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Recognition at Cell Surfaces: Phytohaemagglutinin-Lymphocyte Interaction

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Recognition at cell surfaces: phytohaemagglutinin–lymphocyte interaction

By M. J. Crumpton, D. Allan,† Judy Auger, N. M. Green and V. C. Maino‡
National Institute for Medical Research, Mill Hill, London NW7 1AA

[Plate 2]

Many aspects of cell behaviour are regulated by the interaction of extracellular ligands with specific receptors exposed on the cell surface. The receptors correspond to membrane proteins and especially glycoproteins. A key event in regulation is the transmission across the surface membrane of the information resulting from receptor–ligand interaction. The activation of lymphocytes by *Phaseolus vulgaris* phytohaemagglutinin (PHA) provides a convenient experimental model for the study of the molecular basis of receptor–ligand interaction and the molecular consequences of interaction. The receptor mediating lymphocyte activation by PHA is probably a unique glycoprotein which is present to the extent of about 3×10^4 molecules/cell. The PHA-receptor complex solubilized in 1% sodium deoxycholate has a molecular size of about 3×10^5. The primary event in the activation process is probably an increase in the permeability of the surface membrane to Ca^{2+}. This may be achieved by PHA cross-linking ('patching') the receptors to form a polar channel that permits an influx of Ca^{2+}.

1. Introduction

Various results suggest that a cell's surface structure regulates many aspects of the cell's behaviour. This regulation is believed to be achieved through the interaction of extracellular 'messengers' or ligands with specific 'receptors' exposed on the cell surface. Thus, the cell surface has been implicated in such apparently diverse phenomena as the control of cell growth and division (Burger & Noonan 1972), the regulation of cell recognition, interaction and adhesion (Roth 1973), and the recognition and response to polypeptide hormones and small, pharmacologically active molecules (Cuatrecasas 1974). The hypothesis that cell behaviour is monitored by ligand–receptor interaction was first proposed by Ehrlich (1900). He conceived of the ligand having two regions, the 'haptophore' which was 'adapted to a special side-chain of the cell protoplasm' and the 'ergophore' which on interaction of the above complementary structures initiated the biological events. This view is, in principle, still acceptable, although it is now apparent that the biological consequences of interaction are mediated by the receptor instead of the ligand. Ehrlich's hypothesis has also been modified to accommodate our current view of the molecular structure of the cell membrane and the concepts of allostery and induced-fit. By analogy with other specific molecular interactions whose binding-sites apparently share many structural features (Crumpton 1973), it seems likely that the nature of the combining-sites of cell membrane receptors and the molecular basis of receptor–ligand interaction resemble closely those which have been previously established for antibodies (Poljak *et al.* 1974) and enzymes (e.g. lysozyme; Blake *et al.* 1967). This prediction is supported by the close correspondence between the orders of specificity reported for enzymes and antibodies, and those for the receptors for polypeptide hormones and drugs.

† Present address: Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT.

‡ Present address: Department of Allergy and Clinical Immunology, National Jewish Hospital Research Centre, Denver, Colorado 80206, U.S.A.

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2. Structure of cell surface membrane

A variety of molecular models have been proposed for the structure of cell membranes (Hendler 1971). The most attractive is that due to Singer (1974) who views the cell surface membrane as a two-dimensional solution of oriented globular proteins in a lipid matrix. In this model the lipids are arranged as a discontinuous bilayer with their polar head groups in contact with the aqueous environment. This bilayer provides the basic structural framework of the membrane and functions primarily as a barrier that regulates the flow of ions, nutrients and metabolites between the extracellular and intracellular aqueous compartments. It also provides the location for the cell surface receptors which are identified with the membrane proteins and especially the glycoproteins. These are represented as a heterogeneous collection of globular molecules some of which, the 'peripheral proteins', interact solely with the lipid head groups whereas the remainder, the 'integral proteins', dip into the lipid to varying extents. Although the integral proteins are essentially anchored in the lipid, it appears that under physiological conditions they can diffuse laterally in the plane of the membrane. Some integral proteins traverse the lipid bilayer and are in contact with both aqueous phases. These molecules are of considerable interest since they provide a potential channel across the membrane for the transfer of information resulting from receptor–ligand interaction. They and any other proteins which dip beyond the centre plane of the lipid layer are revealed as intramembranous particles by freeze-fracture electron microscopy. In the case of lymphocytes, the results of freeze-fracture studies (Loor 1973; Matter & Bonnet 1974) indicate that no more than 1% (i.e. 5×10^4 to 10^5 particles/cell) of the total surface membrane protein (equivalent to approximately 10^7 molecules/cell; Crumpton & Snary 1974) spans the membrane.

A variety of results suggest that the surface membrane carbohydrate (i.e. the membrane glycolipids and glycoproteins) also plays a dominant role in determining cell behaviour and that it is located preferentially, if not exclusively, on the cell's outer surface (Bretscher 1973). Thus, the surface carbohydrate has been implicated in cell–cell interaction (Roth 1973), the binding of lectins (Nicolson 1974), the expression of antigenicity (e.g. blood group substances; Hakomori & Kobata 1974) and the differences between normal and neoplastic cells (Hakomori 1970). Also, some cell surface receptors such as the insulin receptor of fat cells (Cuatrecasas 1974) and the B-lymphocyte antigen receptor (Vitetta & Uhr 1974) are glycoproteins.

3. Lymphocyte receptors and lymphocyte activation

Various cell types and ligands have been used to explore the molecular basis of receptor–ligand interaction and the molecular consequences of interaction (Cuatrecasas 1974). Lymphocytes are an attractive cell type for study due, primarily, to two reasons. First, they express a wide spectrum of surface receptors, such as those for antigen (Warner 1974), the Fc-fragment of immunoglobulin (Nussenzweig 1974), the activated C3 component of complement and vasoactive hormones (Bourne *et al.* 1974). These receptors distinguish functionally distinct subpopulations of cells and regulate lymphocyte function (Parish & Hayward 1974; Parish, Kirov, Bowern & Blanden 1975). Second, lymphocytes do not normally proliferate, but division can be induced by the interaction of specific antigen or non-specific mitogens (lectins and various antisera) with the cell surface (Greaves & Janossy 1972). For example, the lectin *Phaseolus vulgaris* phytohaemagglutinin (PHA), which selectively binds *N*-acetylgalactosamine residues

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(Sharon & Lis 1972), activates *T*-lymphocytes to transform into blast cells that subsequently undergo mitosis. Figure 1 shows the capacity of PHA to transform lymphocytes from pig mesenteric lymph node as assessed by the incorporation of [6-^{3}H]thymidine into DNA after 45 h of culture. The results indicate that PHA stimulated a sharp increase in thymidine incorporation when its concentration exceeded a certain critical threshold value and that 2–4 μg of PHA/10^6 cells induced maximal DNA synthesis. PHA-induced lymphocyte transformation mimics closely the effects initiated by specific antigen, but whereas a particular antigen stimulates the specific lymphocytes only (probably $\gtrsim 0.1$ % of the total population) PHA activates a much larger proportion of the cells. As a result, PHA-lymphocyte interaction provides a convenient experimental model not only for antigen receptor–ligand interaction but also for exploring the sequence of molecular events that are initiated by this interaction and that subsequently lead to gene activation.

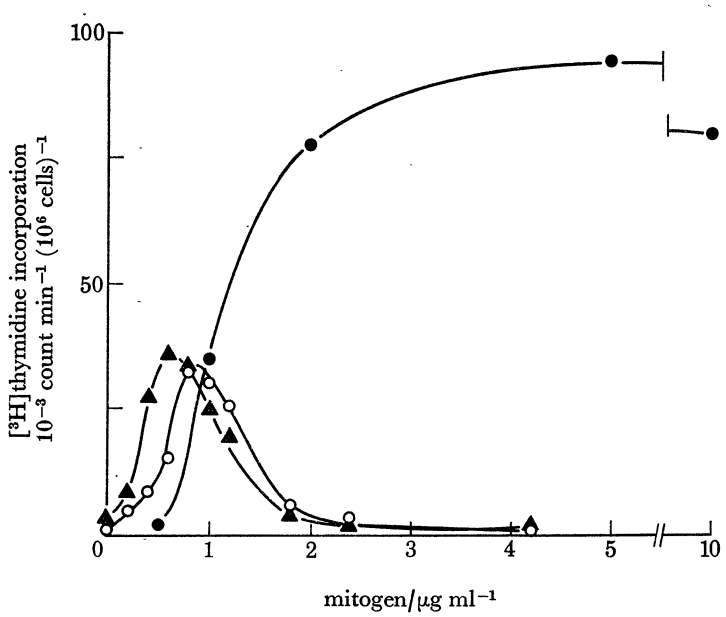

FIGURE 1. Incorporation of [6-^{3}H]thymidine by pig lymphocytes as a function of the concentration of PHA (●) or of A 23187 (○). The effect of sub-threshold amounts of PHA (0.3 μg) on the response to A 23187 is also shown (▲). The assays were performed as described by Maino, Green & Crumpton (1974) using 10^6 lymphocytes from pig mesenteric lymph node in 1 ml of Eagle's medium supplemented with 20 % (v/v) foetal calf serum. A 23187 was kindly donated by Dr R. Hamill (Eli Lilly Research Laboratories, Indianapolis, U.S.A.). Cultures were incubated for 45 h prior to addition of [6-^{3}H]thymidine (1 μCi) and for a further 5 h before recovery of the DNA.

Binding of PHA to lymphocytes and the nature of the receptor for PHA

Figure 2 shows that under the conditions giving maximal activation (i.e. 2 μg of PHA/10^6 pig lymphocytes) 0.3 % only of the added PHA was bound by the cells during the first 24 h of culture. This amount corresponds to about 3×10^4 binding sites/cell. An increase in binding occurred after 24 h but this coincided with the commencement of cell growth and was most probably due to the synthesis of new surface membrane and a consequent increase in the number of receptors for PHA. If this explanation is correct then all the receptors that can interact with PHA under the conditions of the initial phase of culture were saturated with PHA. At much higher PHA concentrations pig lymphocytes bind very much more PHA corresponding

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to a maximum of about 6×10^5 sites/cell (Allan, Auger & Crumpton 1971). These results suggest either that interaction of PHA with a small fraction (about 5%) of the total number of receptors is sufficient to initiate maximal transformation, or that pig lymphocytes possess two types of receptors with different affinities and that only the higher affinity receptor mediates

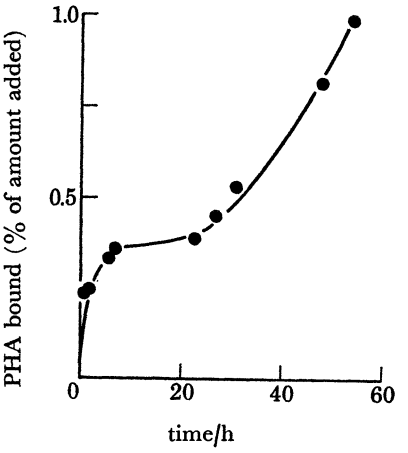

FIGURE 2. Amount of PHA bound by pig lymphocytes under the conditions giving maximal activation, relative to the time of incubation. ^{125}I-labelled PHA (2 μg) was incubated with 10^6 cells in 1 ml of medium. The cells were recovered by centrifuging after various times and the amount of bound PHA assessed by measuring the radioactivity.

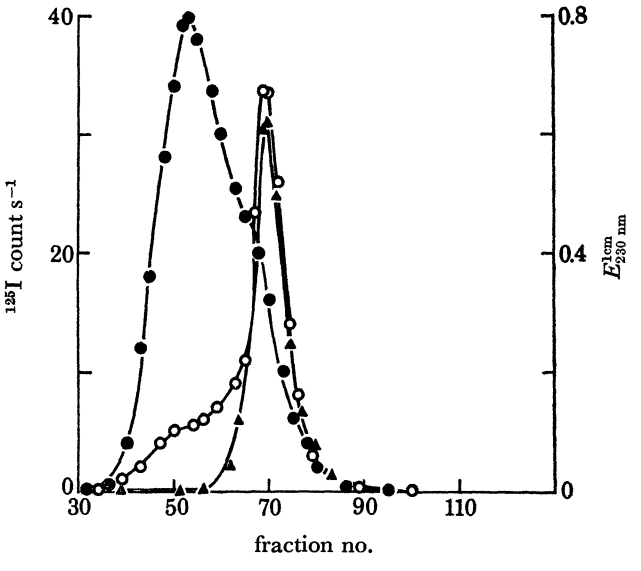

FIGURE 3. Elution of PHA-receptor complex from a column of Sepharose 6B with 1% Na deoxycholate. Pig lymphocytes were incubated with ^{125}I-labelled PHA either under conditions giving maximal activation (●) or with a tenfold excess of PHA (○). After 1 h at 37 °C the washed cells were supended in 1% Na deoxycholate and the soluble fraction was eluted from the column. The elution position of free PHA is also shown (▲).

transformation. The latter suggestion is supported by the results shown in figure 3. This figure compares the molecular size of the PHA-receptor complex formed under optimal-stimulating conditions with that produced using a tenfold excess of PHA; molecular size was assessed after solubilization in 1% Na deoxycholate by elution from a column of Sepharose 6B. The complex formed under optimal conditions was eluted as a broad peak whose position corresponded to a

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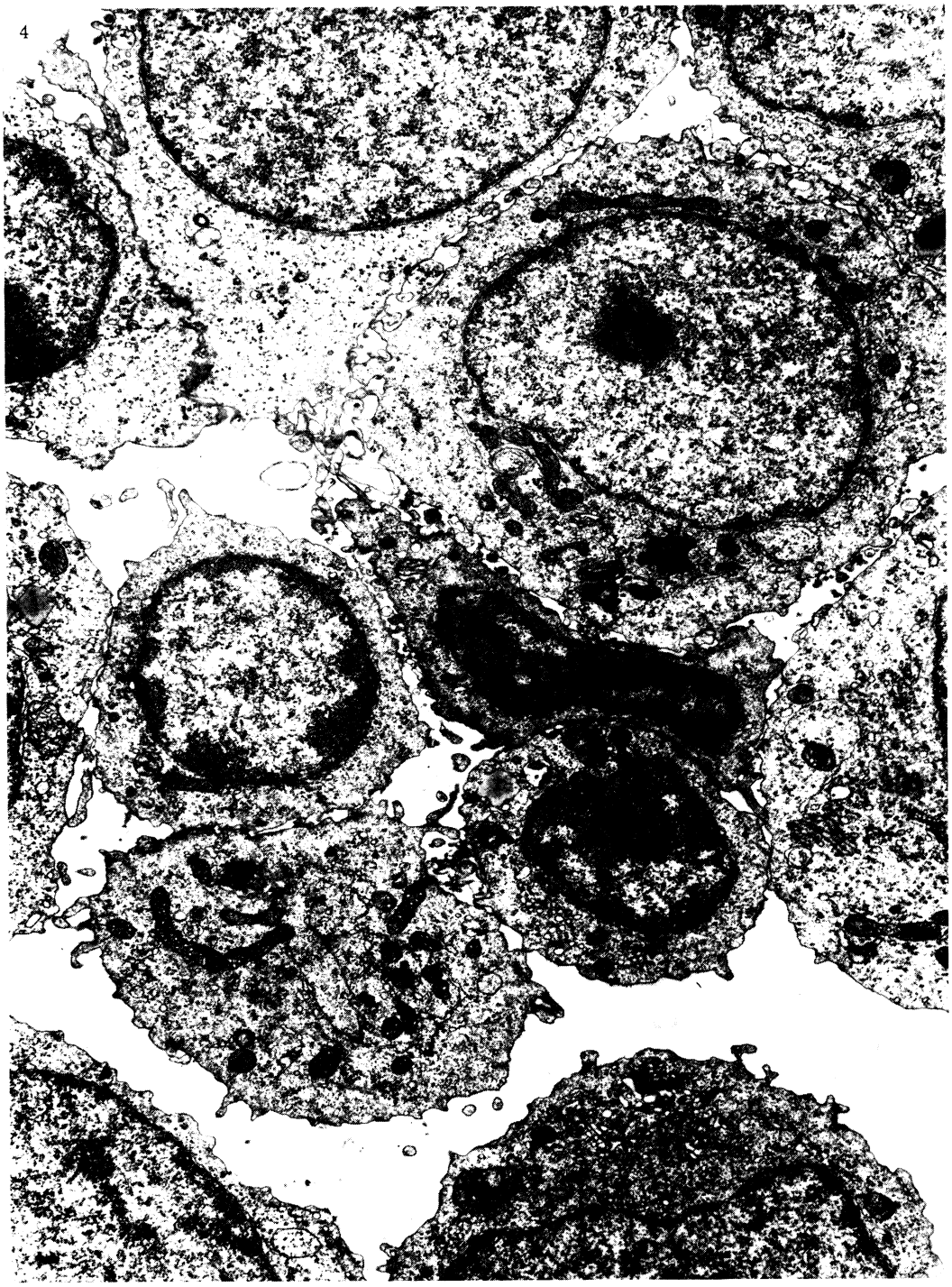

FIGURE 4. Electron micrograph of pig lymphocytes (10^6 cells in 1 ml) incubated for 45 h in the presence of the ionophore A 23187 (0.8 μg/ml). The majority of cells (80–90 %) were transformed but about 10 % resembled unstimulated lymphocytes. In many cells the mitochondria showed dense granules and/or hypertrophied cristae. These aberations were more pronounced in dead cells. (Magnification × 9000.)

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molecular size of about 3×10^5. PHA has a molecular mass of 1.2×10^5 which suggests that the receptor has a molecular size of about 1.8×10^5 or 0.9×10^5 if each PHA molecule cross-links two receptors (see below). In contrast, lymphocytes incubated with excess PHA gave a small shoulder only in the position of the above complex and a major peak that was coincident with free PHA. This result is consistent with the view that in the presence of excess PHA the majority of the PHA was bound by receptors of either low molecular weight such as glycolipids or of low affinity ($K_{\text{dissociation}}$ probably $< 10^{-6}$ M) so that the complex dissociated on solubilization. It was concluded that pig lymphocytes probably possess two types of receptors with different affinities for PHA. Lymphocyte activation is apparently mediated by the higher affinity receptors which correspond to a specific glycoprotein exposed on the cell surface (Allan *et al.* 1971; Allan & Crumpton 1973).

Sequence of biochemical events leading to lymphocyte activation

Interaction of lymphocytes with an optimal stimulating dose of PHA causes the receptors to form clusters ('patches') that may subsequently aggregate into polar 'caps' (Loor 1973). Binding of PHA also rapidly initiates (within 30 min) a series of biochemical changes that are primarily located within the surface membrane. These changes include an increased permeability to ions and metabolites (Greaves & Janossy 1972), a selective incorporation of long chain fatty acids into the plasma membrane phospholipids (Resch & Ferber 1972) and of P_i and *myo*-inositol into phosphatidylinositol (Fisher & Mueller 1971), and an increase in the intracellular concentration of cyclic-GMP (Hadden, Hadden, Haddox & Goldberg 1972). The sequence, relative importance and control of these events has yet to be defined but it would appear that the enhanced turnover of phosphatidylinositol depends on the cross-linkage of the specific receptors and represents an essential early step in the activation process (Maino, Hayman & Crumpton 1975). A critical evaluation of these changes suggests, however, that an influx of Ca^{2+} probably represents the primary event and that all other events are a consequence of the increase in intracellular Ca^{2+} concentration. This evaluation is supported by some preliminary experiments on inhibition by citrate, the results of which indicate that the enhanced turnover of phosphatidylinositol stimulated by PHA is Ca^{2+}-dependent.

Role of Ca^{2+} in lymphocyte activation

Evidence in support of a primary role for Ca^{2+} is provided by the reports that PHA initiates a slow accumulation of Ca^{2+} (Whitney & Sutherland 1972*a*) and that PHA-induced DNA synthesis is inhibited by chelators of Ca^{2+} (e.g. EGTA; Whitney & Sutherland 1973). The most convincing evidence is, however, provided by the demonstration that the ionophore, A 23187, which is specific for divalent cations (Chaney, Demarco, Jones & Occolowitz 1974) induces pig lymphocytes to transform into blast cells (Maino, Green & Crumpton 1974). Thus, electron micrographs of pig lymphocytes which had been cultured for 45 h with ionophore (0.8 μg/10^6 cells in 1 ml) revealed that 80–90 % of the cells were enlarged (> 8 μm diam.) and showed many of the features of PHA-transformed lymphocytes (figure 4, plate 2). For example, the cytoplasm was enlarged and showed marked endocytosis, the plasma membrane had large pseudopodia, there were many polysomes, an increased number of mitochondria and a dispersion of the heterochromatin. In contrast with these enlarged cells, about 10 % of the ionophore-treated cells appeared to resemble unstimulated lymphocytes (figure 4) and the possibility that A 23187 fails to activate a sub-population of lymphocytes cannot be ruled out at present.

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A quantitative measure of activation was obtained by measuring the incorporation of thymidine into DNA after culture with ionophore for 45 h. The response to increasing amounts of A 23187 is compared with that to PHA in figure 1. As noted previously for PHA, the ionophore stimulated a sharp increase in thymidine uptake when its concentration exceeded a certain threshold value. Although A 23187 stimulated a lower maximal incorporation of thymidine than PHA it seems likely in view of the above morphological evidence that this lower incorporation does not represent a smaller proportion of activated cells but is due to the perturbation of the cells' metabolism by the penetration of the ionophore to the mitochondria.

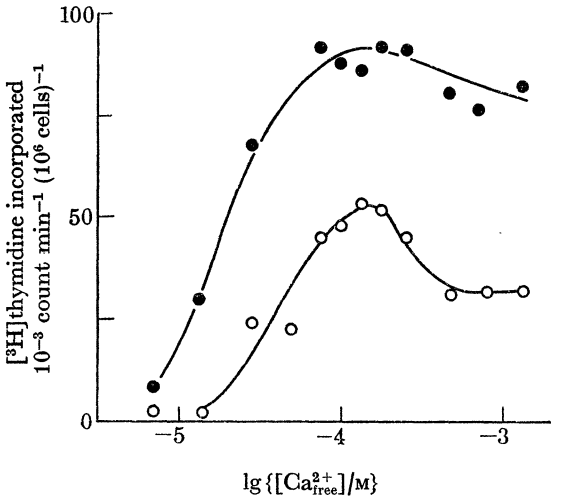

FIGURE 5. Effect of concentration of Ca^{2+} on the response of pig lymphocytes to PHA (●) or A 23187 (○). The Ca^{2+} concentration was controlled by the addition of Ca^{2+} or sodium citrate and was calculated from the total concentration of calcium and magnesium ions, the concentration of citrate and the known dissociation constants (0.5 mM) for calcium and magnesium citrates. The experimental details have been described previously (Maino *et al.* 1974).

If both PHA and A 23187 act by facilitating entry of Ca^{2+} then it should be possible to induce transformation by combining sub-threshold amounts of each. This prediction was confirmed by measuring the capacities of increasing amounts of A 23187 to induce activation in the presence of 0.3 μg of PHA which by itself had a negligible effect (figure 1). This result also suggests that largely overlapping populations of cells were activated by both reagents. The critical role of Ca^{2+} in the responses to A 23187 and PHA was confirmed by lowering its concentration in the medium by addition of citrate. Figure 5 shows that there was a sharp drop in the response to A 23187 when the Ca^{2+} concentration fell below 10^{-4} M which is close to the dissociation constant of the Ca^{2+}-ionophore complex. The PHA response was inhibited when a lower Ca^{2+} concentration was reached; the half maximal response corresponded to a concentration of 2×10^{-5} M which agrees with the results of Whitney & Sutherland (1972*b*). Addition of excess Ca^{2+} restored both responses to the control levels. It was concluded that A 23187 like PHA requires extracellular Ca^{2+} in order to initiate transformation.

The above results indicate that the ionophore initiates a very similar response in pig lymphocytes to that induced by PHA. If the action of the ionophore is effectively restricted to an increase in the intracellular Ca^{2+} concentration then the effects initiated by PHA can be ascribed to the same event. In this case, lymphocyte activation by PHA is the result of a direct

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effect of receptor–PHA interaction on the permeability of the surface membrane to Ca^{2+}. If this interpretation is correct then it is obviously important to determine how the PHA–receptor complex mediates the increase in permeability. One plausible mechanism depends on the possibility that the receptor for PHA spans the lipid bilayer (Loor 1973). In this case, the clusters of receptors formed by interaction with PHA may contain polar channels that permit an influx of Ca^{2+}. This mechanism has the additional attractive feature that it provides a rational explanation for the requirement for multivalency (cross-linkage) which appears to be an essential feature of all mitogens (Greaves & Janossy 1972).

It seems likely that increased permeability of the cell surface membrane to Ca^{2+} represents a general mechanism for the transmission of signals initiated by ligand–receptor interaction. For example, the stimulation of histamine release from mast cells by antibodies to immunoglobulin E can also be induced by A 23187 in the presence of Ca^{2+} (Foreman, Mongar & Gomperts 1973). On the other hand, it would appear that this mechanism is not universally exploited and that alternative methods of informational transfer have been selected for during evolution. Thus, the interaction of glucagon with fat cells probably initiates an allosteric change in the receptor that is ultimately expressed as an activation of the surface membrane-bound adenyl cyclase (Rodbell 1973).

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Discussion

D. Gingell

I am pleased to see that Dr Crumpton thinks that the association of mobile glycoproteins (subunits) in the cell membrane of lymphocytes is instrumental in bringing about an increase in calcium permeability which is thought to initiate transformation.

This, I believe, is an example of a more general phenomenon which I would like to draw to your attention. I have argued that many cellular responses mediated by permeability increase follow a common sequence of events (Gingell 1973). The essence of the argument is as follows. It is known that mobile glycoproteins, probably the particles seen in freeze fracture preparations, bear negative charges which cause them to repel one another as they diffuse in the plane of the lipid bilayer. All *similar* bodies are mutually attracted by van der Waals (electrodynamic) forces, as we heard from Dr Buckingham: consequently sufficient reduction in the electrostatic repulsion between glycoproteins or suitable linking by bridging molecules such as bivalent antibodies will result in what is essentially a two-dimensional colloid precipitation. Pinocytosis in amoebae, accompanied by an increase in membrane permeability, is induced by non-specific salts and not cationic proteins which would reduce the electrostatic repulsion between glycoproteins. One report (Orci & Perrelet 1973) has described subunit aggregation in pinocytosing cells. The lymphocyte capping reaction, a currently fashionable example of pinocytosis, may well follow the same sequence of events. Another example would appear to be the aggregation of membrane subunits to form gap junctions which are widely believed to be the sites of high intercellular ionic permeability.

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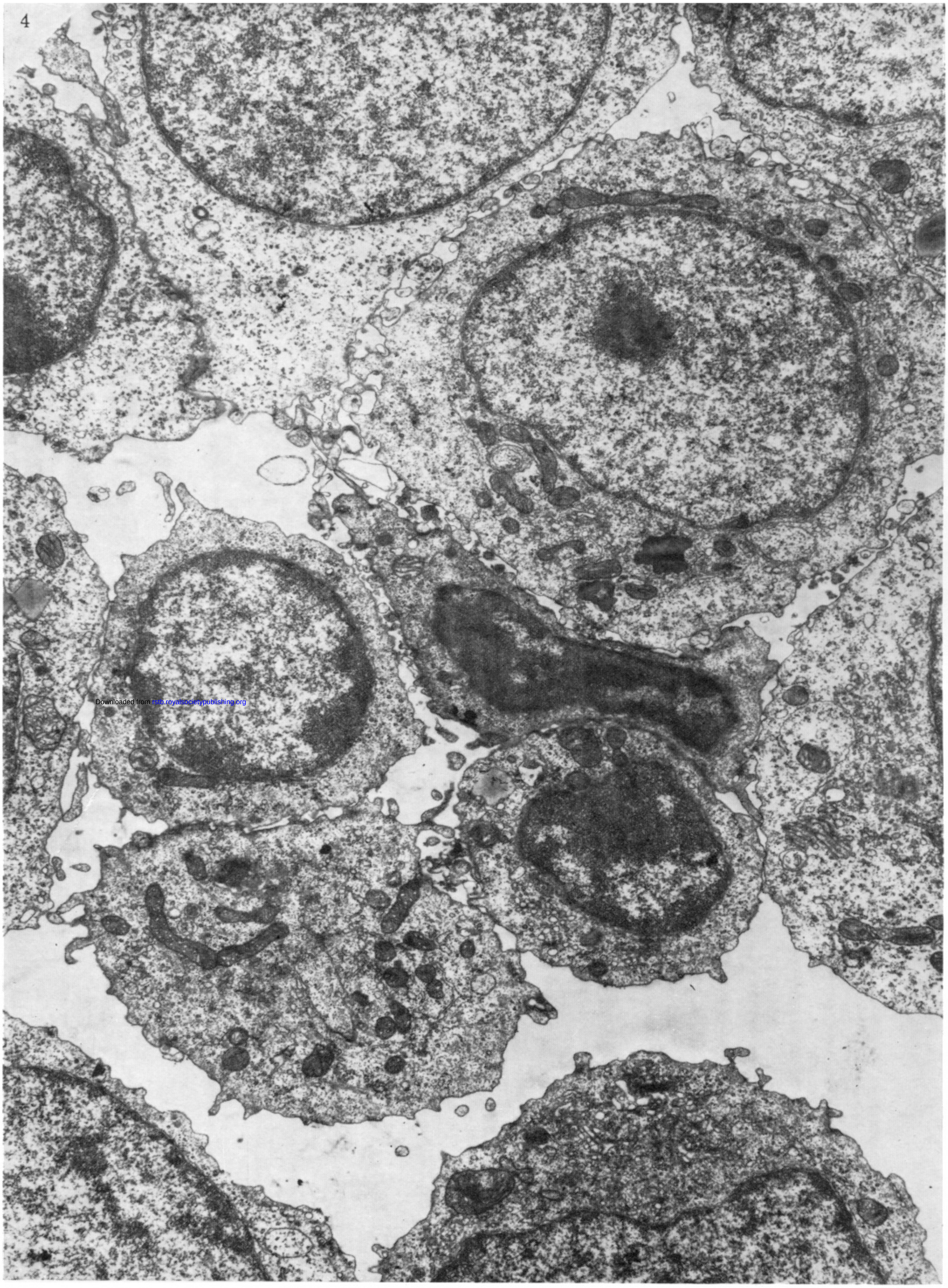

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IGURE 4. Electron micrograph of pig lymphocytes (10^6 cells in 1 ml) incubated for 45 h in the presence of the ionophore A 23187 (0.8 μg/ml). The majority of cells (80–90 %) were transformed but about 10 % resembled unstimulated lymphocytes. In many cells the mitochondria showed dense granules and/or hypertrophied cristae. These aberations were more pronounced in dead cells. (Magnification × 9000.)