Role of Sodium in ADP- and Thrombin-induced Megakaryocyte Spreading

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ABSTRACT We investigated the role of sodium in megakaryocyte spreading induced by thrombin and ADP. We found that if extracellular sodium was replaced by lithium, potassium, or choline, spreading was inhibited. When extracellular sodium was present, amiloride or tetrodotoxin inhibited spreading. Using intracellular recording we found spreading to be associated with a permanent membrane depolarization. The extent and rate of thrombin-induced depolarization was reduced when lithium replaced sodium. Unspread cells had an average membrane potential of −44.8 mV. Spread cells had an average membrane potential of −18.46 mV. When choline replaced sodium, or when in the presence of tetrodotoxin and amiloride, the spread cells repolarized, indicating that the depolarization is due to an increase in sodium permeability. Similar treatments did not change the membrane potential of unspread cells. Incubation of megakaryocytes with A23187 together with monensin or methylamine induced spreading. Methylamine occasionally caused spreading by itself, but neither ionophore alone caused spreading. These results indicate that megakaryocyte spreading induced by ADP and thrombin depends on an increase in sodium conductance.

Platelets and megakaryocytes, the platelets' precursor cells, have a number of physiological characteristics in common. Both are activated by the platelet agonists ADP, thrombin, and arachidonic acid, and the activation of these cells is inhibited by prostaglandin E_1 (PGE_1), tetracaine, and dibutyryl cAMP (1–5). The first step in platelet activation is a change in cell shape: filopodia and membrane protrusions form. Isolated, cultured megakaryocytes also respond to platelet agonists by a change in cell shape; filopodia and a ruffled membrane form and the cells, resting on a surface, become adherent and spread out over the substratum much as platelets spread on glass (4). In both cases, the shape change involves the formation of microfilament nets and bundles from an amorphous precursor material (6; R. M. Leven and V. T. Nachmias, manuscript submitted for publication). The mechanism by which various agents, acting at the cell surface, cause the state of the cytoplasm to change in this way is unknown. However, the close similarities in the responses of these two cell types, and the fact that platelets are themselves fragments of the megakaryocyte cytoplasm, suggest strongly that the mechanism of activation of both platelets and megakaryocytes is very similar, if not the same.

Experiments with platelets have recently suggested a possible role for sodium and pH changes as stimuli for the cytoskeletal reorganization that underlies shape change. Feinberg et al. (7) have shown that ADP causes an increased influx of sodium into platelets, although the correlation of shape change with sodium influx was not studied. Simons et al. (8), using membrane potential and pH sensitive dyes, have shown that the intracellular pH increases ~0.3 pH unit in thrombin-stimulated platelets and that the membrane potential depolarizes from a resting level of −50 to −15 mV after stimulation of platelets with ADP or thrombin. In addition, platelet cytoskeletons in vitro can be transformed from a nonfilamentous state to bundles of microfilaments in the presence of either calcium or magnesium by an increase in pH from 6.8 to 7.6 (9).

To test whether or not sodium movement could serve as a part of the mechanism of stimulation of megakaryocyte spreading we used a combination of intracellular recording, sodium channel blockers, and altered extracellular cations. We found that sodium plays a central role in the stimulation of megakaryocyte spreading induced by platelet agonists.

MATERIALS AND METHODS

ATP, bovine thrombin, methylamine and choline chloride were obtained from Sigma Chemical Co. (St. Louis, MO). Choline chloride was used after twice

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recrystallizing from ethanol. A23187, monensin, and tetrodotoxin were obtained from Calbiochem-Behring Corp. (La Jolla, CA). Amiloride was a gift of Merck, Sharpe and Dohme Laboratories (West Point, PA). Micropipette glass was purchased from WPI Instruments, Inc. (New Haven, CN) and trifluoperazine (TFP) from Smith, Kline & French Laboratories (Philadelphia, PA).

Cultures: Megakaryocytes were isolated and cultured as previously described (4).

Spreading: Megakaryocyte spreading was quantified as previously described (4) and is stated again here. To compare populations objectively, cells were assigned to one of four classes: 0, unspread cells; +1, central raised area of the cell containing the nucleus wider than half the total cell diameter; +2, central raised area of the cell equal to half the total cell diameter; and +3, central raised area of the cell less than half the total cell diameter. At least 100 cells in a dish were assigned before, and then again after, the addition of ADP, and each class was expressed as a percent of the total population. The value for each class before addition of ADP was then subtracted from the value for each class after the addition of ADP so that the data could be expressed as the change of the percent of the total population in each class from before to after incubation with ADP (Δ percent spread). This method allows each dish to serve as its own control and also corrects for a small number of cells that spread spontaneously.

Membrane Potential Recordings: Micropipettes with tip resistances of 10–20 megaohms (MΩ) were pulled on a vertical pipette puller (David Kopf Instruments, Tujunga, CA) and filled with 3 M KCl, 0.15 M potassium acetate. Because of their large size, megakaryocytes were easily penetrated under direct microscopic observation on an inverted phase microscope using a De Fonbrune micromanipulator (Aloe Scientific, St. Louis, MO). Electrical signals were amplified ×10 (Dagan 8100 preamplifier; Dagan Instruments, Minneapolis, MN) and fed into an oscilloscope. Membrane potentials were recorded after a stable reading of ~30 s.

RESULTS

Isolated megakaryocytes have been shown to spread in response to ADP and thrombin, and details of the spreading have been previously described (4). An example of this morphological change is shown in Fig. 1.

Extracellular Na^+ Requirement

Tests of the spreading response of megakaryocytes when external sodium was replaced with other monovalent cations showed that spreading requires extracellular Na^+. Megakaryocytes were incubated in Hanks' balanced salt solution (137 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.33 mM Na_2HPO_4, 4.2 mM $NaHCO_3$, 0.1% glucose, pH 7.4) with sodium phosphate and sodium carbonate replaced by the potassium salts and sodium chloride replaced by choline chloride or lithium chloride. Fig. 2 shows an experiment in which spreading of the megakaryocytes was measured in 100% (control), 10%, 1%, and 0.1% Na+ when lithium was used to replace the sodium. Similar results were obtained with choline.

Inhibitors

The role of extracellular sodium in the spreading response was shown in a different way by using the sodium channel blockers amiloride and tetrodotoxin (TTX). Amiloride at millimolar concentrations has been reported to block a Na^+-H^+ exchange (10). TTX is a neurotoxin with high specificity for sodium channels (11). These experiments were done in normal extracellular sodium. The drugs were added 5 min prior to the ADP. Fig. 3 shows the effect of the channel blocker amiloride. At 1 mM, spreading is almost completely inhibited. Amiloride was not toxic since cells spread after the amiloride was washed out. Fig. 4 shows that tetrodotoxin also inhibits spreading. 2 μM inhibits spreading ~50%. Higher concentrations were not tested to see whether complete inhibition could be attained with TTX. In addition we found that 10 μM TFP blocked ADP-induced spreading. This drug at this concentration inhibits calmodulin-dependent reactions (12).

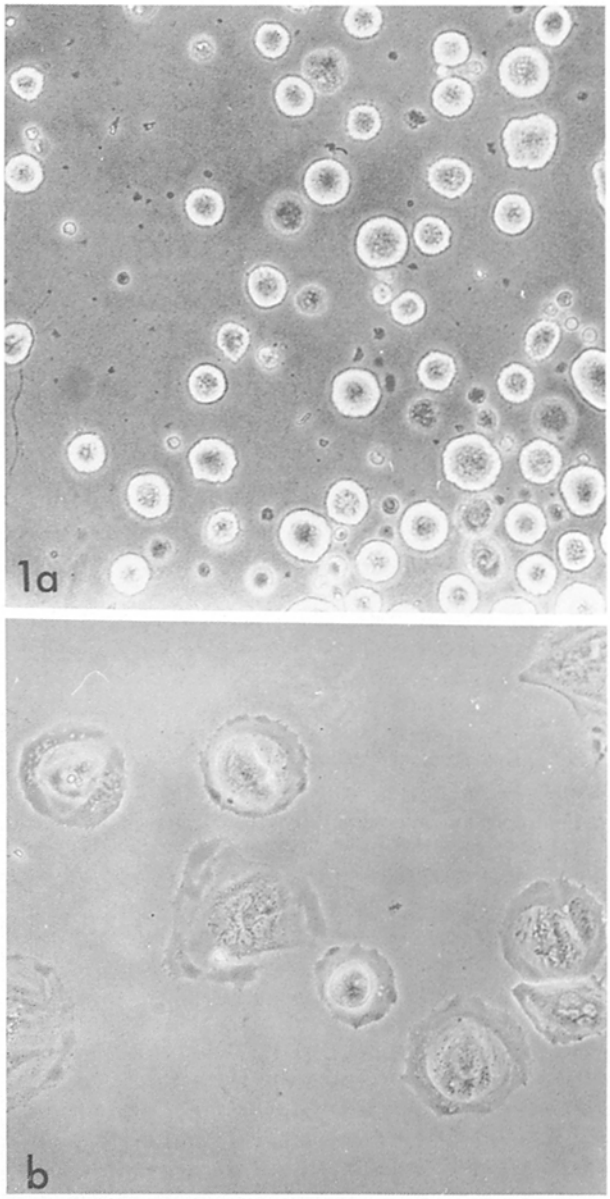

FIGURE 1 Cultured isolated megakaryocytes. (*a*) Resting megakaryocytes which are spherical and slightly adherent to the culture dish. (*b*) An identical culture after 30-min exposure to 10 μM ADP. The cells have now spread and become very adherent. × 265.

Electrophysiology Experiments

Resting megakaryocytes had variable membrane potentials, ranging from about −80 mV to −20 mV (Fig. 5 *a*). We confirmed the previous observation (13) that with a sufficiently large depolarizing current pulse a depolarizing spike occurred (see Fig. 6 *a*). The depolarizing current pulse did not cause cell spreading. The stimulus necessary for this spiking varied from cell to cell but could occur with a pulse of roughly 1–2 nA. We found that replacing Na^+ with Li^+ eliminated this spike (Fig. 5 *b*). We never observed any spiking activity in any spread cells.

Membrane Potentials before vs. after Spreading under Different Ionic Conditions

Since spreading induced by ADP or thrombin required extracellular sodium, there may be a sodium influx in the

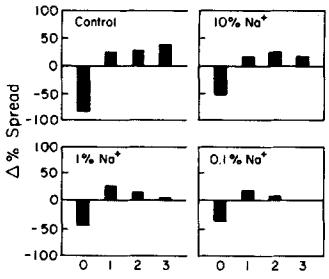

FIGURE 2 Na^+ removal inhibition of spreading. Spreading was measured as described in Materials and Methods. Control is in calcium-magnesium-free Hanks' salt solution. The following panels are spreading measured in decreasing concentrations of sodium. Sodium was replaced by lithium. Spreading was measured after 30-min exposure to 10 μM ADP.

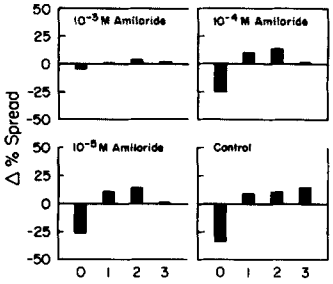

FIGURE 3 Amiloride inhibition of spreading. Inhibition of spreading with increasing concentrations of amiloride added to calcium-magnesium-free Hanks' salt solution. Spreading was measured after the cells were exposed to 10 μM ADP for 30 min.

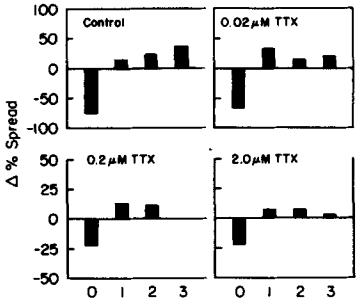

FIGURE 4 Tetrodotoxin inhibition of spreading. Counts of cells spread in response to 10 μM ADP for 30 min after 5-min preincubation in increasing concentrations of tetrodotoxin.

response to these activators. If this is so, one would expect a membrane depolarization to occur, which is what we found. In addition we found that this depolarization was permanent. Membrane potentials of unspread cells and cells previously spread in Eagle's medium were compared after washing in solutions with different cations: isotonic NaCl, KCl, or choline-Cl with 10 mM HEPES buffer adjusted to pH 7.4. The membrane potential of 25 cells was measured in each of the different solutions (Fig. 6). In NaCl the unspread cells had an average membrane potential of −47.7 mV in NaCl (Fig. 6*a*), while the spread cells had a mean membrane potential of −18 mV (Fig. 6*b*). With choline-Cl replacing NaCl, the average membrane potential of unspread cells was −48.4 mV (Fig. 6*c*), an insignificant change from that of the unspread cells in sodium, and the spread cells showed a significant repolarization to an average membrane potential of −35.8 mV (Fig. 6*d*). Finally, we observed that both unspread and spread cells depolarized in isotonic KCl (Fig. 6*g* and *h*). We previously showed that isotonic potassium inhibits spreading (14).

Effect of TTX and Amiloride on Membrane Potential of Unspread and Spread Cells

To further test the sodium dependence of the ADP-induced depolarization, we investigated the effects of sodium channel blockers on the membrane potentials of populations of spread and unspread cells. In some preliminary experiments we found that in calcium-magnesium-free Hanks' salt solution the addition of 2 μM TTX repolarized the average membrane potential

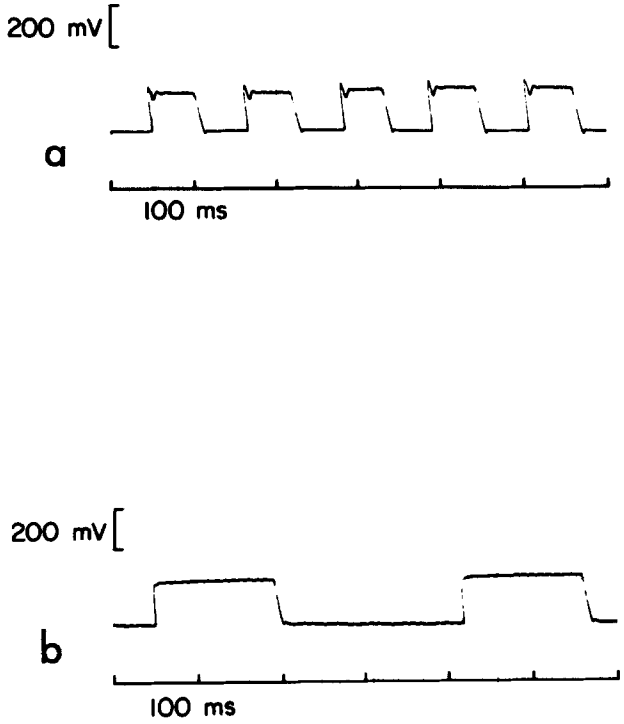

FIGURE 5 Current induced spiking. D.C. potential tracing of megakaryocytes stimulated with a depolarizing current. The abscissa shows the millivolt change from the cell's resting potential; the ordinate is the time base. (*a*) A cell in modified Eagle medium. (*b*) Another cell in calcium-magnesium-free Hanks' salt solution with 5 mM Ca added back and sodium replaced by lithium.

of spread cells from −13.3 to −26.2 mV. The unspread cells had an average membrane potential of −24.4 mV in this experiment. Cells in Dulbecco's modified Eagle medium and 10% serum with the same concentration of TTX and 1 mM amiloride repolarized to an average resting potential in spread cells from −9.0 to −18.4 mV. The unspread cells in the medium with serum had an average membrane potential of −30.2 mV. In a more detailed experiment, in isotonic saline (Fig. 6) 2 μM TTX and 1 mM amiloride repolarized the average potential of spread megakaryocytes from −18.0 to −46.3 mV (Fig. 6*b* vs. *f*). The unspread megakaryocytes in this experiment had an average membrane potential of −47.7 mV (Fig. 6*a*). No significant change occurred in the membrane potential of unspread cells in the presence of these drugs (Fig. 6*a* vs. *e*). It should be noted that the repolarization of spread cells using the Na^+ channel blockers did not cause the spread cells to round up. Statistical comparisons of different populations were made using Student's *t* test (Table I).

Individual Cell Measurements

In addition to comparing populations of cells we also made membrane potential and input impedance measurements on individual cells during the course of spreading in response to ADP and thrombin. While this procedure is more attractive since each cell acts as its own control, it was not used routinely because it was extremely difficult to achieve stable recordings for the time necessary to observe a complete response. When ADP or thrombin was added to megakaryocytes the membrane potential of spreading cells depolarized ~20–60 mV from their resting potential in 1 to 2.5 min after 10 μM ADP (Table II). The response to 1 U/ml of thrombin occurred more rapidly, in

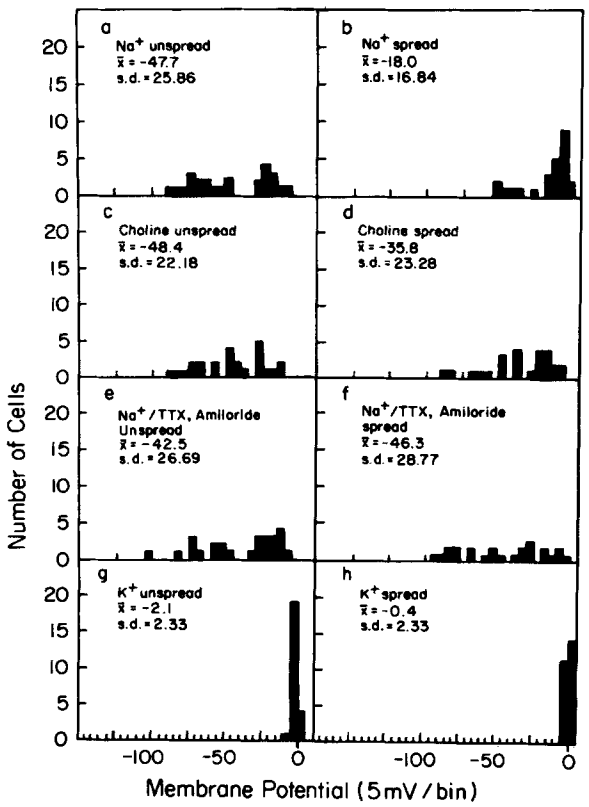

FIGURE 6 Membrane potential distributions. Steady state membrane potentials of populations (25 cells per group) of megakaryocytes in different saline solutions. Cells were in isotonic NaCl, choline-Cl, or KCl buffered with 10 mM HEPES, pH 7.4, as indicated in the panels. The mean ($\bar{x}$) and standard deviation of the population (SD) are given in each panel. *a, c, e,* and *g* are populations of resting megakaryocytes. *b, d, f,* and *h* are populations of megakaryocytes that had spread in response to ADP.

~30 to 45 s, but the magnitude of the depolarization was about the same as with ADP.

Ionophore Experiments

A23187, a divalent cation ionophore, is known to cause platelet shape change (15). We found that exposure of megakaryocytes to 2 μM A23187 caused on occasions some peripheral cytoplasmic ruffling but did not cause spreading. Incubation of megakaryocytes with the monovalent cation ionophore monensin (1 μM), which is most highly specific for sodium (16), also caused no change in megakaryocyte morphology. Incubation of the megakaryocytes in both ionophores together caused almost every cell to ruffle and spread (Fig. 7). Megakaryocytes were also exposed to the weak base methylamine. At neutral pH, methylamine will alkalinize the cytoplasm of cells (17). In two out of five experiments, 5 mM methylamine caused megakaryocytes to spread as with ADP. Many of the cells that spread, spread extensively, but only 30–50% of the cells spread. When added together with 2 μM A23187 the megakaryocytes all spread, as with A23187 and monensin. With both ionophores combined or with A23187 and methylamine together, almost all the cells spread, but individual cells did not spread so extensively as with ADP, thrombin, or methylamine alone.

DISCUSSION

ADP- and thrombin-induced megakaryocyte spreading was inhibited when sodium ions in the medium were replaced by

TABLE I

Statistical Comparison of Megakaryocyte Potentials (From Populations of Cells Shown in Fig. 6)

Na+ unspread (Fig. 6 *a*) −47.7 +/− 25.8	vs.	Na+ spread (Fig. 6 *b*) −18.0 +/− 16.8	0.001
Na+ spread (Fig. 6 *b*) −18.0 +/− 16.8	vs.	Choline spread (Fig. 6 *d*) −35.8 +/− 23.2	0.01
Na+ spread (Fig. 6 *b*) −18.0 +/− 16.8	vs.	Na+/TTX/amiloride, spread (Fig. 6 *f*) −46.3 +/− 28.7	0.001
Na+ unspread (Fig. 6 *a*) −47.7 +/− 25.8	vs.	Choline unspread (Fig. 6 *c*) −48.4 +/− 22.1	ns
Na+, unspread (Fig. 6 *a*) −47.7 +/− 25.8	vs.	Na+/TTX/amiloride, unspread (Fig. 6 *e*) −42.5 +/− 26.9	ns

Statistical comparison of megakaryocyte population membrane potentials. Statistical comparison using Student's *t* test of different megakaryocyte populations from Fig. 6. The first two columns give the two populations being compared for difference between the average membrane potential from those populations. The last column gives the level of significance of the difference between the two populations. The first three groups show a significant difference between the two populations. In the last two groups the populations are not significantly different (*ns*)

TABLE II

ADP- and Thrombin-induced Membrane Potential Changes

Cell	Potential change	Time of change
	mV	*s*
Control		
1	−43 to −52	120
ADP stimulation		
1	−45 to −20	305
2	−62 to −40	54
3	−80 to −35	131
4	−60 to −20	172
5	−67 to −2	149
Thrombin stimulation		
1	−40 to 0	44
2	−60 to +10	45
3	−50 to −2	45

Membrane potentials of megakaryocytes during activation. Membrane potential and input impedance changes and the time of the changes measured in cells during spreading after stimulation with 10 μM ADP or 1 U/ml thrombin.

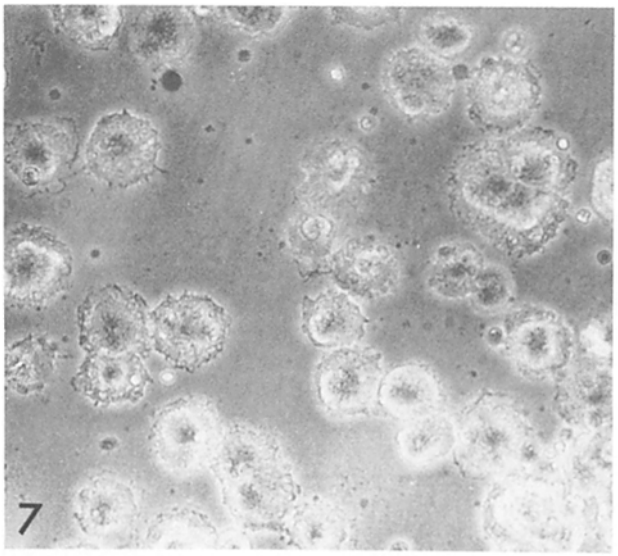

FIGURE 7 Monensin- and A23187-spread megakaryocytes. Megakaryocytes in culture exposed to 10 μM monensin and 2 μM A23187 for 20 min. × 265.

potassium, lithium, or choline, or when sodium channel blockers were present. This suggests that external sodium is required for spreading. The inhibition of spreading in the absence of sodium is probably not due to damage to the cells since we have previously shown that cells that did not spread in the absence of sodium could spread as well as control cells when a normal sodium concentration was restored (14). The electrophysiological studies strongly imply that ADP or thrombin causes an increased sodium conductance. The conductance increase is seen as an increased sensitivity of the membrane potential of spread cells to external sodium. Together these results suggest that there is an increased sodium influx into the megakaryocytes as a result of stimulation with ADP or thrombin.

Since the depolarization of the cells is permanent after spreading, it allowed us to compare populations of spread and unspread cells. If sodium conductance is very low and if sodium is replaced by another ion of low conductance, then no change should occur in the membrane potential. This was in fact observed with unspread megakaryocytes when sodium was replaced by choline. If sodium conductance is high in the spread cells, then their membrane potential should become more negative if sodium is replaced by an impermeant ion such as choline. This is indeed what happened. In isotonic choline chloride the membrane potential of spread cells repolarized an additional −17 mV to reach a value very close to the potential of unspread cells. Therefore these experiments show that spread cells have a greater sodium conductivity than unspread cells and that the increased conductivity could be the source of depolarization in spread cells.

Since TTX and amiloride blocked spreading, it was possible that they did so by blocking the same channels that are the source of the increased sodium conductance. Incubation of cells in TTX and amiloride in isotonic salt solutions repolarized the membrane potential of spread cells so that it was in the same range as that of unspread cells. A smaller effect was seen in tissue culture medium containing serum. This is very likely due to drug binding to the serum proteins which would lower their effective concentrations. This reversal of the depolarization observed in spread cells by sodium channel blockers indicates that the depolarization is due to opening the same sodium channels whose blockage by TTX and amiloride also inhibits spreading. Why both TTX and amiloride affect spreading and membrane potential is not clear. There may be two different sodium channels both of which may open as a result of stimulation with thrombin or ADP.

Ionophore experiments provided further insight into the ionic mechanism of spreading. The calcium ionophore A23187 occasionally caused some ruffling as seen by time-lapse cinematography, but did not cause spreading unless methylamine or the sodium ionophore monensin was also used. This could imply that an increase in cellular calcium is necessary but not sufficient for spreading to occur. Monensin can exchange Na^+ for K^+ or for protons which could cause an intracellular alkalinization. Methylamine will also cause an intracellular alkalinization. This suggests that for spreading to occur the calcium increase must be accompanied by either a Na^+ influx or cytoplasmic alkalinization. Since methylamine itself caused spreading about half of the time when used alone, alkalinization alone may sometimes be enough to cause spreading. This could be because of a direct effect of the pH change on the cytoskeletal proteins or it could be an indirect effect. It has been shown that alkalinization in the physiological range can cause calcium release from sarcoplasmic reticulum vesicles in vitro (18–20). It has been proposed that there are calcium-sequestering membrane vesicles in platelets (21, 22) and therefore perhaps in megakaryocytes as well. It is possible that these vesicles function like the sarcoplasmic reticulum and release calcium in response to an intracellular alkalinization. The alkalinization could come from a Na^+/H^+ exchange. This sort of mechanism has been found to occur during sea urchin egg activation (23, 24) and also as a means of raising intracellular pH during an acid load in a variety of cell types (25–27).

If a sodium-dependent alkalinization leads to calcium release, the next question is: Where exactly could the calcium act? The fact that the spreading of megakaryocytes and other cells (28) is blocked by TFP indicates that a calmodulin-dependent process may be involved in spreading. It has been shown that platelet myosin light chain phosphorylation is mediated through a calcium-calmodulin-dependent process (29–31). This phosphorylation increases the actin-activated ATPase and myosin filament formation (32). Both of these changes in myosin could contribute to the ruffling activity and filament formation that occur during megakaryocyte spreading. This is consistent with our previous observation of myosin in the ruffled membrane of spreading megakaryocytes (4). If there is a requirement for calcium in megakaryocyte spreading, it is unlikely to be from extracellular calcium entering the cells. Since platelets are very similar to megakaryocytes and since platelets do not require external calcium to change shape, by analogy it is very likely that this is also true of megakaryocyte shape change.

The observations are consistent with the following model (Fig. 8). ADP or thrombin binds to its receptor on the megakaryocyte which causes an increase of a TTX- and amiloride-sensitive sodium conductance, presumably opening of sodium channels linked to the appropriate receptor. Sodium enters in exchange for protons. The alkalinization could then lead to

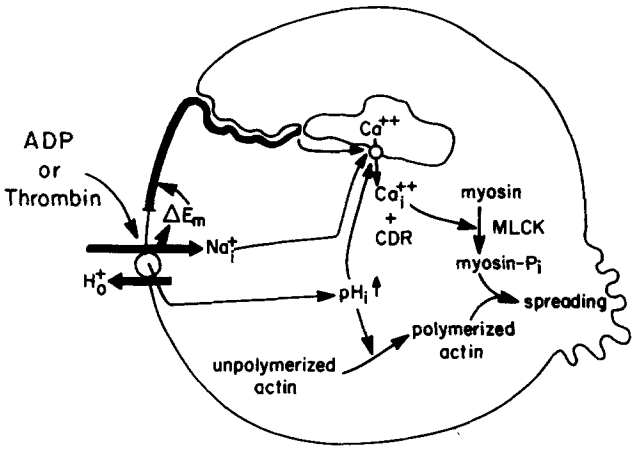

FIGURE 8 Model of the mechanism of ADP- or thrombin-induced megakaryocyte spreading. This model illustrates our model of the ionic mechanism of the stimulus of megakaryocyte spreading. ADP or thrombin binds to a surface receptor which causes a Na^+/H^+ exchange. This leads to a pH change in the cell and to membrane depolarization. Either one of these changes could cause intracellular calcium release. The increased intracellular calcium increase could increase myosin phosphorylation through myosin light chain kinase (MLCK) which would increase its actin-activated ATPase activity and ability to form filaments. The Ca^{++} and pH changes could also cause actin polymerization which is necessary for spreading. Details are given in the Discussion.

release of calcium from a sarcoplasmic reticulum-like storage site in the megakaryocyte. The sodium ionophore monensin by itself does not cause spreading, which appears inconsistent with these first steps in the model. There are some possible reasons for this inconsistency. It could be that since the concentration of K^+ is much higher than that of H^+ inside the cell, monensin may mostly exchange Na^+ for K^+, and cytoplasmic alkalinization would not occur. Alternatively, the monensin may cause a Na^+-H^+ exchange leading to aklalinization but this does not directly cause calcium release. Instead, calcium may be released by a different mechanism linked to the ADP receptor that occurs along with the sodium-dependent alkalinization. This could be effected through release of calcium associated with membrane-bound proteins that could sense the membrane depolarization. Alternatively, the membrane depolarization may carry into the invaginations which form the demarcation membrane system, like the T-tubule of muscle and, by close apposition to an intracellular membrane vesicle, trigger calcium release.

Once calcium is released, this could cause the stimulation of myosin phosphorylation, increasing its contractile activity and ability to form filaments as previously discussed. The pH change may stimulate spreading by causing actin polymerization. The ionic changes deduced are also consistent with the sodium uptake, pH increase and membrane depolarization that occur in ADP-treated platelets (7, 8).

The stimulation of motility by sodium and pH changes may be important in other systems as well. Tilney et al. (33) have found that the acrosome reaction of echinoderm sperm, which most likely occurs by actin polymerization and bundling, can be stimulated by a variety of cation ionophores. This stimulation requires a high external pH, suggesting that protons must leave and cause alkalinization for this process to occur. The motility of polymorphonuclear leukocytes has been shown to be dependent on extracellular sodium (34). Replacement of sodium with sucrose or other monovalent cations decreases random or chemotactic factor-stimulated motility. It has also been shown that chemotactic factor stimulation of neutrophils causes calcium and sodium uptake (35). Sodium uptake in other nonelectrically excitable cells may be similar to that in megakaryocytes since voltage-insensitive TTX channels have been identified in a variety of fibroblastic cells (36–38). Another situation in which intracellular pH has been implicated as a controlling factor is the fertilization-induced microvillar elongation of the new sea urchin egg cortex. It was shown that microfilament bundles were present in cortices of fertilized eggs isolated at pH 7.6 but not at pH 6.8. Also, filament nets formed in cortices which were isolated at pH 6.8 and then transferred to pH 7.6 (39).

The similarity between the ionic dependence of megakaryocyte spreading and such diverse systems as sea urchin gamete activation and leukocyte migration shows that sodium and pH may be of general significance as a mediators of stimulation of nonmuscle cell motility.

While this manuscript was in preparation it was reported that serum or epidermal growth factor induced depolarization of an epithelial cell line was associated with ruffling at the cell periphery (Rothenberg, P., L. Reuss, L. Glaser. 1982. Serum and epidermal growth factor transiently depolarize quiescent BSC-1 epithelial cells. *Proc. Natl. Acad. Sci. USA.* 79:7783–7787).

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