, P., Tong, Y. S., Velasco, P. T., Jonsson, N. A., Mikiver, L. and Moses, P. (1979) Biochemistry **18**, 1756–1765
4 Jeon, W. M., Lee, K. N., Birckbichler, P. J., Conway, E. and Patterson, M. K. (1989) Anal. Biochem. **182**, 170–175
5 Bungay, P. J., Owen, R. A., Coutts, I. C. and Griffin, M. (1986) Biochem. J. **235**, 269–278
6 Davies, P. J. A., Davies, D. R., Levitski, A., Maxfield, R. R., Milhaud, P., Willingham, M. C. and Pastan, I. (1980) Nature (London) **283**, 162–167
7 Fesus, L., Thomazy, V., Autuori, F., Ceru, M. P., Tarcsa, E. and Piacentini, M. (1989) FEBS Lett. **245**, 150–154
8 Aeschlimann, D., Wetterwald, A., Fleisch, H. and Paulsson, M. (1993) J. Cell Biol. **120**, 1461–1470
9 Hand, D., Elliott, B. M. and Griffin, M. (1988) Biochim. Biophys. Acta **970**, 137–145
10 Johnson, T. S., Knight, C. R. L., El Alaoui, S., Mian, S., Rees, R. C., Gentile, V., Davies, P. J. A. and Griffin, M. (1994) Oncogene **9**, 2935–2942
11 Gentile, V., Thomazy, V., Piacentini, M., Fesus, L. and Davies, P. J. A. (1992) J. Cell Biol. **119**, 463–474
12 Martinez, J., Chalupowicz, D. G., Roush, R. K., Sheth, A. and Barsigian, C. (1994) Biochemistry **33**, 2538–2545
13 Achyuthan, K. E. and Greenberg, C. S. (1987) J. Biol. Chem. **262**, 1901–1906
14 Bergamini, C. M. (1988) FEBS Lett. **239**, 255–258
15 Lee, K. N., Birckbichler, P. J. and Patterson, M. K. (1989) Biochem. Biophys. Res. Commun. **162**, 1370–1375
16 Nakaoka, H., Perez, D. M., Baek, K. J., Das, T., Husain, A., Misono, K., Im, M.-J. and Graham, R. M. (1994) Science **264**, 1593–1596
17 Wyllie, A. H. (1987) Int. Rev. Cytol. **17**, 755–785
18 Korner, G., Schneider, D. E., Purdon, M. A. and Bjornsson, T. D. (1989) Biochem. J. **262**, 633–641
19 Takeuchi, Y., Birckbichler, P. J., Maxwell, M., Howell, B., Carter, H. and Patterson, M. K. (1992) FASEB J. **6**, A37
20 Knight, D. E. and Scrutton, M. C. (1986) Biochem. J. **234**, 497–506
21 Swezey, R. R. and Epel, D. (1992) in Guide to Electroporation and Electrofusion (Chang, D. C., Chassy, B. M., Saunders, J. A. and Sowers, A. E., eds.), pp. 347–362, Academic Press, London
22 Jones, P. M., Persaud, S. J. and Howell, S. L. (1992) Biochem. J. **285**, 973–978
23 Wollheim, C. B., Ullrich, S., Meda, P. and Vallar, L. (1987) Biosci. Rep. **7**, 443–454
24 Smethurst, P. A., Bungay, P. J. and Griffin, M. (1993) Biochem. Soc. Trans. **21**, 424S
25 Takahashi, K., Sawasaki, Y., Hata, J.-I., Mukai, K. and Goto, T. (1990) In Vitro Cell. Dev. Biol. **25**, 265–274
26 Yaseen, M. A., Pedley, K. C. and Howell, S. L. (1982) Biochem. J. **206**, 81–87
27 Baudet, S., Hove-Madsen, L. and Bers, D. M. (1994) Methods Cell Biol. **40**, 93–102
28 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
29 Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York

30 Folk, J. E. and Cole, P. W. (1966) J. Biol. Chem. **241**, 5518–5525
31 Eadie, G. S. (1952) Science **116**, 688
32 Geisbuhler, T., Altschuld, R. A., Trewyn, R. W., Ansel, A. Z., Lamka, K. and Brierley, G. P. (1984) Circ. Res. **54**, 536–546
33 Breitweiser, G. E. and Szabo, G. (1988) J. Gen. Physiol. **91**, 469–493
34 Kleineke, J., Duls, C. and Soling, H. D. (1979) FEBS Lett., **107**, 198–202
35 Horie, M. and Irisawa, H. (1989) J. Physiol. (London) **408**, 313–332
36 Howell, T. W., Cockcroft, S. and Gomperts, B. D. (1987) J. Cell Biol. **105**, 191–197
37 Seifring, G. E., Apostol, A., Velasco, P. and Lorand, L. (1978) Biochemistry **17**, 2598–2604
38 Ali, S. M., Geisow, M. and Burgoyne, R. D. (1989) Nature (London) **340**, 313–315
39 Woods, N. M., Cuthbertson, K. S. R. and Cobbold, P. (1986) Nature (London) **319**, 600–602
40 Cheek, T. R. (1989) J. Cell Sci. **93**, 211–216
41 Facchiano, F., Valtorta, F., Benfenati, F. and Luini, A. (1993) Trends Biochem. Sci. **18**, 327–329
42 Maccioni, R. B. and Seeds, N. W. (1986) Mol. Cell. Biochem. **69**, 161–166
43 Fukuda, K., Kojiro, M. and Chiu, J.-F. (1993) Hepatology **17**, 118–124
44 Mian, S., El Alaoui, S., Lawry, J., Gentile, V., Davies, P. J. A. and Griffin, M. (1995) FEBS Lett. **370**, 27–31

Received 24 July 1995/14 September 1995; accepted 25 September 1995