Biochemical Pharmacology, Vol. 57, pp. 491–501, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(98)00311-6

Identification of a Novel Complement-Dependent Serum-Elicited Inward Current in the *Xenopus* Oocyte Provoking Ca^{2+} Influx and Subsequent Activation of Cl^- Channels

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ABSTRACT. The membrane spanning complement channel is assumed to be a nonselective ion 'pore', although little evidence is available to support this hypothesis. In this paper we provide evidence that Ca^{2+} entry and Cl^- exit occur rapidly after complement activation and precede the development of a long-lasting complement-dependent inward current. Addition of rabbit serum (a source of heterologous complement) and mouse anti-human insulin receptor antibody to a single *Xenopus* oocyte expressing human insulin receptor was shown to stimulate an initial hyperpolarising current followed by a sustained depolarising current. On voltage clamping the oocyte, a novel long-lasting inward current generated by serum addition was detected. Complement classical pathway-stimulated calcium influx into the oocyte was directly demonstrated using ^{45}Ca influx measurements. In addition, we found that Ca^{2+} influx was required for the stimulation of the complement alternative pathway-dependent inward current. The novel conductance elicited by the classical pathway was outwardly rectifying, had a reversal potential of -35 ± 8 mV (or -52 ± 7 mV in the presence of chloride channel inhibitors), was inhibited by nifedipine, and was observed in the presence but not in the absence of the pore-forming complement component C9. As overactivation of complement does play a role in many inflammatory or autoimmune diseases, inhibition of early complement-mediated ion flux might restrict tissue damage and aid recovery from such diseases. BIOCHEM PHARMACOL **57**;5:491–501, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. complement; Cl^- channels; Ca^{2+} channels; Ca^{2+} channel antagonists; oocytes

The complement system is composed of more than 20 serum proteins that interact in a precise series of enzymatic cleavage and membrane binding events leading to the generation of products with immunoprotective, immunoregulatory, and proinflammatory properties [1]. Complement can be activated through either of two distinct enzymatic cascades, referred to as the classical and alternative pathways. The classical pathway is generally initiated by the interaction of Clq with antibody/antigen complexes, whereas the alternative pathway is initiated by deposition of C3b on a variety of substrates, including bacterial lipopolysaccharide and cell membranes, which is necessary for the amplification and progression of the complement cascade. C3b is the primary opsonin for many pathogenic microorganisms and, in addition, promotes the clearance as well as solubilisation of immune complexes.

Both the classical and alternative pathways converge at C5, which is cleaved to form a range of fluid-phase and membrane-phase products with multiple proinflammatory effects. C5a is a powerful 'chemotaxin' and activator of both neutrophils and monocytes. C5 cleavage also leads to the formation of C5b-9, or the MAC‖. The mechanism by which complement damages its cellular target *in vivo* is unknown. There is now good evidence that early ion flux generated by the MAC is calcium-specific [2–4], although this contradicts a widely accepted view of a general ion influx followed by colloid osmotic lysis [5, 6]. An intracellular rise in calcium is thought to be responsible for initiating mechanisms leading to cell death [7–11]. However, calcium is also responsible for activating recovery mechanisms [12] in turn responsible for cellular survival following complement attack. This paradoxical role of calcium has been discussed previously [13]. The complement MAC binds to the surfaces of target cells and has been suggested to effect membrane perturbations and even

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Received 25 June 1997; accepted 2 August 1998.

‖ *Abbreviations:* MAC, membrane attack complex; DIDS, 4, 4′-diisothiocyanostilbene-2,2′-disulfonic acid; TEA, tetraethylammonium chloride; and HIR, human insulin receptor.

cytolysis by forming transmembrane pores [14, 15]. 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 [16, 17]. It is still widely believed that MAC is a rigid, hollow structure, like a doughnut [14], that produces a general ion influx followed by colloid osmotic lysis. However, the latter events may only occur in complement-mediated bacterial or erythrocyte lysis. Simple osmotic lysis may not be involved in complement-mediated destruction of nucleated cells which have complex defense mechanisms against complement, such as CD59, a membrane-associated molecule which inhibits MAC assembly. Esser has previously suggested that MAC may precipitate cytolysis by forming a 'leaky patch' in the vicinity of the MAC [18], but has also published data showing the formation of single channels by MAC in artificial bilayer membranes [19], data which support other work showing single channel formation by MAC [20, 21].

Free intracellular calcium ions act as intracellular regulators of cell function [22], 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 via mitochondrial dysfunction and subsequent block of oxidative phosphorylation [23]. Depolarisation of the cell membrane would follow, eventually leading to larger permeability changes, osmotic lysis, and cell death.

Little or no work has previously been done on cellular channel activation immediately subsequent to complement attack. The present work uses the *Xenopus* oocyte to address this question, as it can be easily impaled by microelectrodes required for voltage clamping and the cell can be used to express membrane-bound protein to which an antibody is available, thus providing a mechanism to activate the classical pathway of complement. We have therefore investigated the current generated by either the classical or the alternative pathway of complement, the latter occurring spontaneously after exposure of the *Xenopus* oocyte membrane to heterologous serum.

MATERIALS AND METHODS

Preparation of Oocytes for Experimental Use

Clusters of oocytes were surgically removed from one ovary of an adult female *Xenopus laevis* under tricaine anaesthesia. Frogs were purchased from Xenopus Ltd. Oocytes were collagenase-treated (Sigma, Type 1, 1–2 mg/mL) in groups of approximately 20 follicles in calcium-free Barth's solution (NaCl, 88 mM; KCl, 1 mM; $NaHCO_3$, 2.4 mm; $MgSO_4$, 0.82 mM; HEPES 15 mM, pH to 7.4; penicillin, 50I U/mL, and streptomycin at 50 μg/mL). Oocytes were incubated at room temperature for 2–3 hours and then gently shaken on a Vortex microgenie for 15 min. In some cases, outer epithelia were not shed and were removed later with watchmaker's forceps. This treatment removed epithelial/thecal tissues and a large number of surrounding follicular cells. Treated cells were repeatedly washed and returned to sterile Barth's medium containing $Ca(NO_3)_2$, 0.33 mM and $CaCl_2$, 0.41 mM, and were kept at 18°.

Injection of mRNA

Oocytes were injected with 50 ng of mRNA coding for the HIR or 50 ng of HIR antisense mRNA. The mRNA was prepared from an HIR cDNA clone (obtained from J. Whittaker, University of Chicago) using a transcription and capping kit supplied by Stratagene. Oocytes were then incubated for 48–72 hr in Barth's medium plus 1 mM pyruvate at 18° in a plastic Petri dish treated with Sigmacote (Sigma). Twenty-four hours before electrophysiological studies, 40 nM of monoclonal mouse anti-human insulin receptor antibody, 83-14 [24], was added to the medium surrounding the oocytes.

Electrophysiology

Oocytes were impaled with two glass microelectrodes filled with 3 M KCl (0.5–2.0 MΩ resistance) and were voltage-clamped using an Axoclamp-2A amplifier (Axon Instruments). Oocytes were only used for experimentation if they had good resting membrane potentials (−40 to −70 mV). They were routinely voltage-clamped at −50 mV unless otherwise specified. Current/voltage profiles were established by manually changing the voltage in 10 mV or 20 mV steps from −120 to +40 mV, and recording the current for 1 min at each step. The control (-serum) profile was always subtracted from the serum-stimulated profile for each experimental condition shown. A Gould RS 3200 chart recorder was used to record current and voltage details. The bathing medium consisted of: NaCl, 98 mM; KCl, 2 mM; $NaHCO_3$, 2.4 mm; HEPES, 15 mM, pH 7.4; $MgCl_2$, 2 mM, and $CaCl_2$, 2 mM. Rabbit or human serum was diluted 5-fold into this bathing solution after a steady current recording was established. Rabbit or human serum was sometimes preheated at 50° for 15 min to inactivate factor B, therefore inhibiting alternative pathway activation [25], or 56° for 30 min to totally inactivate complement by denaturing heat labile proteins of both the classical and alternative pathways [1]. Rabbit serum was used for all experiments except C9 depletion and replacement experiments, where human serum was used, as it was possible to obtain both C9-depleted human serum and purified human C9 as a gift from Dr. J. P. Luzio (Dept. of Clinical Biochemistry, University of Cambridge, prepared as described by Morgan *et al.* [26]). Rabbit serum was prepared as described by Cooper [1]. All measurements were made at 22–25°.

^{45}Ca Uptake Assays

Oocytes were injected and incubated as described above. Five oocytes were then incubated per well in a 24-well plate. They were incubated in 500 μL of the same medium as described for the electrophysiological experiments, but

with the addition of 6.5 μCi of ^{45}Ca per well. Rabbit serum (diluted 5-fold) was then added to initiate complement activation. At the end of the incubation period, cells were washed 4 times with calcium-containing 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 using a Packard Tri-Carb 1500 liquid scintillation counter. Drugs, where used, were added 10 min before the addition of serum. All incubations were performed at 22–25°.

Measurement of Monoclonal Antibody Binding to Oocytes Expressing HIR

The anti-human insulin receptor mouse monoclonal antibody 83-14 was of the IgG2a isotype [24] and was a gift from Dr. M. A. Soos. It was iodinated (using 5 μL Na of ^{125}I, 100 mCi/mL) by the iodogen method, for binding studies [27]. Oocytes were injected with insulin receptor mRNA as described above. For binding studies, they were then transferred, 10 per well, into wells of a 24-well plate and were incubated in 500 μL of buffer (NaCl, 120 mm; KCl, 4.8 mM; $MgS0_4$, 1.2 mM; Na_2HP0_4, 8.4 mM; phenylmethylsulfenyl fluoride, 100 μM; BSA, 1%, pH, 7.4) containing 10 nM of [^{125}I] 83-14 antibody (0.5×10^6 cpm/pmol antibody) and various concentrations of unlabelled antibody for 16 hr at 4°. Oocytes were then washed 5 times in buffer without antibody, and were then layered on top of 5 mL of 20% sucrose in a 15 mL tube. After the oocytes had sunk to the bottom of the tube, the tube was frozen in liquid N_2 and the bottom of the tube (containing the oocytes) was removed using a red-hot scalpel. The radioactivity associated with the oocytes was determined using a Nuclear Enterprises NE 1600 counter.

Chemicals and Drugs

Chemicals and antibiotics were obtained from Sigma Chemical Co. or BDH Chemicals. ^{45}Ca (5–50 mCi/mg calcium) was obtained from Amersham International. DIDS, niflumic acid, and TEA were purchased from Sigma Chemical Co.

Statistical Analysis

All results are expressed as means ± SD except where indicated. Statistical comparison was carried out using the students *t*-test.

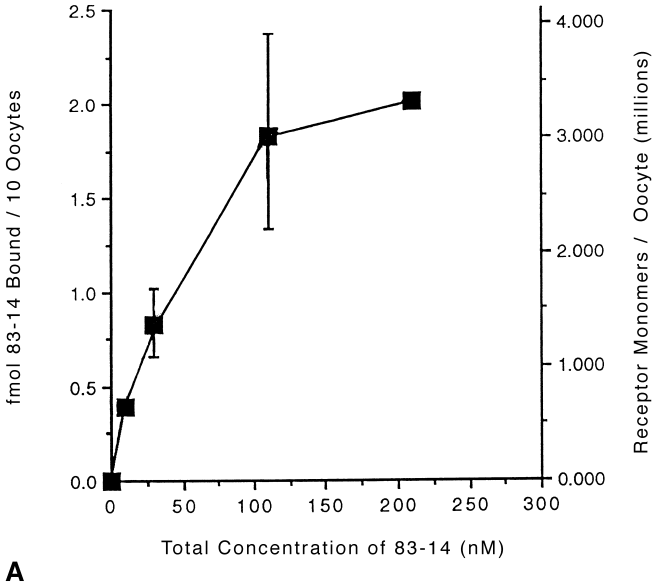
RESULTS

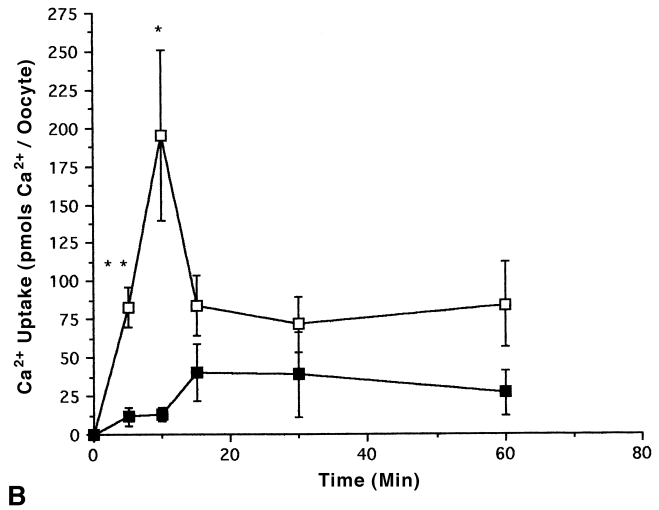
Binding of Mouse Anti-Human Insulin Receptor Antibody to Oocytes Expressing HIR

Oocytes previously injected with mRNA coding for the HIR were incubated in buffered medium (as described in the Methods section) which contained a fixed concentration (10 nM) of [^{125}I] 83-14 monoclonal antibody (specific activity of 5×10^5 cpm/pmol antibody) and various concentrations of unlabelled antibody. A binding curve was constructed after determining the amount of bound antibody at each concentration of total antibody in the medium (assuming that one antibody molecule will bind per monomer of insulin receptor). The curve indicated that the antibody achieved 50% saturation of the expressed receptors at approximately 40 nM and that just over 3×10^6 receptor monomers were expressed per cell (Fig. 1a).

Complement-stimulated ^{45}Ca Uptake into the Oocyte

Rabbit serum addition to oocytes expressing HIR (and previously exposed to anti-insulin receptor antibody) caused a rapid influx of calcium into the cell (Fig. 1b). This effect was substantially reduced by inactivating complement by treating the serum at 56° for 30 min [1]. There was no difference between the calcium uptake stimulated by serum treated at 50° for 15 min (to inactivate Factor B and thus the alternative pathway; [25]) and untreated serum (results not shown), thus implicating the classical pathway of complement activation as an important component of the mechanisms responsible for calcium influx. The difference in calcium influx stimulated by the complement classical pathway and complement-inactivated (56°-treated) serum is statistically significant 5 min after serum addition ($P < 0.01$) and significant 10 min after serum addition ($P < 0.05$). The fall in $^{45}Ca^{2+}$ measured in the oocyte after the peak at 10 min may be due to activation of specific plasma membrane Ca^{2+} pumps [28]. The contribution of endogenous voltage-gated calcium channels to the total calcium influx compared to influx via alternative mechanisms was investigated by altering extracellular potassium concentration to artificially depolarise the cell membrane. An extracellular K^+ concentration of 30 mM would give a membrane potential of approximately −30 mV based on an internal K^+ concentration of 92.5 mM in the oocyte [29]. This degree of depolarisation will activate endogenous voltage-dependent Ca^{2+} channels in oocytes. Thus, the observed complement-induced Ca^{2+} influx may have occurred via secondary activation of endogenous voltage-sensitive Ca^{2+} channels. To determine the relative magnitude of Ca^{2+} influx stimulated via serum-stimulated channels or endogenous Ca^{2+} channels only, we compared the effects of complement-induced depolarisation with that of K^+-induced depolarisation on Ca^{2+} uptake. Complement classical pathway-stimulated $^{45}Ca^{2+}$ uptake was not significantly different from 30 mM K^+-stimulated uptake measured 5 min from the stimulus addition (83.4 ± 22.7 pmol Ca^{2+}/oocyte and 47.7 ± 5.1 pmol Ca^{2+}/oocyte respectively; 3 separate experiments were performed with at least 10 oocytes used in each experiment). Thus, the magnitude of Ca^{2+} influx was similar whether stimulated by serum or by direct depolarisation. To obtain further information on whether endogenous Ca^{2+} channels or other channels may be responsible for the observed serum-stimulated Ca^{2+} influx, the sensitivity of the latter to Ca^{2+} channel antagonists was investigated. The inhibition of 30





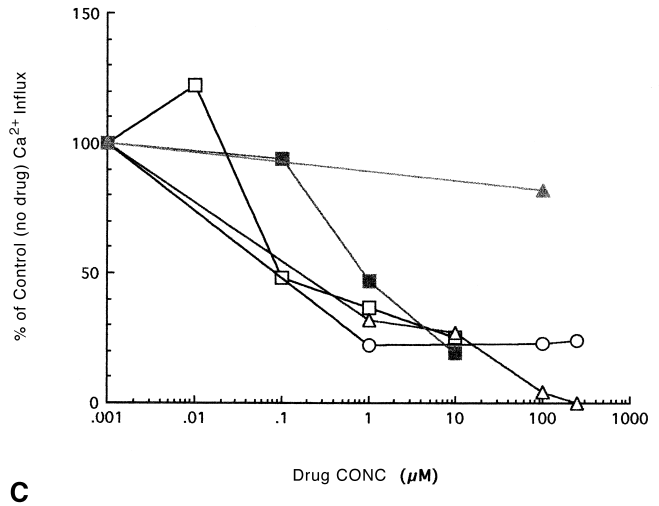


FIG. 1. Measurement of anti-insulin receptor antibody binding to oocytes expressing HIR and serum or KCl-stimulated Ca^{2+} influx into oocytes. (a) Measurement of anti-HIR 83-14 antibody binding to oocytes expressing HIR. Oocytes were injected with insulin receptor mRNA as previously described and were incubated (10/well) in the presence of 10nM [^{125}I] 83-14 antibody and various concentrations of unlabelled antibody for 16 hr at 4°. The radioactivity associated with the oocytes was measured as described in the Methods section, and the concentration of bound antibody was subsequently determined and plotted against total antibody concentration. The error bars represent the SEM values from at least 3 separate experiments. (b) Effect of serum on $^{45}Ca^{2+}$ uptake by the *Xenopus* oocyte. Anti-HIR antibody-coated oocytes (5 per well) were incubated in the presence of 5-fold diluted rabbit serum treated at 50° for 15 min to inhibit the alternative pathway (□) or at 56° for 30 min to inactivate both complement pathways (■). There was no significant difference between the results obtained by using serum treated at 50° for 15 min and those obtained by using untreated serum (data not shown). Methods have been previously described. The error bars represent the SEM values from triplicate determinations from at least 3 separate experiments. P values, where indicated, refer to statistical difference between classical complement pathway-induced Ca^{2+} influx and complement-independent Ca^{2+} influx, $^{**}P < 0.01$, $^{*}P < 0.05$. (c) $^{45}Ca^{2+}$ influx stimulated by 30mM K^+ or serum. Inhibition by Ca^{2+} channel antagonists. The $^{45}Ca^{2+}$ content of oocytes was measured as previously described (see Methods section) after a 5-min incubation with 30mM K^+. The extracellular Ca^{2+} concentration was 2mM. The effect of Ca^{2+} channel antagonists on inhibition of K^+-stimulated Ca^{2+} influx is shown. The L-type Ca^{2+} channel antagonists nifedipine (□), verapamil (○), or diltiazem (△) were used at the stated concentrations. Error bars are not shown as they were <10% of mean. Some oocytes were injected with 50 ng sense or antisense mRNA coding for the HIR as previously described in the Methods section. The oocytes were exposed to 40 nM anti-human insulin receptor antibody 24 hr before $^{45}Ca^{2+}$ measurements. Serum (a source of complement) was added to the bathing medium, constituting 20% of the total volume. The effect of nifedipine on serum-stimulated Ca^{2+} influx (■) or diltiazem on serum-stimulated Ca^{2+} influx (▲) was measured after 5 min. Error bars are not shown as they were <10% of mean.

mM K^+-stimulated $^{45}Ca^{2+}$ influx by nifedipine, verapamil, and diltiazem is shown in Fig. 1c. The concentrations of these drugs that resulted in maximal inhibition of Ca^{2+} uptake via endogenous voltage-sensitive Ca^{2+} channels were 10 μM nifedipine (reduced 30 mM K^+ stimulated $^{45}Ca^{2+}$ influx to 25% of control uptake), 100 μM verapamil (reduced influx to 23% of control uptake) and 100 μM diltiazem (reduced influx to 4% of control uptake). An

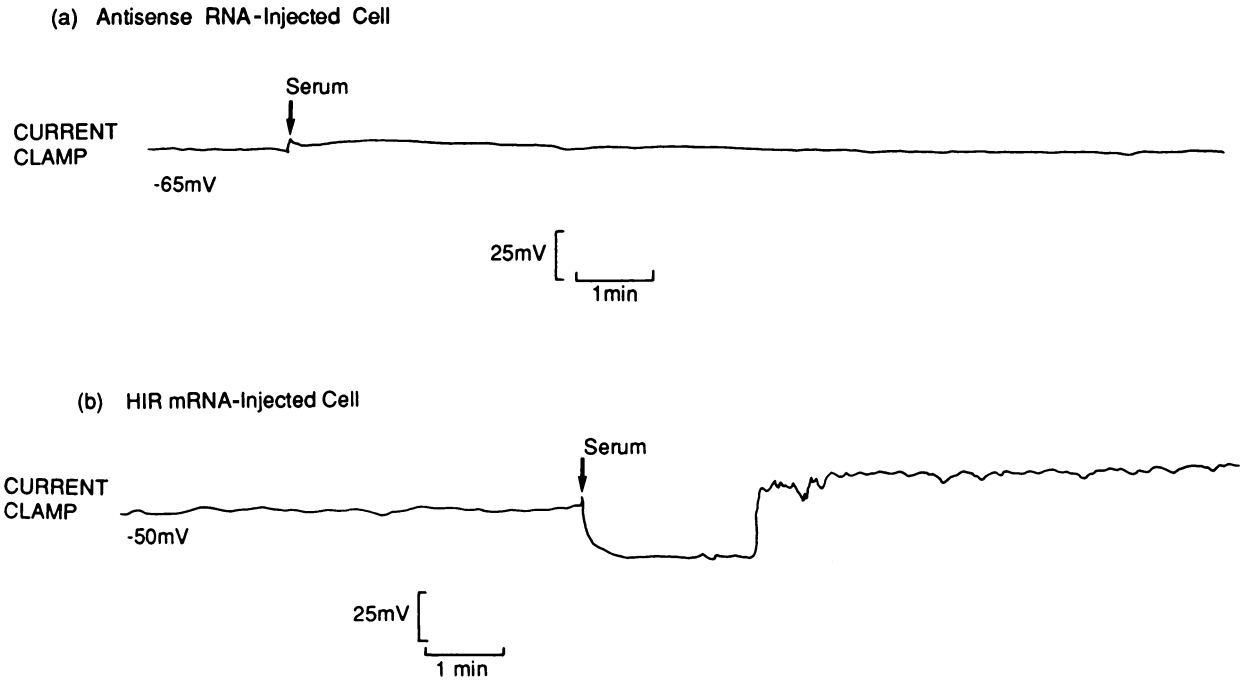


FIG. 2. Serum-elicited changes in membrane potential in the oocyte measured by current clamp. Oocytes were injected with 50 ng sense or antisense mRNA coding for the HIR, as described in the Methods section. The oocytes were exposed to 40 nM anti-human insulin receptor antibody 24 hr before electrophysiological measurements. When impaled by a current measuring microelectrode, it was found oocytes typically had a resting membrane potential of between −40 and −70 mV. Serum (a source of complement) was added to the bathing medium, constituting 20% of the total volume. A typical example of the recording with antisense mRNA-injected (a) and a typical recording with sense mRNA-injected (b) oocytes are shown. In both cases, at least 8 separate recordings were made. TEA, DIDS, or nilumic acid were not present in these experiments.

experiment was then performed to directly compare the ability of nifedipine to inhibit K^+- or complement-stimulated $^{45}Ca^{2+}$ influx measured 5 min after serum addition (Fig. 1c). There was approximately a 10-fold difference in the IC_{50} for nifedipine inhibition of these processes, approximately 0.1 μM for K^+-stimulated flux, and approximately 1 μM for complement-stimulated flux. There was a significant difference in the inhibition of $^{45}Ca^{2+}$ influx at the lower nifedipine concentrations used ($P < 0.01$) at 0.1 μM nifedipine and ($P < 0.05$) at 1 μM nifedipine (N = 3 for each point) compared with inhibition of K^+-stimulated $^{45}Ca^{2+}$ influx. An interpretation of the latter results would be that there are two distinct channels responsible for Ca^{2+} influx, both of which are inhibited by nifedipine but with different specificities. In order to explore this possibility further, we determined the sensitivity of serum- or K^+-stimulated Ca^{2+} influx to diltiazem. The latter drug used at 100 μM potently inhibited K^+-stimulated influx, but did not significantly alter serum-stimulated Ca^{2+} influx (Fig. 1c). The latter data add considerable weight to the argument for the existence of a novel Ca^{2+} permeable channel that is responsible for complement-induced changes in Ca^{2+} permeability.

Effect of Serum on Oocyte Membrane Potential

Rabbit serum (5-fold diluted) was added to oocytes which had been injected with sense or anti sense mRNA coding for the HIR. The oocytes had been exposed to the complement binding anti-human insulin receptor antibody 83-14. On measuring the membrane potential changes of current-clamped oocytes, the initial response on application of serum was either no change in resting membrane potential for antisense mRNA-injected oocytes (Fig. 2a) or immediate hyperpolarisation of the plasma membrane for sense mRNA-injected oocytes (Fig. 2b). The mean resting membrane potential of current-clamped oocytes was −61.0 mV ± 10.1 mV (N = 16) and hyperpolarisation resulted in a membrane potential of −81.0 mV ± 7.7 mV (N = 8). This transient hyperpolarisation was followed by a sudden depolarisation within 2–5 min to −35.7 mV ± 7.6 mV (N = 8, Fig. 2b) which was maintained for at least 30 min. The initial hyperpolarisation may be caused by activation of endogenous cyclic adenosine 3′:5′-monophosphate/cGMP-activated K^+ channels. The K^+ channels which may be responsible for the observed hyperpolarisation may or may not be present in the *Xenopus* oocyte plasma membrane as the latter cells contain few endogenous K^+ channels [30]. However, it is known that K^+ channels exist in surviving follicular cells [30, 31] and that these latter channels can elicit membrane currents within the oocyte through electrical coupling by gap junctions. Thus, complement activation and MAC formation in the locality of the follicular cells via antigen/antibody interacting occurring at the oocyte cell membrane may have activated follicular cell K^+ currents which subsequently elicited a

membrane current in the oocyte. To alleviate the problem of possible non-oocyte hyperpolarising current, all subsequent experiments contained the addition of 20 mM TEA, known to block K^+ conductances from the extracellular as well as the intracellular side of potassium channels (see [32] for review). The addition of 20 mM TEA blocked the serum-elicited hyperpolarising current (results not shown) and was used in all subsequent experiments except where shown.

Serum-elicited Oocyte Chloride Channel Activation

Serum (diluted 5-fold) was added to an oocyte-expressing insulin receptor, in the presence of anti-insulin receptor antibody. A large inward current was generated on the addition of serum measured by voltage clamping the oocyte at a value close to its resting membrane potential, −50 mV. This potential is away from the oocyte's reversal potentials for Na^+ (*ca.* + 50 mV), K^+ (*ca.* − 100 mV) and Cl^- (*ca.* − 30 mV). An example of the inward current generated by serum is shown in Fig. 3a. The amplitude of the current peak generated by serum was 342 ± 169 nA, with 6 oocytes used from 5 different donors). This inward current was abolished by the addition of the chloride channel (and anion transport) inhibitors, DIDS and niflumic acid, at 1 mM and 20 μM, respectively (Fig. 3a), indicating that a chloride conductance is likely responsible for the inward current. These substances have been shown to block anion transport systems and Cl^- channels [33–35], and the presence of both was required to totally abolish the serum-elicited inward current. Evidence that the inward current was due to complement activation is provided by two observations: that destroying heat-labile complement components by treating the serum at 56° for 30 min or removing the anti-insulin receptor antibody essential for classical pathway activation abolished the generation of current (Fig. 3b, the alternative pathway was inactivated under these conditions). An immediate but transient inward current was also observed in several experiments. The origin of this latter current (for example, see the middle trace in Fig. 3b) will be discussed below.

Identification of a Long-lasting Complement-Dependent Tail Current

A long-lasting tail current is also characteristic of the complement-dependent inward current (eg. Fig. 3b). A tail current has not been reported following any complement-independent serum-elicited currents by previous investigators. The tail current reported in this work was complement-(classical pathway) dependent as the omission of 83-14 anti-human insulin receptor antibody from the oocyte bathing medium resulted in an absence of inward current measured 15 min after serum addition (−11 ± 9 nA, N = 6 compared with −40 ± 18 nA in the presence of antibody, N = 6, $P < 0.05$). The alternative pathway had been heat-inactivated in the serum used for the latter experiments. Due to the smooth long-lasting and steady-state nature of this current, the determination of current/voltage profiles was possible for complement-elicited tail currents. These were determined 15 to 20 min after serum addition and are shown in Fig. 3c. The conductance profile of the classical pathway was linear from −120 mV to −20 mV, while at more positive potentials the conductance was strongly outward-rectifying. The conductance was potentiated by the presence of the anti-insulin receptor antibody 83-14, and was substantially reduced by treating the serum at 56° for 30 min, therefore inactivating complement (Fig. 3c). The reversal potential of the classical pathway-induced conductance (open squares) was −35 mV ± 5 mV (mean, ±SD, N = 6), very close to the calculated reversal potential of oocyte chloride conductance of −33 mV, based on the known extracellular chloride concentration (120 mM) and the value given for intracellular chloride, 33.4 mM [29]. However, the nonlinear current/voltage profile associated with the classical pathway of complement (open squares, Fig. 3c) was not typical of that obtained following normal Cl^- channel activation, which was linear from −150 mV to +50 mV (see [36] for example). Thus, the observed complement-associated I/V curve may only be partly explained by activation of chloride channels.

Nifedipine was shown to reduce the amplitude of the complement-specific current in a dose-dependent manner (Fig. 3d), virtually abolishing the current at 10 μM. The latter result supports the concept of Ca^{2+} influx playing a crucial role in subsequent stimulation of ion movement in response to complement. The presence of the chloride channel inhibitors DIDS and niflumic acid in the latter experiments (to minimise the contribution of Cl^- channels to the observed current) shifted the reversal potential of the complement classical pathway-induced current from −35 ± 5 mV to −52 ± 7 mV (N = 6), thus raising the possibility that endogenous Cl^- channels are active during the long-acting phase of complement-induced conductance changes. The shift in reversal potential to −52 mV suggests that K^+ ions quantitatively make a significant contribution to the complement-induced changes in conductance described above.

We also investigated the current/voltage profile elicited by the alternative pathway of complement. This pathway was nonantibody-dependent and was stimulated by exposure of non heat-treated serum to a heterologous cell membrane, in this case the *Xenopus* oocyte cell membrane. The profile obtained had a reversal potential of −25 mV and was similar in shape to that generated by the classical pathway (described above), except that the conductances generated by activation of either of the two pathways were different (Fig. 4a). The alternative pathway conductance was 1.18 ± 0.2 μS (mean ± SD, N = 4), but during the outward rectification phase this became 8.125 ± 0.7 μS. The equivalent figures for the classical pathway were 2.5 ± 0.3 μS and 26 ± 2.0 μS (N = 4), respectively. The differences between the alternative pathway and classical pathway conductances are significant ($P < 0.001$) for

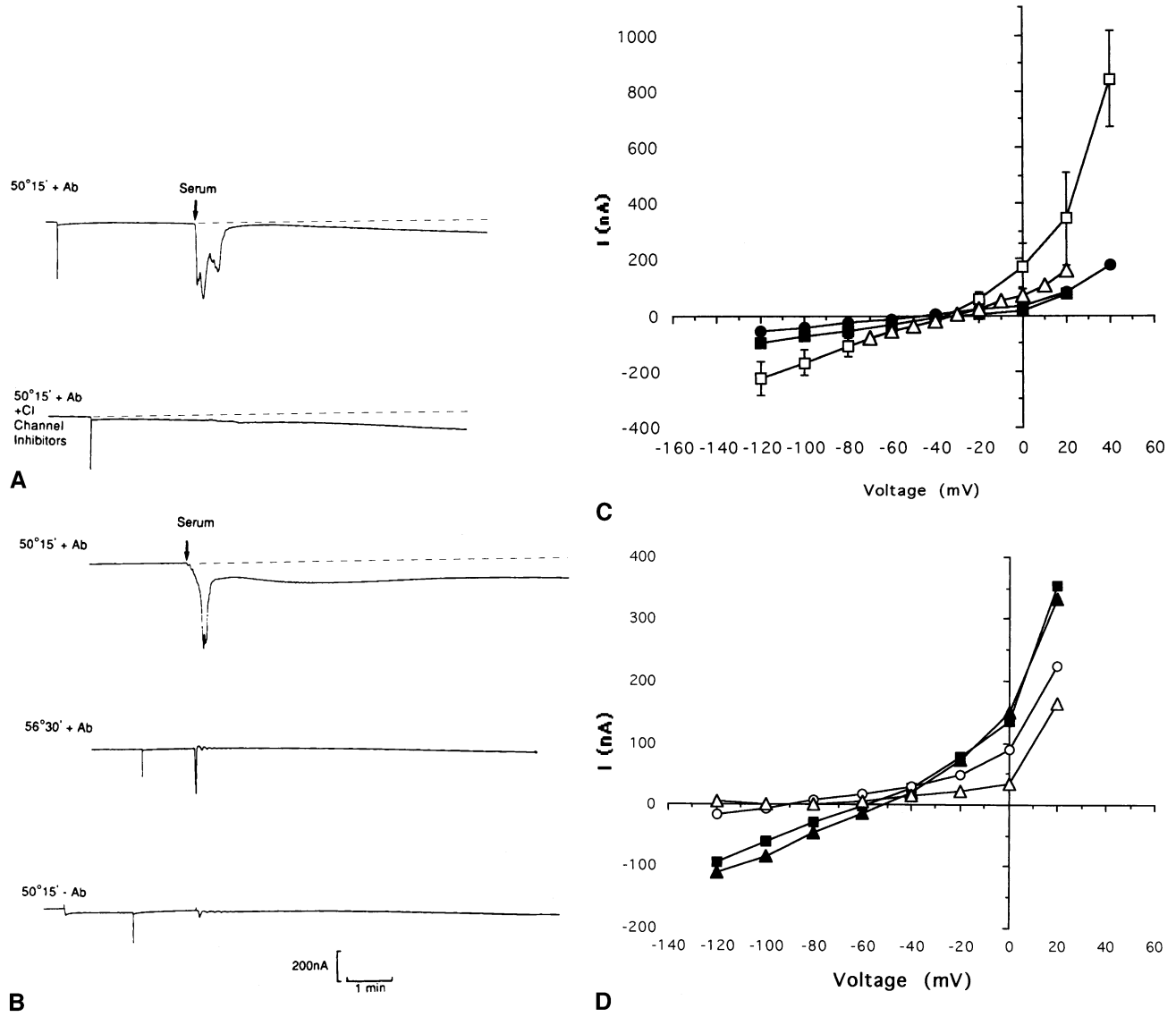


FIG. 3. Complement classical pathway-induced currents in the oocyte, measured by voltage clamp. Oocytes were pretreated as described in the legend of Fig. 2. The oocytes were voltage-clamped at −50 mV. Serum (a source of complement) was added to the bathing medium, constituting 20% of the total volume. Serum was treated at 50° for 15 min to inhibit the alternative pathway or at 56° for 30 min to inactivate both complement pathways. Twenty mM TEA was present in all experiments except where indicated. DIDS and niflumic acid were not present unless otherwise indicated. (a) The effect of 1 mM DIDS and 20 μM niflumic acid upon serum-elicited currents and (b) the effect of heat treatments for anti-HIR antibody on serum-elicited currents. Serum was heated to destroy labile complement components of the alternative pathway (50° for 15 min) or both the alternative and classical pathway (56° for 30 min) prior to dilution and addition to the oocyte. (c) The effect of heat treatment or TEA on current/voltage relationships from serum-elicited currents in oocytes. (□) Antibody present and serum treated at 50° for 15 min, (●) no antibody present and serum treated at 50° for 15 min, (■) antibody present and serum treated at 56° for 30 min, (△) antibody present and serum treated at 50° for 15 min but in the absence of TEA. The error bars represent the SEM values from single determinations from at least 3 separate experiments. The protocol used to generate I/V data has been described in the Methods section. (d) Classical pathway-induced currents in oocytes and inhibition by nifedipine. The dose-response effect of nifedipine on complement-specific current/voltage profiles in the presence of DIDS and niflumic acid, measured approximately 15 min after serum addition. Serum only added (▲), plus 0.1 μM nifedipine (■), plus 1.0 μM nifedipine (○), plus 10.0 μM nifedipine (△). The error bars are not shown as they were <5% of the mean of single determinations from at least 3 separate experiments.

both conductance phases. The alternative pathway of complement activation is not dependent on extracellular Ca^{2+}, but does require extracellular Mg^{2+} [37]; thus, the contribution of calcium to the observed complement-induced conductance may be determined (Fig. 4a). For this experiment, conditions were as described in the Methods section except that 2 mM calcium was replaced with 10 mM EGTA and magnesium was increased in concentration from 2 to 10 mM. No conductance increase was detected at potentials between −80 mV and +20 mV. This result implicates extracellular calcium as having a direct role in the generation of complement alternative pathway-induced

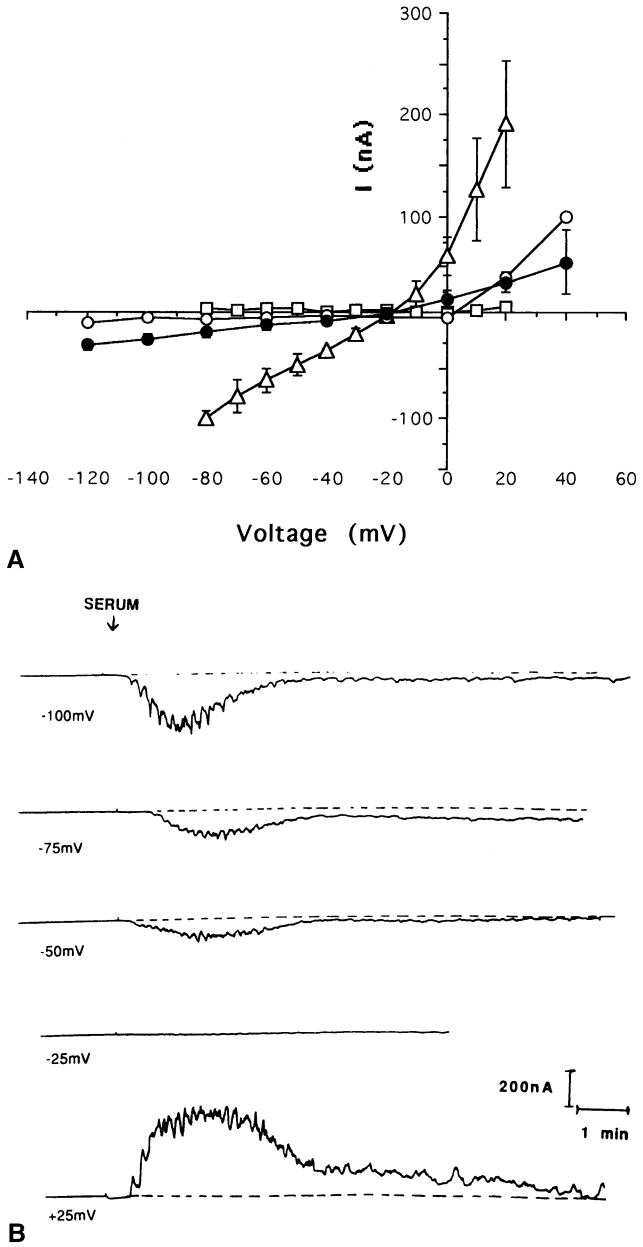


FIG. 4. Characterisation of alternative pathway-elicited currents in the oocyte. Oocytes were voltage-clamped at −50 mV. Serum (a source of complement) was added to the bathing medium, constituting 20% of the total volume. Twenty mM TEA was present in all experiments. DIDS and niflumic acid were not present unless otherwise indicated. (a) The effect of heat treatment or EGTA on current/voltage profiles from serum-elicited currents in oocytes. The following conditions were used: (△) untreated serum was added; (□) untreated serum was added in the presence of 10 mM EGTA/ 10 mM Mg^{2+}; (○) untreated serum was added that had been previously treated at 50° for 15 min; (●) untreated serum was added that had been previously treated at 56° for 30 min. The error bars represent the SEM values from single determinations from at least 3 separate experiments. (b) The effect of 25 mM extracellular Ca^{2+} on the amplitude and duration of the serum-elicited current in oocytes at various membrane potentials was determined. The bathing medium used for this experiment consisted of $MgCl_2$, 2 mM; K_2CO_3, 8.5 mM; Tris Cl, 98 mM; HEPES, 15 mM, pH 7.4; $CaCl_2$, 25 mM; DIDS, 1 mM; niflumic acid, 20 μM.

conductance increases. This result may be contrasted with the complement-independent chloride current elicited by serum, which is independent of extracellular calcium and is mediated by mobilisation of intracellular IP_3 [38, 39]. The effect of increasing the extracellular Ca^{2+} concentration to 25 mM and exposing noninjected oocytes to 5-fold diluted rabbit serum elicited a more prolonged inward current at −50 mV than that described in Fig. 3, one that was increased in amplitude at voltages more negative that −50 mV, reversed at approximately −25 mV, and outward in direction at +25 mV (Fig. 4b). The concentration of 25 mM of Ca^{2+} was chosen as this provoked reproducible effects.

The Effect of C9 Depletion and Replacement on Serum-elicited Inward Current

The effect of serum depleted of the (membrane spanning) complement component C9 on an HIR-expressing oocyte voltage clamped at −50 mV in the presence of 83-14 antibody is shown in Fig. 5a. The inward current typical of whole serum was not seen until the addition of 36 μg/mL of C9 (physiological concentration). The current generated was nonoscillating in contrast to that generated by whole serum and did not return towards baseline values so rapidly. There was no period of latency to the onset of the response after the addition of C9, as C5b–C8 complexes are bound to the cell membrane, thus allowing C9 to bind to C5b–C8 and form a membrane spanning ion-conducting channel [16, 40].

The data presented in Fig. 5a provide an opportunity to directly compare the whole serum, complement-independent, but IP_3/intracellular Ca^{2+}-dependent chloride current (described in more detail below) with the novel C9-dependent current (compare the short-lasting current observed after addition of C9-depleted serum with the current observed after addition of purified C9). The current/voltage profiles (Fig. 5b) were obtained by establishing the conductance profile with C9-depleted serum and then, using the same oocyte, establishing a profile after the addition of 36 μg/mL of C9. The conductance profile was similar to that described in Fig. 3c for classical pathway-elicited conductance changes. Human C9 addition resulted in a conductance of 2.0 ± 0.4 μS (N = 3) in the linear region of the I/V profile and a conductance of 16.8 ± 3.1 μS (N = 3) during outward rectification at potentials more positive than −20 mV. The reversal potential of this current was −35 ± 8 mV (N = 3).

DISCUSSION

The sequence of events proposed to account for the observations documented in this paper are: (i) initial influx of Ca^{2+} due to channel/pore formation by the MAC; (ii) activation of calcium-gated chloride channels within the oocyte; (iii) depolarisation of the plasma membrane; (iv) activation of endogenous voltage-gated Ca^{2+} channels; and

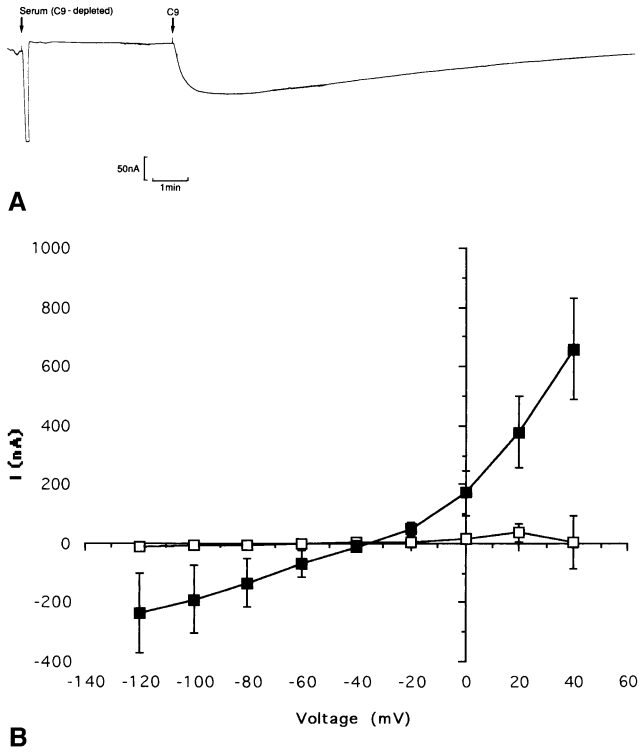


FIG. 5. Measurement of C9-independent and C9-dependent currents in the oocyte. Twenty mM TEA, 1 mM DIDS and 20 μM niflumic acid were present in all experiments. (a) Oocytes were pretreated as described in the legend of Fig. 2. Human serum was used for these experiments as C9-depleted human serum and purified human C9 were available due to a generous gift from Dr. J. P. Luzio (Department of Clinical Biochemistry, University of Cambridge). The oocytes were voltage-clamped at −50 mV. C9-deficient serum was added to the bathing medium, constituting 20% of the total volume, where indicated. Serum was treated at 50° for 15 min to inhibit the alternative pathway of complement. C9 was added at a concentration of 36 μg/mL where indicated. (b) The effect of C9 replacement on current/voltage profiles from C9-deficient serum-elicited currents in oocytes. (□) C9-depleted serum was treated at 50° for 15 min prior to addition to oocytes, (■) C9-depleted serum was treated at 50° for 15 min prior to addition to oocytes, and 10 min prior to C9-dependent I/V determination, 36 μg/mL of C9 was added. The error bars represent the SEM values from single determinations from at least 3 separate experiments.

(v) establishment of a long-lasting complement-dependent current that maintains membrane depolarisation. The characteristics of the latter current are not clear and further experiments are required to determine the involvement of the MAC and/or other channels.

Evidence that the MAC can act as a Ca^{2+} permeable channel, at least initially, is reported in this paper. For example, the difference in potency of nifedipine inhibition of K^+ or complement-stimulated calcium influx coupled with the fact that diltiazem did not significantly inhibit the initial phase of complement-stimulated calcium entry (up to 5 min after serum addition) but did inhibit K^+-stimulated Ca^{2+} influx by 96% (Fig. 1c) suggests that nifedepine binds to and inhibits two distinct channels: endogenous calcium channels and complement-dependent Ca^{2+} permeable channels. Further evidence that MAC may form a novel Ca^{2+} permeable channel is provided in Fig. 4b. Complement-stimulated Ca^{2+} influx was not voltage-dependent and reversed at approximately −25 mV. Evidence has previously been presented that early complement-stimulated ion flux is calcium-specific [2–4]; thus, the data presented here support those obtained by the latter authors who used different target cells, i.e. erythrocytes and neutrophils, for complement attack.

Calcium-activated chloride channels in the membrane have previously been described for the oocyte [35, 41, 42]. Indeed, previous work has shown that a serum factor, identified as lysophosphatidate, can induce calcium-activated chloride conductances in oocytes [43, 38] and calcium-activated cation conductances in human skin fibroblasts [44]. However, there appear to be two major differences in the characteristics of the inward current generated by serum as described previously and as described here as complement-dependent. First, previous studies have shown that treating the serum to denature heat-sensitive protein factors, e.g. boiling for 30 min, reduced but did not abolish the serum-stimulated inward current [43, 38], while in another study boiling for 5 min had no effect on the inward current [44]. Second, previous studies have also shown that very low concentrations of serum (up to 1000-fold diluted) can elicit activation of endogenous chloride channels. More recently, the major heat stable factor(s) responsible for the activation of chloride channels was reported to be lysophosphatidate, which activated a phosphatidylinositol/Ca^{2+} second messenger system in *Xenopus* oocytes resulting in activation of endogenous chloride channels [39]. In contrast, the complement-dependent current reported here was abolished by mild heat treatment (56°) and could not be measured at concentrations of serum below 50-fold diluted, being maximal at 5-fold diluted (results not shown). The initial calcium activation of endogenous chloride channels, by previously described (lysophosphatidate) mechanisms, is a brief event, and as shown in Fig. 5a (start of trace), activation and inactivation occur within 1 minute of serum addition to the oocyte. However, complement-stimulated membrane depolarisation does not occur until 2–5 minutes after serum addition (Fig. 2b) and is antibody-dependent (Fig. 3b), suggesting that the lysophosphatidate/IP_3/Ca^{2+}-stimulated endogenous chloride channels do not play a major role in complement-stimulated membrane depolarisation. The addition of the 'pore forming' complement component C9 to oocytes previously exposed to C9-depleted serum gave rise to a sustained inward current (Fig. 5a). Thus, sustained oocyte membrane depolarisation is probably a consequence of ion movement through novel channels formed by MAC or/and activation of endogenous longer-acting chloride channels.

As overactivation of complement contributes to the pathophysiology of many inflammatory diseases (see [45] for review) including a recently reported role in the pathogenesis of Insulin-Dependent Diabetes Mellitus [46, 47], then inhibition of complement mediated ion fluxes, metabolic

disturbances, and/or lysis might restrict tissue damage and aid recovery from disease.

We thank Dr. J. Paul Luzio for provision of C9-depleted human serum, purified C9, and helpful comments and suggestions. This work was supported by a grant from the British Diabetic Association. All experimental work described in this paper was carried out in the Department of Clinical Biochemistry, University of Cambridge, by PN who was in receipt of a Medical Research Council Training Fellowship.

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