INHIBITION OF MACROPHAGE SPREADING BY ANTAGONISTS OF CELLULAR CALCIUM

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Received 10 March 1981; revised version received 21 April 1981

1. Introduction

The spreading of macrophages on glass is an energy-dependent process analogous to phagocytosis [1,2]. In simple salt solutions, spreading is stimulated by proteolytic enzymes including subtilisin [3]. Such stimulated spreading is insensitive to the extracellular concentration of free calcium [3]. To determine whether cellular calcium might play a role in the process of macrophage spreading, we have assessed the ability of several calcium antagonists to inhibit spreading in the absence of extracellular calcium. 8-(*N,N*-diethylamine)-Octyl-3,4,5-trimethoxybenzoate–HCl (TMB-8), an inhibitor of calcium movement across membranes in skeletal and smooth muscles [4], inhibits spreading in a dose-dependent manner. This inhibition is relieved by addition of extracellular calcium. Lanthanum and verapamil, blockers of plasma membrane calcium channels in a variety of cells [5], also inhibit macrophage spreading. Electron microscopy demonstrates that the peripheral lamellipodia of macrophages, where spreading is actively occurring, are virtually devoid of membranes other than the cell surface membrane. We suggest from these data that the spreading of macrophages depends on the local movement of calcium from a sequestered site on the external side of the plasma membrane, through calcium channels in the membrane, to its site of action in the cytoplasm.

2. Materials and methods

Macrophages of normal Balb C/Baily J mice were collected by intraperitoneal lavage and cultured on glass cover slips as in [3] in 0.154 M NaCl, 2 mM $MgCl_2$, 5 mM KCl, 2.5 mM imidazole–HCl (pH 7.0) (SIM) containing 50 μg/ml subtilisin Carlsberg (protease: Type VIII, Sigma, St Louis Mo). After incubation for 20 min, the cultures were fixed in neutral buffered formalin and were then dehydrated in increasing concentrations of ethanol, from which they were dried in air. The dried cells were photographed using phase-contrast optics on a Zeiss photoscope I. The negatives were then printed to a final magnification of 800, and the areas of at least 20 cells were accurately determined using a Ladd digitizer interfaced with a Monroe 1860 calculator.

Additions to the culture medium were present during the entire incubation period. 8-(*N,N*-diethylamine)-Octyl-3,4,5-trimethoxybenzoate–HCl (TMB-8) was a gift of Dr P. W. O'Connell, The Upjohn Co. (Kalamazoo MI). Verapamil–HCl was obtained from Knoll Pharmaceutical (Whippany NY). Ethyleneglycol-*bis*-(β-aminoethyl ether) *N,N'*-tetracetic acid (EGTA) was obtained from Sigma. All other reagents were of reagent quality. Deionized water was used throughout.

Electron microscopy was as in [6].

3. Results

The extent to which macrophages spread under the conditions used in these studies is independent of free $[Ca^{2+}]$ in the medium (fig.1). This confirms [3,7]. However, addition of the Ca^{2+}-antagonist TMB-8 inhibits the spreading process in a dose-dependent manner (fig.2). The dose required for 50% inhibition of spreading is $\sim 1.8 \times 10^{-4}$ M. This inhibitory effect of TMB-8 is not due to a general toxic effect on the cells; they rapidly resume spreading upon removal of the drug. It is apparently not due to an inhibition of glycogen metabolism, since the addition of 8 mM glucose to

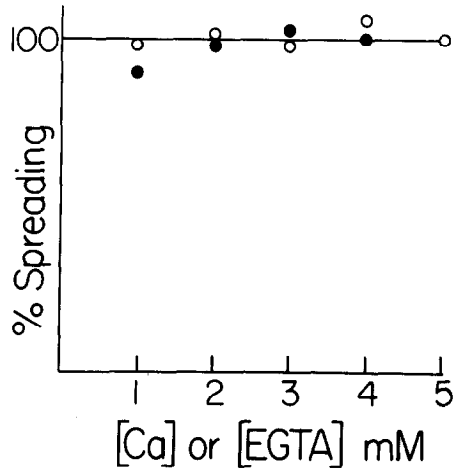

Fig.1. The extent to which macrophages spread in the presence of $CaCl_2$ (•) and EGTA (○). Percent spreading is defined as: (mean area covered by experimental cells/mean area covered by control cells) × 100.

the incubation medium does not abrogate the inhibition of spreading by TMB-8. The inhibition of spreading by TMB-8 is abrogated by the addition of $CaCl_2$ to the incubation medium (fig.3): the greater the inhibition effected by TMB-8, the greater $[CaCl_2]$ required to reverse it.

Since TMB-8 is a drug which inhibits the passive movement of Ca^{2+} across cell membranes, including internal membranes such as the sarcoplasmic reticulum

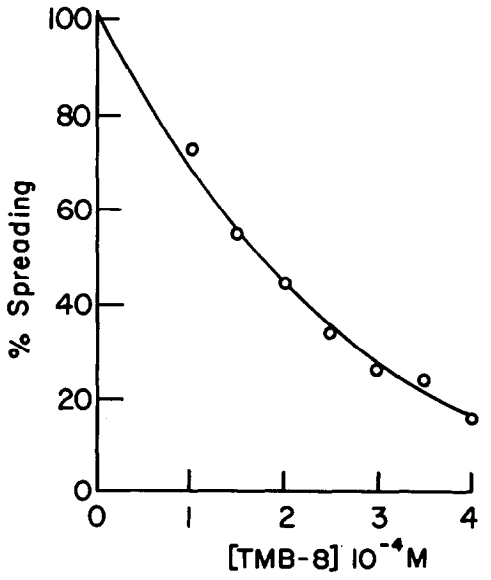

Fig.2. Inhibition of macrophage spreading by TMB-8. Percent spreading is defined in the legend to fig.1.

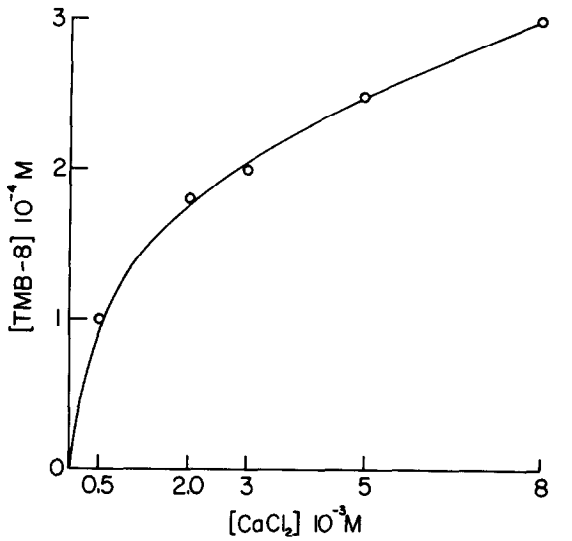

Fig.3. Abrogation by $CaCl_2$ of the TMB-8-induced inhibition of macrophage spreading. Various $[CaCl_2]$ were present during exposure of the cells to different amounts of TMB-8. The $[CaCl_2]$ indicated on the abscissa were those required to effect a 50% abrogation of the TMB-8-induced inhibition at each dose of TMB-8.

[4], and since the spreading of macrophages is not inhibited by 5 mM EGTA in the bulk medium, the above results suggest that TMB-8 inhibits macrophage spreading by inhibiting the movement of calcium across a membrane from a sequestered site within the cell. However, two classic inhibitors of calcium channels in the cell surface membrane [5], verapamil and $LaCl_3$, were also found to inhibit the spreading of macrophages (fig.4). The dose required for 50% inhibition was ~2.7×10^{-4} M in each case.

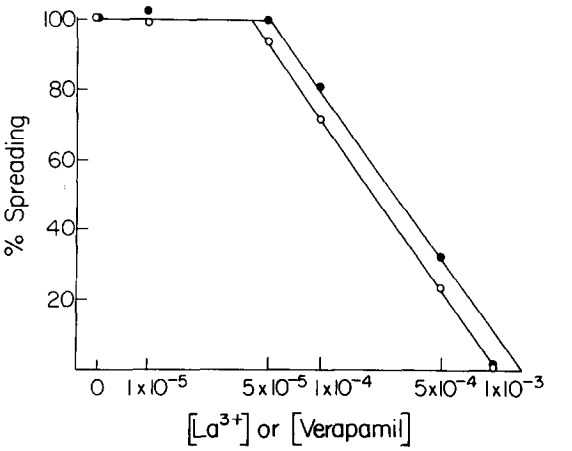

Fig.4. Inhibition of macrophage spreading by verapamil (•) and lanthanum (○) (mol/l).

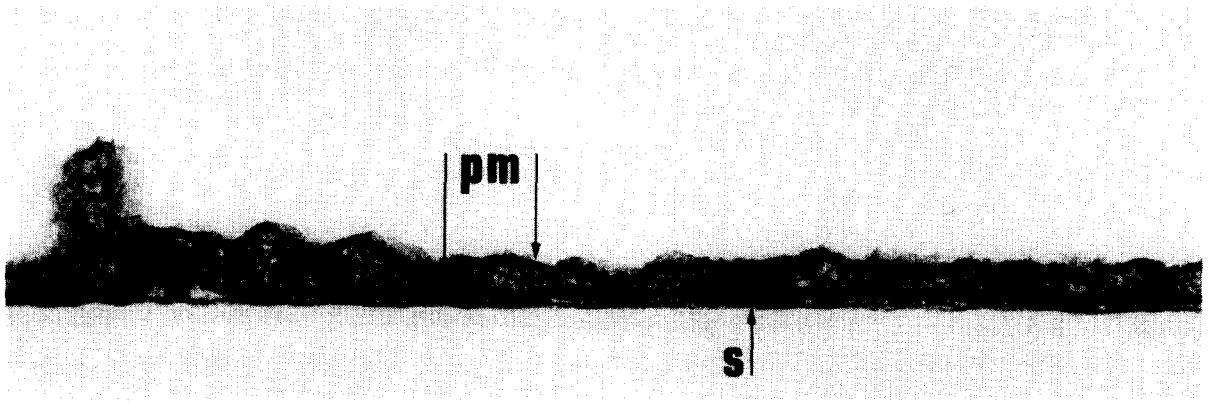

Fig.5. Representative electron micrograph of the peripheral part of a spreading macrophage. Plane of section is normal to the plane of the substratum (S). The only membrane visible is the plasma membrane (PM). ×55 000.

Such studies suggest that if the inhibition of macrophage spreading by TMB-8, verapamil and lanthanum, in the absence of extracellular calcium, is due to the blockage of calcium channels in membranes, then the membrane being blocked is the plasma membrane. This suggestion is supported by our electron microscopic examination of the actively spreading lamellipodia of the macrophages (fig.5). In the majority of micrographs, the only membrane visible is the cell surface membrane.

4. Discussion

These results are consistent with the plasma membrane of macrophages serving as a site of membrane-sequestered calcium. This calcium would have to be tightly bound or protected from rapid exchange with calcium in the medium since it can not be removed by brief exposure to 5 mM EGTA. Yet this calcium would also have to be bound at an external site of the plasma membrane, since the calcium-channel blockers verapamil and lanthanum, as well as TMB-8 (an inhibitor of calcium release from SR membranes and movement across cell membranes), all inhibit the process of spreading. According to this hypothesis, contact of the plasma membrane with an appropriate surface would cause the local movement of calcium into the cytoplasm where it would exert highly localized effects In the absence of continued stimulation, the Ca^{2+} would be actively pumped out of the cytoplasm by a Ca^{2+}-activated ATPase in the membrane.

Data supporting this hypothesis are available from studies on several cell types including platelets, neutrophils, macrophages and others. Calcium fluxes play a significant role in neutrophil secretion, motility, phagocytosis and chemotaxis [8–16], and these calcium fluxes are related to changes in the amount of membrane-associated calcium [8,10,11,17]. Significantly, TMB-8 inhibits neutrophil secretion over $\sim 1.76–7.04 \times 10^{-4}$ M [18]. The platelet release reaction is similarly dependent on the release of membrane-bound calcium [19], and is blocked by TMB-8 [20]. Macrophages also show calcium dependence of motility [1,21,22] and a stimulus-dependent calcium flux [23]. In addition, macrophages [24,25], platelets [26,27] and neutrophils [28] have a membrane associated Ca^{2+}-activated ATPase which, in the case of macrophages and platelets, functions as a calcium pump [25,26]. The macrophage enzyme is known to be associated with the plasma membrane [25]; that of platelets is associated with microsomes [26,27], but these may be composed of plasma membranes [27], especially since the plasma membrane of platelets is continuous with an extensively ramified tubular system within the platelet [29].

The studies cited above generally consider the membrane-bould calcium to be intracellular calcium. However, none of the data excludes the cell surface membrane as the principal source of intracellular membrane-bound calcium. In mouse L-cells it is postulated that a pool of calcium which does not readily exchange with the free calcium in the medium is tightly bound at the cell surface, and, upon stimulation, is released

into the cytoplasm where it may 'trigger' a more generalized release of calcium from the endoplasmic reticulum [30]. A pool of calcium tightly bound to the cell-surface membrane of neutrophils has been demonstrated [17]. In neither case is the calcium readily removed by exposure to EGTA [17,30]. These considerations lead to the postulate that the cell-surface membrane of non-muscle cells may be analogous to the sarcoplasmic reticulum or the T-tubules of striated muscle cells, both of which contain a transmembrane ATP-dependent calcium pump [31,32] and a high-affinity calcium-binding protein [33]. The cell surface membrane could thereby control with exquisite precision both the temporal and spatial distribution of cytoplasmic calcium. Since phagocytosis and cell spreading are thought to be dependent on highly localized, Ca^{2+}-dependent response of the cortical cytoskeletal system to insoluble stimuli [1,8,34], the advantages of having the calcium control at the level of the plasma membrane are obvious. Moreover, during phagocytosis [1] and spreading, as shown here, the pseudopods and lamellipods of macrophages are devoid of membranous organelles other than the plasma membrane [35]. If the drugs used in the present studies are actually blocking membrane channels for calcium, as is thought to be the case, then the calcium channels are most likely part of the plasma membrane.

Acknowledgements

This work was supported by a grant from the American Lung Association, and by a stipend to Ray Quintana from the Southwest Resource Center for Science and Engineering. The authors thank Judi DeLongo for producing the ultrathin sections and Suzanne Newell for typing the manuscript.

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