

PSEUDPODIUM INDUCTION BY THE ACTION OF QUATERNARY AMMONIUM IONS ON *AMOEBA PROTEUS*

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SUMMARY

Some aliphatic long-chain quaternary ammonium compounds and choline derivatives have been examined for their ability to induce pseudopodia from *Amoeba proteus*. The reaction involved in pseudopodium induction is believed to be between the quaternary nitrogen cations and polysaccharides on the cell surface. Not all of the carboxyl groups of the polysaccharide are involved in this reaction. The mechanism of pseudopodium induction is discussed in terms of changes in the surface charge and the permeability of the cell membrane.

INTRODUCTION

The chemotactic response of *Amoeba proteus* to a number of basic proteins applied at a distance from the cell surface was described by Jeon & Bell (1965). It was proposed that the compounds were adsorbed to the mucopolysaccharide coat of the cell where they produced an electrostatic imbalance by reaction with carboxyl groups. This electrostatic imbalance was considered to be the signal which initiated the formation of new pseudopodia.

The use of poorly defined proteins by these workers was the major disadvantage encountered in the detailed interpretation of their results. The main purpose of the present work was to extend the above observations to the response of amoebae to well-defined compounds of known properties. Our attention was directed to quaternary ammonium compounds by supposing that surface-active compounds with long hydrocarbon chains might affect the functioning of the membrane by dissolution in the lipid-layer of the membrane.

MATERIALS AND METHODS

Culture methods

A. proteus, strain X 65 (Ord, 1968), was used exclusively throughout this work. Cells were grown in mass culture in Chalkley's solution with *Tetrahymena pyriformis* as food organisms (Griffin, 1960). Before use, the cells were starved (24 h) and washed at least five times in Chalkley's solution to remove contaminating materials.

Chalkley's solution was of the following final composition: NaCl, 1.37 mM; NaHCO₃, 4.76×10^{-2} mM; KCl, 2.68×10^{-2} mM; Na₂HPO₄, 2.79×10^{-3} mM; CaHPO₄, 7.35×10^{-3} mM; MgCl₂, 4.92×10^{-3} mM.

Chemicals

All chemicals were of the best grade available. Long-chain quaternary ammonium compounds were obtained from K and K Laboratories Inc.; acetyl choline, benzoyl choline and succinyl dicholine were obtained from Koch-Light Laboratories Ltd.

Technique for the demonstration of pseudopodium induction

The technique of Jeon & Bell (1965) was used. The chamber was constructed of pieces of glass slide cemented to a larger slide with Araldite. The chamber so formed had a depth of approximately 1 mm. Amoebae were introduced into the chamber with sufficient Chalkley's solution to produce a flat upper surface.

Micropipettes, tip diameter 10 μ , were filled with a solution of the compound to be tested in Agarose (0.5%) by compressed air. After gelation had occurred, the tip of the pipette was brought near an amoeba and its effect on the production of pseudopodia was noted. If no response was obtained, at least ten different pipettes were used on two separate occasions before the result was recorded as negative. Compounds which elicited pseudopodia were repeatedly tested in the same way. Cinematography was used to record these experiments.

RESULTS

Two main groups of compounds containing quaternary nitrogen atoms were tested for their ability to induce pseudopodia from amoebae; these were cationic detergents of the type $C_nH_{2n+1}(CH_3)_3N^+$ and choline derivatives. The compounds were used at concentrations of 10^{-3} M in Agarose gel (0.5%). It should be emphasized that the effective concentration of the solute at the cell surface is much less than its concentration in the micropipette; a simple calculation suggests an effective concentration of 10^{-6} M would be obtained in these experiments at a distance of 50 μ from the pipette.

If the compound was active in pseudopodium induction, pseudopodia were formed towards the pipette. Pipettes filled with inactive compounds had no discernible effect on the cells which continued to form pseudopodia and to move in directions unrelated to the position of the pipette. The first step in the formation of pseudopodia was cessation of the movement of cytoplasmic particles immediately below the area of stimulation, followed rapidly by the formation of a small hyaline bleb (Jeon & Bell, 1965). Cytoplasmic streaming into this bleb then followed and the newly formed pseudopodium advanced towards the micropipette. In most cases, further stimulation led to the appearance of a food cup which completely engulfed the tip of the pipette. Once the formation of a food cup had been initiated by a pipette, it continued to develop even after the stimulating pipette was removed. The outside of the food cup was also sensitive to pseudopodium induction and several large food cups could be induced consecutively from the same amoeba.

Micropipettes containing dodecyl, tetradecyl, hexadecyl or eicosanyl trimethylammonium salts (abbreviated to C_{12} quat, C_{14} quat, C_{16} quat and C_{20} quat respectively) or succinyl dicholine (10^{-3} M in 0.5% Agarose) were able to induce pseudopodia when

placed 40–50 μ from the cell. Tetramethylammonium bromide, acetylcholine and benzoylcholine at the same concentration (10^{-3} M) were ineffective and amoebae were indifferent to micropipettes containing these compounds placed as close as 10 μ from the cell surface. The response of amoebae to pseudopodium-inducing agents is graded along the length of the cell and decreases towards the tail, where only the most active agents will induce pseudopodia (Jeon & Bell, 1965). By finding the point nearest the tail from which a pseudopodium may be induced, a crude estimate of the activity of the compound may be obtained. Thus, the C_{12} quat and C_{14} quat salts were able to induce new pseudopodia from retreating pseudopodia or from approximately two-thirds of the cell length from the advancing front. The C_{16} quat and succinyl dicholine were able to induce new pseudopodia from half-way along the cell but the C_{20} quat was only effective in the front third of the cell, the region most sensitive to pseudopodium induction. Hexadecylpyridinium bromide was similar in activity to C_{16} quat. The negatively charged analogue, hexadecare sulphonic acid, failed to induce pseudopodia in this system and the nature of the charged group in the molecule was clearly the most important factor in determining pseudopodium-inducing activity.

Cells immersed in solutions of cationic detergents have deranged locomotory patterns or are immobilized, depending on the concentration and chain length of the detergent. Amoebae will survive immersion in 10^{-3} M tetramethylammonium bromide solution, in 10^{-4} M C_{12} quat solution or in 10^{-5} M solutions of higher members of the series. Higher concentrations than these are lethal and lead rapidly to cell lysis. The time taken for amoebae to recover their normal locomotory pattern following immersion in the above concentrations of detergent and after washing with Chalkley's solution, varied according to the chain length of the detergent. Cells originally immersed for 2.5 h in C_{12} quat (10^{-4} M) or C_{14} quat (10^{-5} M) solutions recovered within 15 min of washing with fresh Chalkley's solution, those immersed for the same time in C_{16} quat solution (10^{-5} M) recovered in 18 h whereas those immersed in C_{20} quat solution (10^{-5} M) failed to recover even after 48 h in fresh Chalkley's solution. Amoebae immersed in tetramethylammonium bromide solution (10^{-4} M) move normally and pseudopodia may be induced from cells in this condition by micropipettes containing C_{12} quat, C_{14} quat or C_{16} quat gels. It is apparent that the binding of these compounds to the cell surface is a function of the length of the hydrocarbon chain and that the C_{16} and C_{20} quats bind far more strongly than do the C_{12} and C_{14} quats. Tetramethylammonium salts do not induce pseudopodia and appear to have no effects on the cells which might be attributed to binding of the cations to the surface.

The way in which quaternary ammonium cations bind to the cell has parallels in the way in which these ions react with acidic polysaccharides. Scott (1955) reported that C_{16} quat salts precipitate a number of acidic polysaccharides and this reaction has been developed as a means of separating mixtures of these compounds (Antonopoulos *et al.* 1961). We have investigated the reaction of these detergents with a number of polysaccharides and polyanions, which might represent those species present on the cell surface. The metachromatic dye, toluidine blue, was used as an indicator for the presence of anionic polyions. Detergent solution (10^{-2} M) was added to polysaccharide solution (1 ml; 3 mg/ml) with toluidine blue (0.5 ml; 0.01%) and the volume was

adjusted to 3.5 ml with Chalkley's solution. The end-point was determined spectrophotometrically and expressed as moles detergent/mole anionic groups on the polysaccharide. The results are presented in Table 1.

As can be seen, it was not necessary to add sufficient detergent to react 1:1 with all the anionic groups on these polysaccharides before the metachromatic colour of the toluidine blue was discharged, i.e. the charge on the polyanion was not reduced to zero at this stage. It was not possible to obtain a clear end-point for two of the reactions involving C₁₂quat; similarly it was not possible to obtain an end-point in reactions between tetramethylammonium cations and any of the polyanions tested.

Table 1. *Number of moles of quaternary ammonium cation required to react with one mole of anionic groups on the polyanions*

	Poly-L-glutamic acid	Polygalacturonic acid	Chondroitin sulphate	Heparin
C ₁₆ quat	0.68	0.45	0.54	0.64
C ₁₄ quat	0.76	0.56	0.63	0.62
C ₁₂ quat	No end-point	No end-point	0.66	0.66

Attempts were made to suppress the metachromatic staining of the cell surface by toluidine blue by treating the cells with C₁₆quat solution (10⁻⁵ M) (Quintarelli, Scott & Dellovo, 1964). However, cells treated with the detergent stained metachromatically and the stained cells retained the metachromatic colour after immersion in detergent solution. Similarly it was not possible to suppress the staining of the surface by alcian blue in this manner. This stain is also believed to react with the polysaccharide coat of the cell (Nachmias, 1966). It should be realised that only small numbers of anionic groups are required to produce the metachromatic shift in the spectrum of toluidine blue and that the reaction of cations of this kind with polysaccharides is competitive. Also the concentration of C₁₆quat used by Quintarelli, Scott & Dellovo (1964) to block metachromatic staining of polysaccharides was approximately 5 × 10⁻² M, which is at least 100 times greater than the concentration which will lyse the cells immediately. Our results suggest that if the quaternary ammonium cations are in fact reacting with carboxyl groups on the cell surface not all of these groups are involved in the reaction.

The competitive nature of the reaction of quaternary ammonium cations with polysaccharides was demonstrated by experiments in which heparin was shown to displace polygalacturonic acid from the precipitate formed between this polysaccharide and C₁₆quat. When heparin solution (3 mg/ml) was added to a suspension of the precipitate formed by the reaction of an equivalent amount of C₁₆quat solution (0.7 ml; 10⁻² M) with polygalacturonic acid solution (1 ml; 3 mg/ml) in the presence of toluidine blue (0.5 ml; 0.01%), the characteristic metachromatic shift caused by heparin could not be detected until enough heparin had been added to react with all the C₁₆quat present in the original precipitate. Heparin may also be capable of displacing the polysaccharide from the complex formed between the cell coat and C₁₆quat. If cells treated with C₁₆quat solution (10⁻⁵ M) for 15 min are washed with heparin solution

(0.1 mg/ml), after removal of the excess C_{16} quat, the cells fail to recover their normal pattern of movement and remain rounded up. Control cells, similarly treated with C_{16} quat solution but washed in Chalkley's solution, recover within 2.5 h. This result may be accounted for in terms of the formation of a heparin- C_{16} quat complex by the reaction of heparin with C_{16} cations displaced from the complex with the cell-surface.

DISCUSSION

The part played by the cell envelope in controlling the cell's response to its environment is becoming increasingly clear and the importance of the mucopolysaccharide components is of some interest (Bell, 1962). The importance of molecules adsorbed at the cell surface in the induction of pseudopodia (Jeon & Bell, 1965) has been further substantiated by the present work. The quaternary compounds used by us have the considerable advantage over the proteins originally described as pseudopodium inducers (Jeon & Bell, 1965) in that their molecular structures and properties are precisely known. It has been possible to study the way in which variations in the molecular structure of these compounds affect their adsorption to the cell and their ability to induce pseudopodia.

All those compounds found to induce pseudopodia in our system were positively charged ions, although it must be emphasized that not all such ions were able to induce pseudopodia. Thus, although succinyl dicholine was active in this respect, the smaller, univalent analogues, acetyl choline and benzoyl choline were ineffective in our system. The presence of two or more basic groups is not a prerequisite for pseudopodium-inducing activity since the univalent cations of C_{12} quat, which are of approximately the same molecular dimensions as succinyl dicholine, are extremely active pseudopodium-inducing agents. The role of the size of the inducing ions in this process will be discussed below.

Although we have shown that amoebae are unaffected by local concentrations of tetramethylammonium bromide cations near to the cell surface, we wish to draw attention to the presence of this compound in hydra (Welsh & Prock, 1958). An extract from hydra was one of the biological materials described by Jeon & Bell (1965) as being particularly active in the induction of pseudopodia from *A. proteus*. Welsh & Prock (1958) suggested that in the living hydra tetramethylammonium cations could be adsorbed to protein. Their suggestion was made to account for differences between the biological activity of tetramethylammonium salts and the activity of a material extracted from the hydra. It could also explain the pseudopodium-inducing activity of the extract obtained by Jeon & Bell (1965).

Hexadecane sulphonic acid, which has the same chain length as C_{16} quat, does not induce pseudopodia at the equivalent concentration. If the action of these long-chain compounds depended on their adsorption and insertion into the lipid layer, then a charge difference should be of relatively little importance, but as the negatively charged long-chain compound is inactive it appears that the reactivity depends on the nature of the charged group. Also it seems unlikely that the adsorption of C_{12} and C_{14} quat cations would be readily reversible if these compounds were adsorbed into

the lipid layer of the cell membrane. We therefore propose that the quaternary ammonium compounds are more likely to be adsorbed to the mucopolysaccharide coat of the amoeba. The reaction between quaternary nitrogen compounds and acidic polysaccharides has been studied for hexadecyl-chain detergents and curariform drugs (Scott, 1955; Ehenpreis & Fishman, 1960; Ehenpreis & Kellock, 1960). Hassón (1962) showed that strong interactions could occur between a synthetic curariform drug and an acidic polysaccharide without precipitation. Our results with C_{12} and C_{14} quat compounds suggest that their action does not involve surface precipitation because their action is easily reversed by washing.

Our studies of these interactions with polysaccharides which may resemble those at the cell surface have used the metachromatic shift in the spectrum of toluidine blue as an indicator for the presence of polyanions. The reaction of $C_nH_{2n+1}(CH_3)_3N^+$ cations with the carboxyl or sulphate groups of these acidic polysaccharides abolished the metachromatic shift. Estimates of the number of moles of detergent reacting with each mole of charged groups could be made knowing the number of charged groups on the repeating unit of the polysaccharides. Of the compounds investigated, polygalacturonic acid is probably the most similar to the mucous material present at the surface of the amoeba. Although very little information about the composition of the mucous coat is available, the results of O'Neill (1964) suggest that polymannuronic acid may be an important component.

By using models of polyhexauronic acids and assuming that each detergent molecule reacts with two carboxyl groups, as in the case of polygalacturonic acid, it can be shown that the hydrocarbon chains of these detergents are likely to interact strongly with each other only when the chain contains at least 16 carbon atoms. On this basis, the adsorption of C_{16} and C_{20} quats involves hydrophobic interactions between the hydrocarbon chains of the detergent ions as well as electrostatic binding of the quaternary nitrogen atom to carboxyl groups. This type of hydrophobic interaction means that the molecules co-operate effectively to form a polycation. Their adsorption would be expected to be much less readily reversible than the adsorption of C_{12} and C_{14} quats, which cannot interact in this way.

The adsorption of positively charged proteins to the surface has been recognized as the first step in the induction of pinocytosis by these compounds (Brandt, 1958; Schumaker, 1958). Jeon & Bell (1965) also demonstrated this to be the site of adsorption of their papain-fraction, which was able to induce pinocytosis by general application and pseudopodia when applied locally. Experiments in which amoebae are immersed in potentially active solutions cannot lead to conclusions about pseudopodium induction, because they ignore the fact that the inducing agent must be polarized with respect to the cell.

The way in which the adsorption of cations to the mucopolysaccharide coat of the amoeba is able to direct pseudopodium formation remains a problem. Recent experiments on compounds which induce pinocytosis (Josefsson, 1966; Brandt & Freeman, 1967) and with amphibian eggs (Gingell & Palmer, 1968) suggest that adsorption of cations leads to an increase in the permeability of the cell membrane. Bingley & Thompson (1962) have suggested that permeability changes might also govern pseudo-

podium formation. Josefsson (1966) reports that acetyl choline, protamine and alcian blue cause membrane depolarization and suggests that the permeability of the membrane is governed by the adsorption of cations to negatively charged groups in the mucopolysaccharide layer of the cell envelope. Our unpublished results show that both alcian blue and protamine are capable of inducing pseudopodia in our system but acetyl choline is ineffective. The precise significance of Josefsson's (1966) results is difficult to evaluate as it is not clear whether his experiments were carried out on amoebae immersed in Pringsheim solution, containing Ca^{2+} (0.85 mM), K^+ (0.35 mM) and Na^+ (0.22 mM) as the chief cations, or in glass-distilled water.

A relationship between the physical structure of the membrane and its permeability state has been demonstrated by electron microscopy (Brandt & Freeman, 1967); the highly permeable state was characterized by a doubling of the thickness of the lipid layer. Such an increase in the thickness of the cell-membrane clearly involves a substantial micellar reorganization. This arrangement could lead to the activation of enzymes on the inner face of the membrane which might, for example, initiate cytoplasmic streaming. This suggestion receives support from the work of Jones & Wakil (1967), who showed that micelles of phospholipids are required to activate an enzyme involved in a microsomal electron-transfer reaction.

A number of basically similar ways in which changes in the cell surface could provide the stimulus for local changes in membrane function have been suggested. Thus Bell (1962) has proposed that the binding between polysaccharides on the cell surface and an underlying protein layer may be affected by compounds which react with the polysaccharide. The resultant changes in the protein may then be transmitted to deeper layers of the membrane thus affecting its functioning. Kavanau (1963) has particularly emphasized a general role of cations in governing the configuration of lipids in the membrane and suggested that small changes in the numbers or kinds of cations associated with the membrane could have large effects on its behaviour. A general treatment of the ways in which the surface potential of cells may be altered by changes in the environment has been given by Gingell (1967). Unfortunately, estimates of the potential change required to elicit a stimulus would be very difficult to make. There is, however, no reason to suppose that reversal of the sign of the net negative charge is necessary and the suggestion of Gingell (1967) that a large change in the properties of the membrane may be produced by a change of only a few per cent in the surface charge may well be correct. The presence of carboxyl groups after the reaction with applied cations is shown by our results; concentrations of quaternary ammonium compounds ten times greater than those involved in pseudopodium induction were insufficient to react with all the carboxyl groups on the cell surface.

A major problem is how to explain the passage of a signal from the outer layer of mucopolysaccharide through hundreds of Ångstrom units to the cell membrane.

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