Complement-induced Ca^{2+} influx in cultured fibroblasts is decreased by the calcium-channel antagonist nifedipine or by some bivalent inorganic cations

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The effects of different extracellular cations or organic Ca^{2+}-channel modulators on complement-induced changes in intracellular Ca^{2+} and cell death have been investigated in the transfected NIH-3T3 HIR 3.5 cell line, which overexpresses the human insulin receptor. Cells were incubated with mouse anti-(human insulin receptor) monoclonal antibodies before exposure to rabbit or human serum (sources of heterologous complement). Changes in intracellular Ca^{2+} were complement-dependent (measured by influx of ^{45}Ca), as was cytotoxicity (monitored by leakage of lactate dehydrogenase into the culture supernatant). Addition of a dihydropyridine Ca^{2+}-channel antagonist (nifedipine) or some bivalent inorganic cations caused inhibition of ^{45}Ca entry via a novel channel distinct from endogenous voltage-gated Ca^{2+} channels. Nifedipine decreased, but conversely the addition of a phenylalkylamine Ca^{2+}-channel antagonist (verapamil) or the inorganic Ca^{2+} agonists Ba^{2+} and Sr^{+} increased, complement-induced cytotoxicity. These agents had no effect on cell viability at the studied concentrations, in the absence of complement. It is concluded that complement-induced cytotoxicity is mediated by Ca^{2+} influx through novel specific transmembrane channels which are sensitive to the Ca^{2+}-channel antagonist nifedipine, but otherwise show little resemblance to L- or T-type voltage-gated Ca^{2+} channels.

INTRODUCTION

The membrane attack complex (MAC), formed by the self-polymerization of the terminal components of the complement cascade, binds to the surfaces of appropriate target cells and has been suggested to effect cytolysis by forming transmembrane pores [1,2]. An alternative hypothesis is that of Esser, who has suggested a 'leaky patch' formation in the vicinity of MAC [3]. However, Esser's group has recently published data showing the formation of single channels by MAC in artificial bilayer membranes [4], data which support other recent work showing single channel formation by MAC [5,6]. These studies have indicated that there is selectivity for cations over anions upon formation of the channel. It is thought that the binding and insertion of complement component C9 to the membrane-attached C5b–8 complex is responsible for the actual pore formation [7].

The mechanism of MAC-induced cell death is uncertain, though it has been shown that MAC insertion is associated with a transient Ca^{2+} influx and increased cytosolic Ca^{2+} concentration [8–10], which may be followed by large membrane permeability changes [11] and cell death. However, the transient rise in intracellular Ca^{2+} may be followed by cell recovery via MAC shedding in the case of neutrophils, oligodendrocytes and platelets [10–12]. It is possible that a rise in intracellular Ca^{2+} is, under certain circumstances (e.g. higher complement concentrations) ultimately responsible for cell lysis. Free intracellular Ca^{2+} ions act as intracellular regulators of cell function [13], and excessive increases in intracellular concentration may precipitate cell death by a multitude of events, including activation or inhibition of key enzymes, organelle disruption, protein denaturation and depletion of ATP. More specifically, phospholipase activation would stimulate membrane breakdown and loss of ionic gradients [14], and Ca^{2+}-induced dissipation of mitochondrial membrane potential would consequently block oxidative phosphorylation [15]. Cellular ATP content would subsequently fall, and original ionic gradients across the plasma membrane will not be regenerated; thus depolarization of the cell membrane will occur, eventually leading to larger permeability changes, osmotic lysis and cell death. The requirement for Ca^{2+} in the complement-mediated lytic process has still not been proved, and it is still widely believed that MAC is a rigid hollow structure, like a doughnut [1], that produces a general ion influx followed by colloid osmotic lysis. However, various studies using alternative lytic stimulators have confirmed, for example, that the lethal effect of A23187 is due to the rise in intracellular Ca^{2+} [16] (an essential event that appears to represent a final common pathway in toxic cell death [17]. Early complement studies [18,19] have shown that, if high concentrations of EDTA (30 or 90 mM) are added to cells after reaction with complement, then lysis was blocked. However, use of chelating agents at such high concentrations makes clear interpretations of results difficult.

We have used cultured NIH 3T3 HIR 3.5 cells, which express high levels of the human insulin receptor [20], and complement-fixing anti-(insulin receptor) monoclonal antibodies [21] as a model system in which to investigate Ca^{2+} influx during complement-mediated cell attack, possible blocking agents of this ion influx, and whether Ca^{2+} entry is a key event in stimulating cell lysis.

MATERIALS AND METHODS

Reagents, cells, culture media

Tissue-culture medium, supplements and sterile plasticware were obtained from Gibco, Paisley, Scotland, U.K. Intravenous-grade nifedipine was obtained from Bayer (Newbury, Berks, U.K.), and stored in the dark at 4 °C until use. Unless otherwise stated, all other chemicals were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.), BDH Chemicals (Poole, Dorset, U.K.), Roche Products (Welwyn, Herts., U.K.) and SmithKline

Abbreviations used: MAC, membrane attack complex; DMEM, Dulbecco's modified Eagle's medium; LDH, lactate dehydrogenase.

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Beecham (Welwyn, Herts., U.K.). The NIH 3T3 HIR 3.5 mouse fibroblast cell line was kindly given by Dr. J. Whittaker [20].

NIH-3T3 3.5 HIR cells were propagated in 75 cm^2 tissue-culture flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal-calf serum, 2 mM glutamine, 50 i.u./ml penicillin and 50 μg/ml streptomycin. The cells were cultured in an atmosphere of air/CO_2 (19:1) within a humidified tissue-culture incubator at 37 °C. The culture medium was changed every 3 days, and cells were serially passaged on attaining ~ 80% confluence.

The serum used for a source of complement for these studies was derived from non-immune Dutch rabbits or from a human source (P.N.). The blood was allowed to clot at room temperature for 45 min, and was then centrifuged at 980 *g* for 20 min. Serum was stored at −70 °C for up to 1 month before use.

Antibodies

The anti-(human insulin receptor) mouse monoclonal antibodies 18-44 and 83-14, which were of the IgG2b and IgG2a isotypes [21] respectively, were purified from ascites fluid by $(NH_4)_2SO_4$ precipitation, followed by chromatography on a hydroxyapatite column as described previously [22]. A mouse IgG1 anti-insulin antibody, 14B [21], and a mouse IgG1 monoclonal antibody recognizing an intracellular epitope on the b-chain of the human insulin receptor, CT1 [21], were used as negative controls. Preliminary experiments were performed to determine which were the most cytotoxic anti-insulin-receptor antibodies from the batch initially provided, as well as the most potent serum dilutions to use with these antibodies [23].

Complement cytotoxicity assay

In the standard procedure, NIH-3T3 3.5 HIR cells were seeded into 24-well culture dishes at a cell density of 5×10^4 cells/well, and allowed to reach ~ 80% confluence over 48 h. The cells were rinsed twice with DMEM (without fetal-calf serum), before incubation with 300 μl of 40 nM antibody in DMEM for 30 min at 37 °C. The cells were rinsed again with DMEM to remove unbound antibody, before incubation at 37 °C for 60 min in 300 μl of non-immune rabbit serum, diluted 1:5 in a Hepes buffer (containing 88 mM NaCl, 2 mM KCl, 2.4 mM $NaHCO_3$, 2 mM $MgCl_2$, 2 mM $CaCl_2$ and 15 mM Hepes, pH 7.4). The reaction was rapidly terminated by transferring the plates to an ice chamber. Supernatant (200 μl) was removed from each well, centrifuged at 500 *g* for 10 min to remove cell debris and stored at −70 °C until required. Lactate dehydrogenase (LDH) assays were performed in triplicate to assess cell viability, by the method of Wroblewski and Ladue [24]. For the assessment of spontaneous LDH release, the supernatant obtained from target fibroblasts incubated with control antibodies/rabbit serum was similarly assayed. Total LDH was measured after lysis of parallel wells by 15 s sonication in lysis buffer [containing 50 mM Hepes, pH 7.6, 30 mM sodium pyrophosphate, 10 mM EDTA, 10 mM NaF, 1% (w/v) BSA, 1% (w/v) Triton X-100, 1 mM phenylmethanesulphonyl fluoride, 20 mM leupeptin, 100 mM *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane]. Percentage LDH release (as a measure of cytotoxity) was calculated by the formula:

$$\text{LDH release (\%)} = 100 \times \frac{\text{experimental release} - \text{spontaneous release}}{\text{total LDH content of target cells}}.$$

Correlation of LDH release with Trypan Blue exclusion

After incubation of antibody-coated target cells with complement, both LDH assays and Trypan Blue dye exclusion were performed to determine the correlation of enzyme leakage with residual cell viability. LDH release into the supernatant showed a close positive correlation with Trypan-Blue dye staining (correlation coefficient, $r = 0.97$).

Measurement of ^{45}Ca uptake

3T3 HIR cells were cultured and seeded into 24-well culture dishes as described above. Before addition of $^{45}Ca^{2+}$, cells were incubated with 300 μl of 40 nM antibody 83-14 in DMEM for 30 min at 37 °C. The cells were rinsed again with DMEM to remove unbound antibody, before incubation at 37 °C in 300 μl of human serum (source, P.N.) diluted 1:5 in a Hepes buffer (containing 88 mM NaCl, 2 mM KCl, 2.4 mM $NaHCO_3$, 2 mM $MgCl_2$, 2 mM $CaCl_2$ and 15 mM Hepes, pH 7.4), but with the addition of 6.5 μCi of ^{45}Ca per well. At the end of the incubation period, cells were washed four times with buffer (minus ^{45}Ca) and then lysed by addition of buffer + 1% Triton X-100, followed by sonication. Cellular debris was removed by centrifugation, and the radioactivity in the supernatant was measured with a Packard Tri-Carb 1500 liquid-scintillation counter.

Pathway of complement activation

Antibody 18-44 (300 μl of 10 nM) was added to NIH 3T3 HIR 3.5 cells, and incubated as described above. Thereafter, target cells were further exposed to 300 μl of non-immune rabbit serum diluted in Hepes buffer at 37 °C for 60 min. Parallel samples using rabbit serum preheated at 50 °C for 15 min to inactivate factor B [25] were set up, and incubated as described above. The supernatants were removed for LDH assay at the termination of incubation.

Effects of channel modulators on cell lysis

All drug-containing media used for experimentation were prepared from fresh aqueous stock solutions. Non-immune rabbit or human serum as a source of complement was added to the Hepes buffer described above containing the various agents under test at specified concentrations. Each drug, diluted in buffer, was also tested in the absence of serum over 100 min, to exclude an independent non-immune cytotoxic effect on target cells.

For experiments where inorganic ions were substituted for Ca^{2+}, rabbit serum was diluted as described in Ca^{2+}-free Hepes buffer, Ca^{2+} being replaced by Mn^{2+}, Ni^{2+}, Sr^{2+}, Ba^{2+}, La^{2+}, Co^{2+} Cd^{2+}, Zn^{2+}, Cu^{2+} and Mg^{2+} (as the chloride salts). The final concentration of Ca^{2+} (derived from serum) was approx. 0.3 mM in these experiments.

Presentation of data

Results presented, unless indicated otherwise, are as means ± S.D. of quadruplicate wells, and are representative of at least three separate experiments.

RESULTS

The classical pathway of complement activation elicits Ca^{2+} entry into and LDH release from 3T3 HIR cells

LDH release in antibody-coated fibroblasts was found to be dependent on the activation of the classical pathway of complement (Figure 1). Destruction of Factor B (and thus the

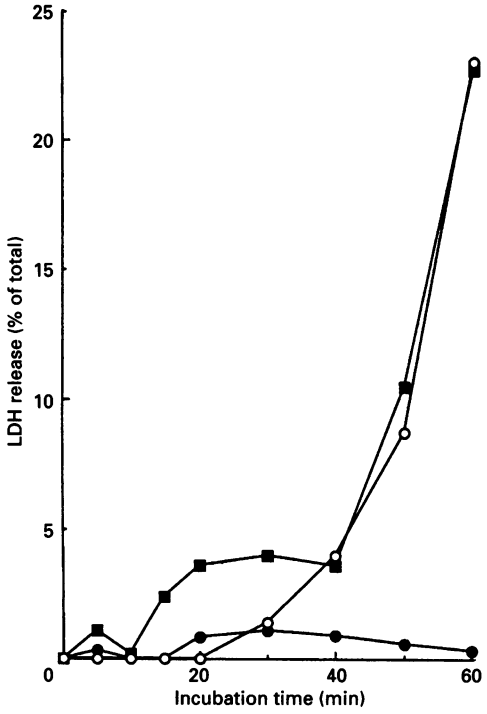

Figure 1 Pathway of complement activation

LDH release from 3T3 HIR cells (as % of total cell LDH) was measured in culture supernatant as described in the Materials and methods section, after exposure to antibody and diluted rabbit serum (■) or rabbit serum preheated at 50 °C for 15 min to inactivate factor B before dilution in Hepes buffer (○) or rabbit serum diluted in Ca^{2+}-chelated Hepes buffer, containing 10 mM EGTA/7 mM Mg^{2+} to allow activation of the alternative pathway while inhibiting the classical pathway (●). The S.D. at each point was less than 3.5% of the mean value.

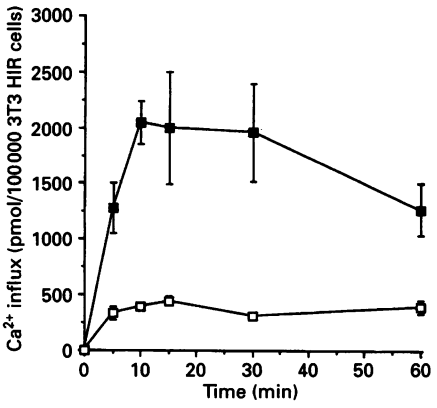

Figure 2 Effect of human serum on $^{45}Ca^{2+}$ uptake

Antibody-coated 3T3 fibroblasts were incubated in the presence of 5-fold-diluted human serum treated at 50 °C for 15 min to inhibit the alternative pathway (■), or at 56 °C for 30 min to inhibit both complement-activation pathways (□). There was no significant difference between the results obtained by using serum treated at 50 °C for 15 min or those obtained by using untreated serum (results not shown). Methods have been described previously.

alternative pathway of activation) by treating the serum at 50 °C for 15 min [25] did not inhibit LDH release at the 40 and 60 min time points. The fact that there was no difference in LDH release at these time points between untreated and 50 °C-heated serum suggests that the classical pathway is solely responsible for lysis.

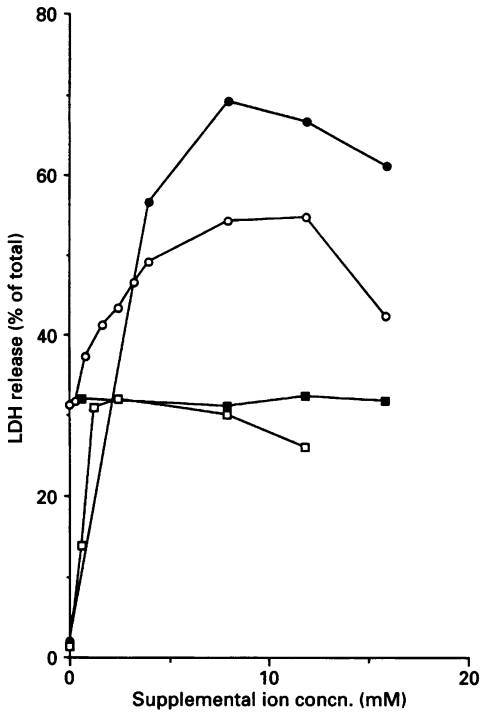

Figure 3 Effects of cation concentration on cytotoxicity

Antibody-coated fibroblasts were incubated in Hepes buffer with final concentrations of either Ca^{2+} (□) or K^+ (■) altered over a total range of 0–17 mM, as described in the Materials and methods section. Ba^{2+} concentrations were similarly altered in both Ca^{2+}-free (●) and normal (○) Hepes buffer over a similar concentration range, and complement-induced cytotoxicity was assessed as described previously, in quadruplicate wells (S.D. of mean at each point less than 3.5%). Osmolarity was maintained in each case by adjusting the NaCl concentration appropriately.

The addition of 10 mM EGTA + 7 mM $MgCl_2$ to the buffer used to dilute the rabbit serum (which allowed alternative-pathway activation while inhibiting the classical pathway) resulted in inhibition of LDH release. The implication of this result is addressed in the Discussion section, as it provides evidence for the essential role of Ca^{2+} in the lytic process. LDH release after 60 min was relatively low in this experiment, compared with those shown in Figures 3 and 5 and Table 1. This finding may have been a consequence of switching from New Zealand White rabbit to Dutch rabbit serum for the latter experiments.

Ca^{2+} entry into 3T3 HIR cells was also stimulated by the classical pathway of complement activation (Figure 2). Complete destruction of heat-labile proteins in both complement-activation pathways by treating serum at 56 °C for 30 min [26] resulted in low fluxes of Ca^{2+} into these cells. Rabbit serum treated at 56 °C for 30 min to inactivate complement [26] totally abolished LDH release from the 3T3 HIR cells over the 60 min incubation period (results not shown).

Effects of bivalent cations on ^{45}Ca entry and LDH release

There was a concentration-dependent increase in LDH release with increasing extracellular Ca^{2+} (Figure 3). A similar cytotoxicity profile was observed with the Ca^{2+} agonist Ba^{2+}, though this had a higher peak than Ca^{2+} alone, which was diminished by addition of 2.0 mM Ca^{2+}. It is known that Ba^{2+} can replace Ca^{2+} as a charge carrier and that it has a greater conductance through L-type calcium channels [27]. Conversely, there was no significant

Table 1 Effect of inorganic cations on cytotoxicity

Experimental details are given in the Materials and methods section. 3T3 HIR cells were incubated over 60 min in 5-fold-diluted rabbit serum diluted in the standard buffer minus Ca^{2+}. The ions listed below were added at a final concentration of 3 mM. The results expressed are normalized to the LDH release occurring in the presence of Ca^{2+} only. This was 38% of total cellular LDH. S.E.M. was less than 10% of the mean value for all results shown ($n = 9$). The final Ca^{2+} concentration in all but the Ca^{2+}-only condition was 0.3 mM.

Inorganic cation	LDH release (%)
Ba^{2+}	176
Sr^{2+}	155
Ca^{2+}	100
La^{3+}	71
Co^{2+}	50
Cd^{2+}	32
Mn^{2+}	31
Ni^{2+}	13
Zn^{2+}	8

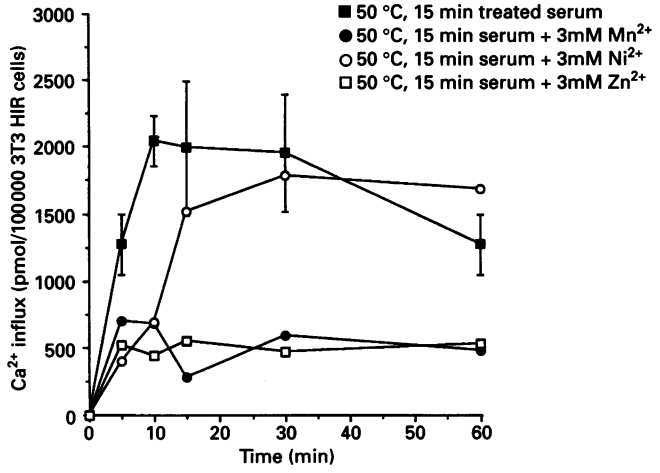

Figure 4 Effect of Mn^{2+}, Ni^{2+} or Zn^{2+} or serum-induced Ca^{2+} influx

Antibody-coated 3T3 fibroblasts were incubated in the presence of 5-fold-diluted human serum treated at 50 °C for 15 min to inhibit the alternative pathway of complement activation (■). Inorganic ions were added at a final concentration of 3 mM, and Ca^{2+} was always present at a final concentration of 2 mM. Methods have been previously described.

change in percentage LDH release on increasing K^+ concentration up to 17 mM (Ca^{2+} was held constant at 2 mM). The result of increasing extracellular K^+ to 17 mM will be to depolarize membrane potential from approx. −100 mV to approx. −55 mV (calculated from the Nernst equation), a substantial decrease but insufficient to activate endogenous voltage-gated Ca^{2+} channels. There was no enhancement in LDH release on depolarizing the resting membrane potential via altering the extracellular K^+ concentration, suggesting the cytolytic properties of MAC in the presence of 2 mM Ca^{2+} are not altered by membrane potential over this range.

The potency of polyvalent cations in stimulating LDH release were in the order: $Ba^{2+} > Sr^{2+} > Ca^{2+} > La^{3+} > Co^{2+} > Cd^{2+} > Mn^2 > Ni^{2+} > Zn^{2+} > Cu^{2+}$, as shown in Table 1. These data indicate the cytolytic properties of the above ions, which may result from ion and accompanying water entry into the cell via complement-dependent channel formation and subsequent colloid osmotic lysis. Ion entry is unlikely to be a result of flux via endogenous Ca^{2+} channels, as the potent Ca^{2+}-channel blocker La^{3+} can stimulate LDH release to 70% of that stimulated by Ca^{2+}.

The high concentrations of bivalent cations required for stimulation or inhibition of complement-dependent cytotoxicity argues against these profiles occurring as a result of altered $C1r_2C1s_2$ structure (Ca^{2+} binding, which occurs at low Ca^{2+} concentrations, is required for this tetrameric complex formation [28]). It is a possibility that other bivalent cations may compete for Ca^{2+}-binding sites in this structure, thus inhibiting Ca^{2+} binding and hence classical-pathway activation, but this argument must then be applied to all the bivalent cations studied. The results described in Table 1 demonstrate that Ba^{2+} and Sr^{2+} actually enhance cell lysis. The classical pathway of complement activation does not require either of these cations for its activity. Thus cell lysis via colloid osmotic lysis is the likely explanation for the effects of these ions on cytotoxicity. Additionally, experiments performed with *Xenopus* oocytes have shown that ionic currents elicited by complement can be inhibited by addition of Zn^{2+} after development of the inward current, ruling out Zn^{2+} inhibition of the early activation of the classical pathway (P. Newsholme, unpublished work).

The failure to elicit LDH release by Mn^{2+} or Zn^{2+} may be a consequence of blocking the complement-dependent channel or failure to elicit osmotic lysis if cell membrane penetration had been achieved. The inhibition of Ca^{2+} entry into 3T3 HIR cells by the addition of 3 mM Mn^{2+} or 3 mM Zn^{2+}, measured by ^{45}Ca uptake, is shown in Figure 4. The results indicate that Mn^{2+} or Zn^{2+} can block the complement-dependent channel, thus inhibiting complement-elicited Ca^{2+} entry. At a concentration of 3 mM, Ni^{2+} only transiently blocked Ca^{2+} entry; Ca^{2+} influx approached control values at 30 min after serum addition. The extracellular Ca^{2+} concentration was maintained at 2 mM in these latter experiments.

Effect of channel modulators on ^{45}Ca entry and LDH release

The effects of organic channel antagonists on LDH release are illustrated in Figure 5. The voltage-dependent L-type Ca^{2+} channel antagonist nifedipine [29] exhibited a concentration-dependent inhibition of LDH release at micromolar concentrations. It was not possible to use concentrations above 40 μM, owing to insolubility of the drug at high concentrations. Although trifluoperazine (which blocks Ca^{2+} entry and inhibits Ca^{2+}/calmodulin-dependent processes) [29] was able to inhibit LDH release at 100 μM, the L-type Ca^{2+}-channel antagonist verapamil [29] and the T-type Ca^{2+} channel antagonist phenytoin [27] actually enhanced LDH release at both 100 and 500 μM concentrations. The third class of L-type Ca^{2+}-channel antagonist, the benzothiazepine diltiazem [29] was not used in the present study, since, after 15 min incubation in the presence of this drug, cells no longer remained attached to the tissue-culture dish. However, up to 15 min Ca^{2+} influx was not different from control values (results not shown).

The effect of nifedipine and verapamil on complement-stimulated ^{45}Ca influx is shown in Figure 6. There is a distinct correlation between the concentration of nifedipine needed to decrease Ca^{2+} influx (40 μM) and that required to decrease LDH release by 42%. Also, at 100 μM, verapamil not only enhances Ca^{2+} influx but also enhances LDH release, although the enhancement seen in either case is not statistically significant.

Effect of C9 depletion on ability of serum to stimulate ^{45}Ca influx

Human serum was used to provide complement in the ^{45}Ca-influx experiments, as C9-depleted human serum and purified

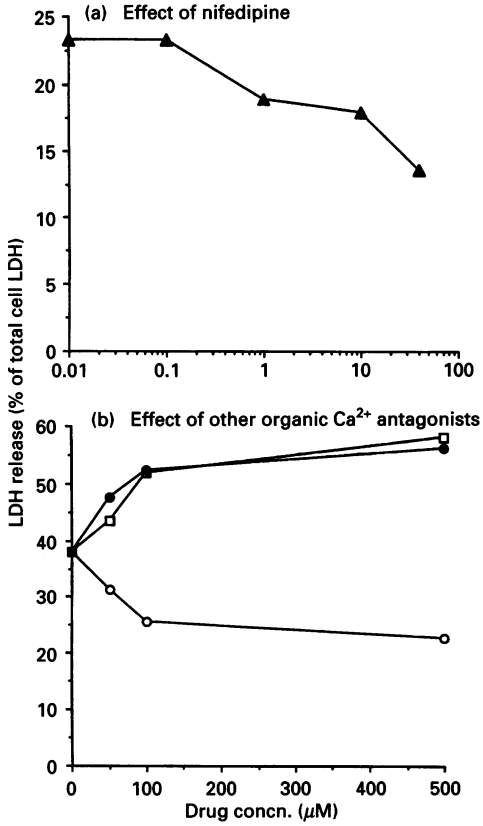

Figure 5 Dose–response of organic Ca^{2+} antagonists on cytotoxicity

Antibody-coated fibroblasts were incubated in a 1:5 dilution of rabbit or human serum in Hepes buffer, supplemented with different concentrations of the test agents: verapamil (□), trifluoperazine (○), phenytoin (●) and nifedipine (▲). The cells were incubated for 60 min at 37 °C, and assessed for complement-dependent LDH release (S.D. of mean at each point is less than 3.5%). The nifedipine experiment was performed with human serum, and the other drug experiments were with rabbit serum.

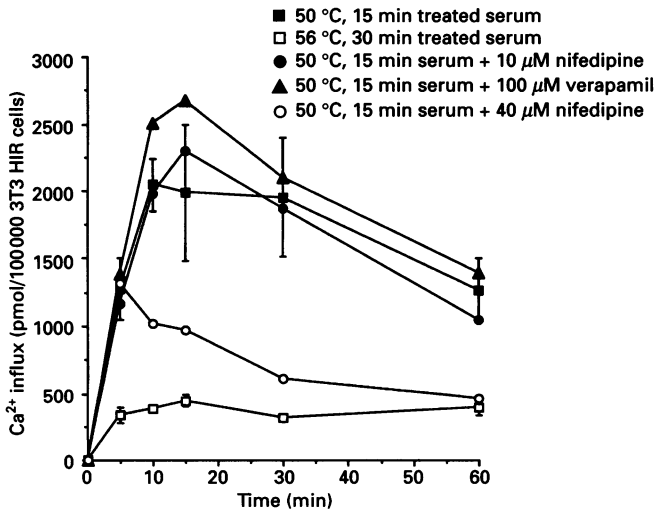

Figure 6 Effect of nifedipine or verapamil on serum-induced Ca^{2+} influx

Antibody-coated 3T3 fibroblasts were incubated in the presence of 5-fold-diluted human serum treated at 50 °C for 15 min to inhibit the alternative pathway (■), or at 56 °C for 30 min to inhibit both complement-activation pathways (□). Nifedipine was used at 10 or 40 μM and verapamil at 100 μM, concentrations known to inhibit endogenous Ca^{2+} channels.

Table 2 Effect of C9 depletion on serum-stimulated $^{45}Ca^{2+}$ uptake

The incubation period for cells exposed to either whole serum or modified serum was 10 min. Results are normalized to $^{45}Ca^{2+}$ uptake after 10 min exposure to whole serum. This value was 2.045 nmol of Ca^{2+} per 100000 3T3 HIR cells. S.E.M. was less than 10% of the mean for all results shown ($n = 9$). Heating the serum for 50 °C for 15 min will inactivate factor B and therefore the alternative pathway [25], whereas treating the serum at 56 °C for 30 min will inactivate both classical and alternative pathways by denaturation of heat-labile proteins [26].

Treatment of serum (human)	Normalized $^{45}Ca^{2+}$ uptake
Untreated serum	100
Serum treated at 50 °C, 15 min	99
Serum treated at 56 °C, 30 min	19
Serum − C9	40
Serum − C9, + C9 (40 μg/ml)	99

human C9 were available for use (a gift from Dr. J. P. Luzio [30]). The relative potency of rabbit versus human serum on LDH release is 1.4 in favour of rabbit serum. The results of an experiment performed after a 10 min incubation (the point at which Ca^{2+} influx is at a maximum) in the presence of C9-depleted serum and depleted serum to which C9 had been replaced at 40 μg/ml are shown in Table 2. For C9-depleted serum, it is shown that Ca^{2+} influx can be decreased to 40% of that stimulated by normal serum. This can be compared with 19% when complement is destroyed by treating serum at 56 °C for 30 min. The discrepancy arises as C5b–C8 complexes have been shown to stimulate Ca^{2+} influx, but at a much lower rate than C5b–C9 complexes [31].

DISCUSSION

The role of Ca^{2+} in the lytic process caused by complement activation, insertion of MAC into target cell membranes and subsequent ion flux across the membrane remains controversial. Ca^{2+} is absolutely required for activation of the classical pathway [28], and thus formation of MAC, but the question remains unresolved as to whether Ca^{2+} is required for the lytic process to proceed. Evidence has previously been presented that the early complement-generated ion flux is Ca^{2+}-specific [8–10], though this contradicts a widely accepted view of a general ion influx followed by colloid osmotic lysis [32,33]. Morgan has presented data to show that Ca^{2+} influx is essential for nucleated-cell recovery from complement attack [34]. Increased cytosolic Ca^{2+}, required for cell recovery, is only transiently increased [10,11], reaching a peak after 1 min for neutrophils and 2 min for oligodendrocytes, while Ca^{2+} returns towards basal levels approx. 5 min from the initial stimulation. The present study has used ^{45}Ca as a marker to measure the influx of Ca^{2+} into the fibroblast cell line 3T3 HIR. The measurement of ^{45}Ca uptake unfortunately gives no indication of free cytosolic Ca^{2+} concentration, as a proportion of cellular Ca^{2+} will reside in intracellular stores such as mitochondria and the endoplasmic reticulum. However, the data presented in Figure 2 suggest that the Ca^{2+} concentration within the cell rises steadily after initial complement attack and reaches a peak after 10 min. It is extremely unlikely that all the Ca^{2+} entering the cell will all be immediately sequestered within intracellular stores, especially as Ca^{2+} has been shown to be released from intracellular stores during complement attack [10,11].

LDH release from the 3T3 HIR cell is indicative of breakdown of membrane integrity, to such an extent that cytoplasmic enzymes can leak out of the cell. LDH release from the 3T3 HIR

Table 3 Effect of nifedipine or verapamil on K^+-stimulated $^{45}Ca^{2+}$ influx

Results are normalized to the $^{45}Ca^{2+}$ uptake stimulated after a 10 min incubation in the presence of 30 mM extracellular K^+. This was 37.7% of that stimulated by 5-fold-diluted human serum. S.E.M. was less than 10% of the mean value for all results shown ($n = 9$). N.D., not determined.

Concn. of drug (μM)	Nifedipine	Verapamil
0 (30 mM K^+)	100	100
0.1 (30 mM K^+)	107.0	N.D.
1.0 (30 mM K^+)	36.4	61.2
10.0 (30 mM K^+)	25.0	41.6
100.0 (30 mM K^+)	N.D.	23.0
0 (60 mM K^+)	125	125

cell reaches a peak approx. 100 min after addition of serum, approx. 30% greater than LDH release at 60 min [23].

The release of [^{14}C]sucrose from, or uptake of propidium iodide into, cells undergoing complement-stimulated cytolysis has previously been documented [35,11]. Ca^{2+} uptake from extracellular sources preceded membrane permeability to large molecules by approx. 15 min in both the cited studies. For lysis of erythrocyte ghosts [35], cell recovery mechanisms have never been documented, whereas lysis of oligodendrocytes [11] was stimulated by a concentration of serum 3-fold that required to cause the Ca^{2+} transient and cell recovery via MAC removal from the membrane. Thus the higher concentration of serum required to stimulate lysis of oligodendrocytes may well have led to greater amounts of MAC being inserted into the cell membrane and possibly a longer intracellular Ca^{2+} transient.

The present study provides evidence that Ca^{2+} is essential to the lytic process initially stimulated by complement. In Ca^{2+}-free conditions, where alternative-pathway activation can occur but the classical pathway is inhibited (EGTA present), and thus MAC can still be generated, LDH release was fully inhibited (Figure 1). Evidence that ion fluxes were generated in the absence of Ca^{2+} (equivalent conditions to those described for Figure 1) via activation of the alternative pathway was obtained from experiments that were designed to detect alternative-pathway-dependent ion fluxes in voltage-clamped *Xenopus* oocytes using electrophysiological methodology (P. Newsholme, unpublished work).

Nifedipine had an inhibitory effect on complement-dependent cytotoxicity at a concentration of 10 μM (Figure 5), a concentration which does not effect complement-elicited Ca^{2+} influx (Figure 6). At high concentrations, nifedipine may sufficiently stabilize the membrane via a non-specific mechanism to allow cell recovery mechanisms to operate more effectively. The decrease in Ca^{2+} influx at 40 μM nifedipine (Figure 6) may further aid cell recovery, as lower intracellular Ca^{2+} concentrations are less likely to stimulate cytotoxic mechanisms and are more likely to stimulate recovery mechanisms [34].

The inhibitory effects of nifedipine on complement-elicited Ca^{2+} influx, it may be argued, could be due to direct inhibition of endogenous Ca^{2+} channels which may be opened in response to plasma-membrane depolarization due to MAC-stimulated ion fluxes. However, in an experiment where 3T3 HIR cells were artificially depolarized by addition of 30 mM extracellular K^+, the ^{45}Ca influx thus stimulated was inhibited substantially by 10 μM nifedipine (Table 3). Data also presented in this paper (Figure 6) shows that, at 10 μM nifedipine, complement-stimulated ^{45}Ca influx is not inhibited. Also 100 μM verapamil substantially inhibited K^+-stimulated ^{45}Ca influx (Table 3), but did not inhibit complement-stimulated ^{45}Ca influx (Figure 6). ^{45}Ca influx stimulated by 60 mM extracellular K^+ was only 47% of that stimulated by complement (Table 3). The concentration of nifedipine required to decrease cytotoxicity by approx. 50% and also to decrease Ca^{2+} influx in these studies was 40 μM (Figure 5). This elevated concentration is above the generally accepted value for inhibition of voltage-sensitive calcium channels (10–100 nM), and other membrane activities may be altered at this concentration. However, the cell line used in these studies also had an elevated IC_{50} for the inhibition of endogenous Ca^{2+} channels (0.6 μM; Table 3). With *Xenopus* oocytes as an alternative cell for experimentation, nifedipine had an IC_{50} for inhibition of endogenous calcium channels of 0.1 μM. The IC_{50} was also determined for complement-elicited Ca^{2+} influx in the oocyte and was determined as 1.0 μM (P. Newsholme, unpublished work). Complement-elicited Ca^{2+} influx in the oocyte was not altered by the presence of 100 μM diltiazem, a concentration that was shown to inhibit endogenous Ca^{2+} channels fully (results not shown). Thus the evidence presented in this paper is strongly suggestive that Ca^{2+} influx due to complement occurs via a channel/pore that is independent of endogenous Ca^{2+} channels, but that shares a common property, its sensitivity to nifedipine inhibition.

The profile of ion-stimulated LDH release ($Ba^{2+} > Sr^{2+} > Ca^{2+} > La^{3+} > Co^{2+} > Cd^{2+} > Mn^{2+} > Ni^{2+} > Zn^{2+} > Cu^{2+}$), as documented in Table 1, is consistent with the entry of Ca^{2+} or other ions through a specific membrane channel/pore, rather than non-selective permeabilization (leaky patches) of the plasma membrane. As there is a low extracellular Ca^{2+}-concentration in this experiment (0.3 mM), cytoxicity may occur via colloid osmotic lysis caused by movement of Ba^{2+} or Sr^{2+} into the cell accompanied by water, eventually leading to cell bursting, or inhibition of cytotoxicity may occur due to block of the channel/pore, thus avoiding osmotic changes. Other work has shown that complement-induced membrane damage may be blocked by some bivalent cations [36]. Ca^{2+} is less likely to be directly involved in colloid osmotic mechanisms, as it will be buffered by intracellular stores and will also be pumped out of the cell by specific plasma-membrane Ca^{2+} pumps [37], and thus may not reach the likely intracellular concentrations achieved by Ba^{2+} and Sr^{2+}.

That the complement-induced ^{45}Ca influx is almost fully inhibited by Mn^{2+} and Zn^{2+} and partially by Ni^{2+} (Figure 4) indicates that the MAC channel/pore is discriminating between ions of similar ionic radii, with respect to Ca^{2+}. Ni^{2+}, however, only inhibited Ca^{2+} entry for 10 min (Figure 4), perhaps indicating a change in pore structure after this time period. This hypothesis would support that of Stanley et al. [7], in which they argue that C9 is inserted in a two-step manner, the first associated with Ca^{2+} influx and the second leading to C9 polymerization. There is evidence that voltage-gated Ca^{2+} channels are potently blocked by Co^{2+} and Ni^{2+} [38], thus providing further evidence that MAC has characteristics that are not typical of endogenous Ca^{2+} channels, as data in this paper indicate that Ni^{2+} does not inhibit complement-induced Ca^{2+} influx, after an initial lag.

In conclusion, it appears that the lytic effects of MAC which we have observed are dependent on the presence of novel, initially Ca^{2+}-selective, channels on the cell surface, leading to an influx of Ca^{2+} into the cell. These channels show little, if any, similarities to either T- or L-type calcium channels. The precipitous rise in intracellular Ca^{2+} sets in motion events leading to cell death, while over-riding protection mechanisms. The results are consistent with the hypothesis of a threshold for cell death proposed by Campbell [13]. The paradoxical role of Ca^{2+}-stimulating recovery mechanisms and cell lytic processes has been previously discussed [31]. That paper provides evidence

that Ca^{2+} is essential for complement-dependent cytotoxicity (alternative-pathway activation in the presence of EGTA did not lead to cell lysis). If physiological concentrations of extracellular Ca^{2+} and Mg^{2+} are maintained, and if the initial Ca^{2+} influx is inhibited by blocking agents, cytolysis is also inhibited. These results raise the possibility that drugs may be produced which specifically block the Ca^{2+}-influx component of complement attack, with potential benefit for treatment of complement-dependent diseases, e.g. multiple sclerosis, myasthenia gravis and membranous glomerulonephritis [34,39].

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