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Evidence for a K^+ Channel Requirement in Spreading of Rat Basophilic Leukemia Cells on Fibronectin-Coated Surfaces

Paola Zigrino,* Guido Gaietta,* Alberta Zambonin-Zallone,† Anthony J. Pelletier,*,[1] and Vito Quaranta*

*The Scripps Research Institute, Department of Cell Biology, 10666 North Torrey Pines Road, La Jolla, California 92037; and †Institute of Human Anatomy, University of Bari, P.zza G. Cesare, 70100 Bari, Italy

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We investigated the ionic requirements for the early events of cell–extracellular matrix interactions leading to cell spreading. We found that potassium ions were required specifically in several cell types. Adhesion to fibronectin- (FN) coated surfaces was independent of K^+ in the medium. In contrast, cells that adhered to FN in the absence of K^+ failed to spread. This requirement for K^+ occurred only during a discrete time frame: in the first 15 minutes following adhesion. Moreover, we identified a specific trans-membrane flux of the radioactive K^+ analog $^{86}Rb^+$, the kinetics of which correlated with this requirement. Both this ion flux and cell spreading were blocked by the K^+-channel inhibitors tetraethylammonium (TEA) and 4-aminopyridine (4-AP). Our results suggest that this K^+ ion flux and the channels that regulate it are important in regulating the initial responses to adhesion that lead to spreading.

Cell adhesion and spreading requires the regulation of several events. Adhesion is mediated primarily by integrins, which are receptors for the various extracellular matrix (ECM) proteins (1,2). Several signaling molecules such as kinases FAK, src, MAPk and others have been implicated in regulating events downstream of cell adhesion involved in cell growth and differentiation (3), but little is known about initial signals regulating early events such as reorganization of the membrane skeleton and recruitment of integrins to the site of adhesion.

Cations have been suggested to play a role in some of these early events. For example, cells adhering to fibrinogen via the integrin αIIbβ3 initiate oscillations in intracellular Ca^{++} (4), and these oscillations were suggested to be required for re-organization of the actin cytoskeleton. Schwartz and coworkers reported alkalinization of cells following adhesion to FN, which was due to activation of the Na^+/H^+ ATPase (5).

Modulation of potassium in cells requires a complex balance of active and passive transport mechanisms. These include the Na^+/K^+ ATPase, which is present in all eukaryotic cells and is responsible for establishing and maintaining the Na^+/K^+ gradient across the membrane, and multiple classes of K^+ channels (6,7). Although extensively studied in excitable cells, these K^+ channels are also present in non-excitable cells, where their role in regulation of cellular functions are poorly understood (6,7).

However, in the mast cell derived cell line RBL-2H3 (RBL) antigenic stimulation causes a biphasic response of membrane potential with an initial depolarization followed within 3–4 min by repolarization. The latter stage of the response is associated with efflux of K^+ ions (8,9) that was required for the antigen-stimulated secretory response (10). In erythroleukemia cells, a hyperpolarization was detected following adhesion to FN that was mediated by K^+ channels (11,12). Thus, it appears that K^+ channels may play several roles related to structural changes in the cell membrane related to secretion and adhesion.

In the present work, we examined the ion requirements for initial events leading to cell spread-

[1] Corresponding author. The Scripps Research Institute, 10666 North Torrey Pines Road, SBR-12, La Jolla, CA 92037. Fax: (619) 554-6251; E-mail: anthonyp@scripps.edu.

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ing. We found that K^+ was required during the first 10–15 minutes following cell adhesion. Using the radioactive potassium analog $^{86}Rb^+$ (9,13) we identified a K^+ flux that occurs during this same time frame. Moreover, both the $^{86}Rb^+$ flux and cell spreading were inhibited by the specific K^+ channel inhibitors 4-AP and TEA. Our data suggest a role for K^+ channels in initial signals following cell adhesion that lead to spreading.

MATERIALS AND METHODS

Cell culture. HeLa (ATCC CCL 2, human cervix epitheliod carcinoma), HT1080 (ATCC CCL 121, human fibrosarcoma), WI38 (ATCC CCL 75, lung, diploid, human) and HaCat (human keratinocytes) (14) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Bio-Wittaker, Walkersville, MD) supplemented with 10% fetal calf serum, penicillin and streptomycin at 100 mg/ml. RBL (2H3) cells (Rat Basophilic Leukemia) were the kind gift of Dr. F. Letourneur (National Institute of Child Health and Human Development, Bethesda, MD) and were grown in the same medium except with 20% FCS. M21 cells (Human Melanoma) were from Dr. D.L. Morton, (John Wayne Cancer Institute, Santa Monica, CA) and were grown in RPMI-1640 supplemented with 10% fetal calf serum and antibiotics as described above. Cells were maintained at 37°C in a 5% CO_2 atmosphere. Confluent cultures of RBL cells were split 1:2 twentyfour hours prior to adhesion assays in order to obtain consistent populations of cells for each assay.

Adhesion assay. Cells were detached with trypsin-EDTA, (Sigma, St Louis, MO) (0.05%), trypsine was inactivated by incubation with trypsine inhibitor, (Sigma) (1mg/ml). Detached cells were washed with Hepes buffered saline, pH 7.4 (HBS) (20 mM Hepes, 150 mM NaCl and 1 mM Glucose) then resuspended in the HBS at 2.5×10^5 cells/ml. $MgCl_2$, $CaCl_2$ and KCl were added to a final concentration of 0.8 mM, 1.8 mM and 5.4 mM, respectively (or as indicated). Non tissue culture treated 48-multiwell plates (Corning Costar Co, Livermore, CA) were coated with bovine fibronectin (FN) (Sigma; 30 μg/ml, in pH 7.4 buffer), or Poly-L-Lysine (Sigma; diluted 1:10 in distilled water) for 2 hours at 37°C. Non-specific binding sites were blocked by a one-hour incubation with heat-denatured BSA, (Sigma) (1% solution in HBS) at 37°C. Cell suspension was added and plates were then placed at 37°C, 5% CO_2 in humidified atmosphere. After 1 hour of total incubation (or as indicated) at 37°C, non-adherent cells were removed by washing twice with HBS. Adhered cells were fixed with 3% formaldehyde in Dulbecco's modified Phosphate Buffered Saline (DPBS, Bio-Wittaker), stained with 0.5% crystal violet in 20% methanol and photographed under phase-contrast microscopy. The inhibitors were used as follows: tetraethylammonium (TEA, from 1 to 50 mM), Ba^{++} ($BaCl_2$, from 0.1 to 50mM), 4-Amynopyridine (4-AP, from 0.1 to 20mM), Apamin (from 5 to 30nM), all from Sigma.

Image analysis. Cell images were recordered by a light microscope, Zeiss Axiovert 35, connected with a CCD camera (Photometric CH250, type KAF1400), with an usable pixel array of 1320 × 1034 and pixel size of 6.8 × 6.8 microns. The A/D conversion was 12-bits. The software used for image acquisition was Mirage (a custom software program for image acquisition and analysis provided by Gary Fan at the National Center for Microscopy and Imaging Research). Projected cell area was determined by using NIH Image (15). Projected cell area of 200–300 cells for each sample was determined in 3 separate experiments. Data presented represent mean (μm^2) ± SE.

Rubidium uptake assays. Potassium transport was assayed by measuring the uptake of the K^+ analog $^{86}Rb^+$ (specific activity 10 mCi/ml, NEN) in the presence or absence of specific inhibitors, tetraethylammonium, Ba^{++} and 4-Amynopyridine. Cells were prepared as previously described (see adhesion assay method) and resuspended in HBS containing 0.8 mM $MgCl_2$ and 1.8 mM $CaCl_2$, at the density of 10^6 cells/ml. Cells were then plated on FN or Poly-L-Lysine coated wells in a non-tissue culture treated 96-multiwell plate (Falcon) (coated as described above) and incubated in the same buffer plus 5mM K^+ at 37°C. At time points following adhesion, the cells were pulse-labeled with 1μCi/ml $^{86}Rb^+$ for two minutes. At the indicated times, medium was removed and cells were washed 3× with ice-cold 0.1 M $MgCl_2$. Cells were allowed to air dry for 1 hour and cell extract obtained using 0.1% SDS. SDS-extracts were made and internalized $^{86}Rb^+$ was quantitated by liquid scintillation and normalized to protein concentration determined from SDS-extracts of parallels wells, (by the microBCA assay, PIERCE).

RESULTS

Adhesion of RBL cells to fibronectin (FN) can be blocked by short synthetic peptide containing the sequence Arg-Gly-Asp (RGD), therefore is mediated by surface receptors belonging to the integrin family (16). We found that when RBL cells were plated on FN in DMEM in the absence of serum, they adhered and spread rapidly. However, when plated in HBS, these cells adhere to, but did not spread on, FN.

In order to determine the requirements for cell spreading, we systematically added components of DMEM to HBS and assayed for cell spreading. We found addition of the metal-ion components of DMEM (5mM K^+, 1.8 mM Ca^{++}, 0.8 mM Mg^{++}) to HBS was sufficient to induce spreading. We then tested the metal ions individually and in combinations for their effect.

In order to do so, we first needed a quantitative definition of cell spreading. Cells were allowed to adhere for 1 hour in HBS containing 5mM K^+, 1.8 mM Ca^{++}, 0.8 mM Mg^{++} on either Poly-L-Lysine or FN, fixed and analyzed at the N.I.H. sponsored imaging facility, The National Center for Microscopy and Imaging Research, as described in Materials and Methods. Projected area from more than 200 cells in each condition was determined. For RBL cells, the mean area of cells adhered to Poly-L-Lysine was $94\mu m^2$ +/− standard deviation of $29\mu m^2$. We defined a cell as spread on FN, if the projected area was greater than 1.5 standard deviations above the mean value on Poly-L-Lysine. Therefore, for RBL cells, any cell with a projected area greater than $137\mu m^2$ is considered to be spread.

As shown in Table 1, when K^+ ions were omitted from the medium containing Ca^{++} and Mg^{++} alone, only 2% of RBL exceeded the minimum defined area for spreading. However, when K^+ was included, 73% of cells spread. Calcium and Magnesium were required for adhesion (data not shown), and therefore their influence on spreading *per se* could not be measured. However, cells adhered equally in the presence or absence of K^+ when Ca^{++} and Mg^{++} were included. Fig. 1 shows the morphology of various cell types adhered to Poly-L-Lysine, or to FN in the presence or absence of K^+. Among the cells adhered to FN in the presence of K^+, there are cells whose spreading appears to be limited by crowding from the confluent monolayer. This accounts for most of the cells that fail to reach the defined minimal spreading area.

Other cell types were tested in these assays although cell-type-dependent variation existed, HeLa, HaCat and M21 all showed a clear dependence of spreading on K^+ (Table 1 and Fig. 1). Of those cells tested, only fibroblasts (HT1080 and WI38) showed no dependence on K^+. Because the most dramatic effects were detected with RBL and because we have characterized these cells previously, we chose to focus on them for subsequent experiments.

We next wanted to determine whether K^+ was required continuously throughout spreading, suggesting it was part of a general requirement for cell fitness, or during a discrete time frame, suggesting it was part of a specific signal cascade required during spreading. Fig. 2 shows the results of reciprocal-shift time-course experiments.

When K^+ was provided to cells during the first 15 minutes following cell attachment and then washed out of the medium, cells proceeded to spread nearly as well as those which remained in K^+ throughout the experiment (Fig. 2). This indicated that K^+ was required only in this period. Since no attempt was made to synchronize adhesion, the fifteen minute time point included many cells that had only recently come in contact with the plate. This is evidenced by the fact that many cells were washed off when the K^+ was removed (Fig. 2). Conversely, cells plated in the absence of K^+ proceeded to spread if potassium was added subsequently (not shown). This indicated that cells adhering in the absence of K^+ were arrested in an early step required for cell spreading, and that the addition of K^+ allowed them to continue defining a window for K^+ requirement in the first 15 minutes after attachment.

Based on this, we hypothesized a specific K^+ flux across the cell membrane during the first 15

TABLE 1

	% of cells exceeding minimum spread area[a]	
Cell type	$-K^+$	$+K^+$
RBL-2H3	2	73
HeLa	29	75
HaCat	22	79
M21	54	81

[a]See test for definition (for each condition at least 200 cells were measured randomly).

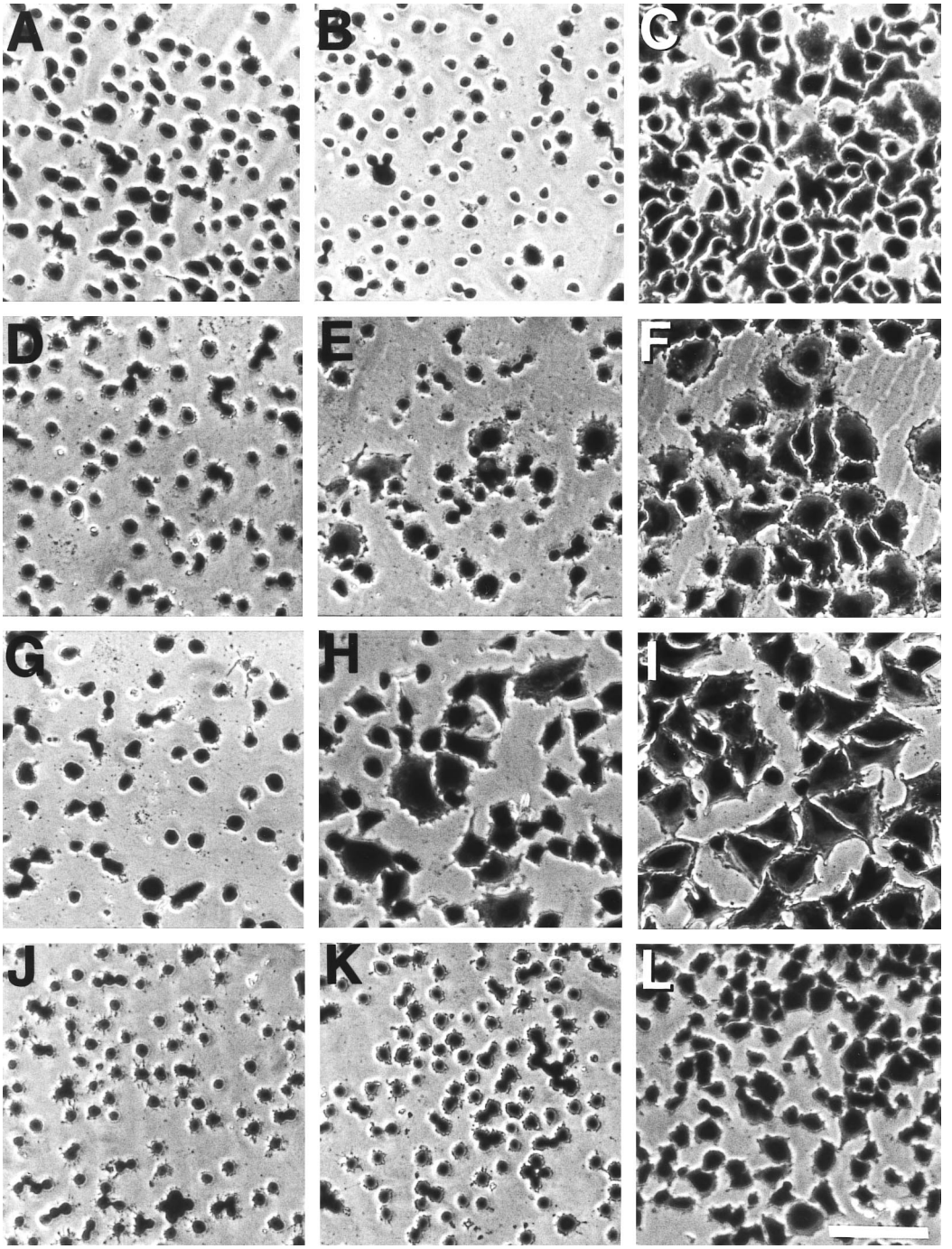

FIG. 1. Spreading of different cell types on fibronectin-coated surfaces is influenced by potassium ions. Cells resuspended in HBS containing Ca^{++} and Mg^{++}, were plated on surfaces coated with Poly-L-Lysine only (A,D,G,J), or fibronectin (all others) and incubated for 60 minutes at 37°C either in the presence (C,F,I,L) or absence (B,E,H,K) of K^{+}. RBL cells (A,B and C), HeLa cells (D,E and F), M21 cells (G,H and I) and HaCat cells (J,K and L) all showed enhanced spreading in the presence of K^{+}. Bar = 0.1mm.

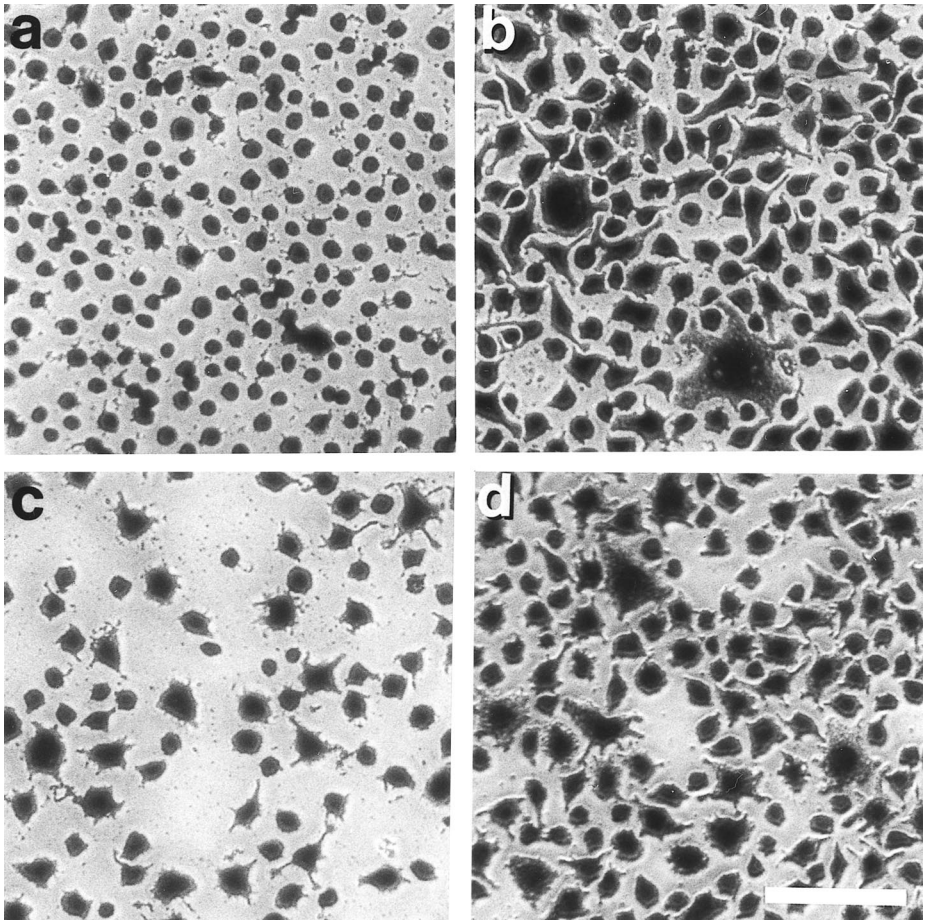

FIG. 2. Potassium is required only during the first 15 min following adhesion. RBL cells were plated on fibronectin-coated surfaces in HBS containing Ca^{++} and Mg^{++} in absence (a) or presence (b) of K^+, and incubated for 60 minutes at 37°C. For c and d, cells were washed with K^+-free medium at fifteen (c) or thirty (d) minutes following plating, and incubated for the remaining time (total of 60 minutes) in K^+-free medium. Bar = 0.1mm.

minutes after cell adhesion. To test this, we used the radioactive K^+ analog $^{86}Rb^+$, as tracer for the K^+ flux (9,13). Cells were allowed to attach to FN in the presence of K^+. At time points following the initial adhesion, cells were pulse labeled with $^{86}Rb^+$ and internal uptake was quantitated as described in "Materials and Methods". The results in figure 3 show a nearly 4-fold increase in $^{86}Rb^+$ uptake, peaking at 10 minutes following adhesion and returning to baseline within 15 minutes. This time-course agrees well with the time window for K^+ requirement as determined in the reciprocal-shift experiments described above.

Many types of K^+ channels have been described, regulating both influx and outflux of K^+, and each category of channel shows different sensitivities to inhibitors (6,7). 4-aminopyridine (4-AP), which is membrane-permeant, blocks voltage-gated K^+ channels and is most specific for the "A-current" subtype of voltage-gated channels. Tetraethylammonium (TEA), when applied externally, also blocks voltage-gated K^+ channels, including the "delayed rectifier" and "A-current" (6,7,17,18). In contrast, externally applied Ba++ blocks inward rectifier K^+ channels (6,7) and apamin, a honeybee-venom-derived neurotoxin, blocks calcium-dependent K^+ currents (6,7,19).

To identify the type of K^+-channel involved in cell spreading, we used these specific inhibitors in an attempt to block the flux. Figure 4A shows the effect of 4-AP on $^{86}Rb^+$ uptake following cell

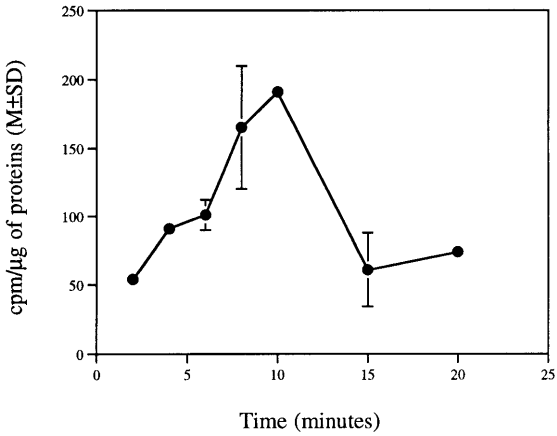

FIG. 3. Adhesion of RBL cells to fibronectin results in uptake of $^{86}Rb^{+}$. RBL cells were plated on fibronectin-coated surfaces and at time points following the initial adhesion, $^{86}Rb^{+}$ was added for a two-minutes pulse-label. The data are from a single representative experiment. Each point is the mean of three samples ± one standard deviation.

adhesion. At 10 mM, the flux is reduced substantially and is abolished by 20 mM. We also examined the ability of this drug to inhibit cell spreading and the results, shown in Figure 4B, show reasonable correlation between inhibition of the K^{+} flux and inhibition of cell spreading. Similar results were obtained with TEA (Table 2). In contrast, barium (up to 50 mM) and Apamin (up to 30 nM) had no effect on either the $^{86}Rb^{+}$-flux or cell spreading (Table 2). Taken together, these data implicate a voltage-gated K^{+} channel in the early $^{86}Rb^{+}$-flux we detected, and suggest that this flux is responsible for the K^{+} requirement of cell spreading.

Measurements of $^{86}Rb^{+}$ uptake were made for cells plated on Poly-L-Lysine, which induces adhesion via an integrin-independent mechanism. Although cells do not spread on this substrate (fig. 1), a measurable increase in $^{86}Rb^{+}$ uptake was detected (data not shown). This response did not show the same pharmacology of inhibition as that seen on FN and may be due to an effect of the poly-ionic substrate on the cell's electrical gradient.

DISCUSSION

It is generally recognized that spreading of cells on extracellular matrix proteins requires signals that follow adhesion. The nature of these regulatory signals is not yet well characterized. In this work, we present evidence that spreading requires an early trans-membrane K^{+} flux that follows adhesion RBL cells plated on FN in HBS including magnesium, Ca^{++} and K^{+}, adhered and spread within one hour. In contrast, those plated in the same medium lacking K^{+} adhered well, but failed to spread. Reciprocal-shift time-course experiments indicated that K^{+} was required only during a specific fifteen-minute time frame immediately after adhesion. Experiments following $^{86}Rb^{+}$ flux

TABLE 2

Inhibitors	$^{86}Rb^{+}$-uptake Inhibition (effective concentration)	Spreading Inhibition
4-AP	yes (20mM)	yes (1–10mM)
TEA	yes (1mM)	yes (40mM)
Apamin	n.d.	no (up to 30nM)
Ba^{++}	no (up to 50mM)	no (up to 50mM)

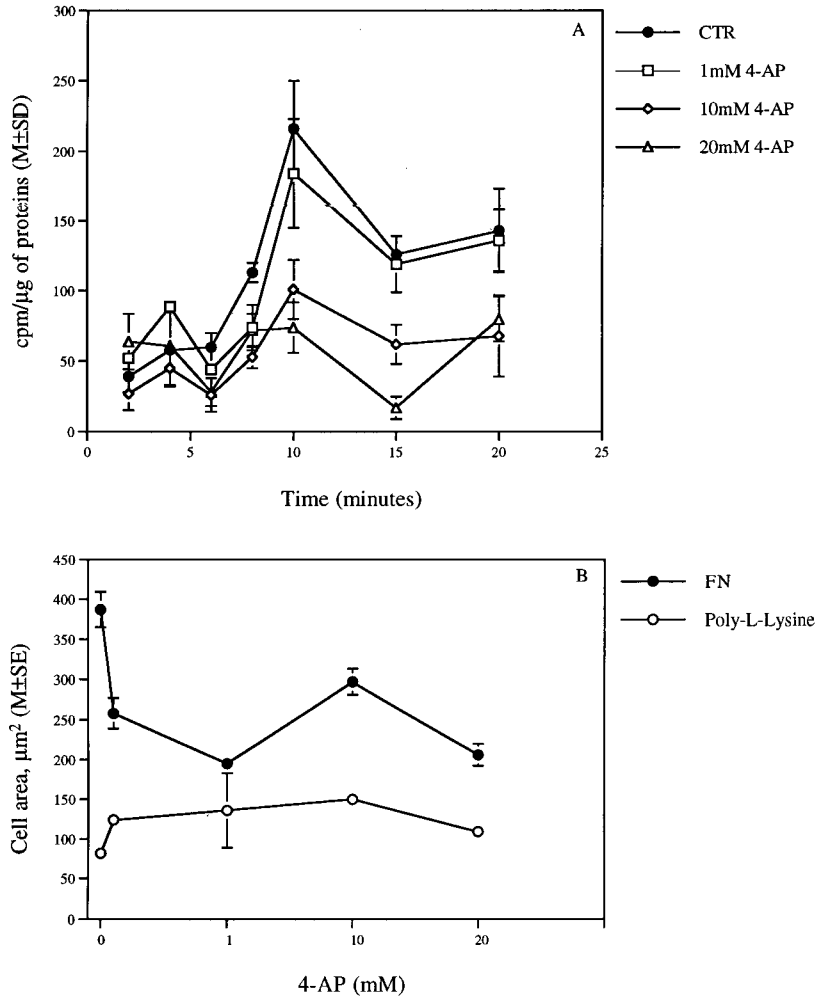

FIG. 4. 4-AP inhibits $^{86}Rb^{+}$ uptake and cell spreading. Varying doses of 4-AP were added to RBL cells suspended in complete buffer prior to plate on fibronectin-coated surfaces. (A): effect of 4-AP on $^{86}Rb^{+}$ uptake; (B): dose response curves for the effect of 4-AP on projected cell area on FN or Poly-L-Lysine. The data in panel A are from a single representative experiment. Each point is the average of three samples. The data in panel B represent the mean area of 200 for each point ± standard error.

suggested an influx of K^{+} within the same time period. Furthermore, we found that the inhibitors of K^{+} channels capable of inhibiting the $^{86}Rb^{+}$ flux also inhibited cell spreading, while those that failed to inhibit the $^{86}Rb^{+}$ flux had no effect on cell spreading.

Two types of K^{+} channels have been identified previously on RBL cells: Lindau and coworkers (20) identified a K^{+}-selective inward rectifier that functions in maintaining the resting potential of the RBL cells at −80mV; Labrecque and coworkers (8) provided evidence for an outward-directed K^{+} channel responsible for repolarization following antigen-induced depolarization.

The pharmacology of inhibition that we observed is most consistent with voltage-gated channels conducting the K^{+} current, since TEA and 4-AP inhibited both $^{86}Rb^{+}$ flux and cell spreading. These channels generally regulate an outward flux of K^{+} to repolarize membranes (6,7). However, here these inhibitors appear to inhibit an inward flux. Barium, an inhibitor of inward rectifier K^{+} channels, was unable to inhibit the uptake. We initially attempted to measure an outflux of K^{+}

directly by pre-loading the cells with $^{86}Rb^+$ prior to plating, but were unable to do so perhaps because of the limited sensitivity of this type of assay.

It is possible that the influx we measured was actually responsible for re-establishing the chemical gradient, and its sensitivity to 4-AP and TEA was due to inhibition of an earlier (outward) repolarization flux by these reagents. In this hypothesis, adhesion results in depolarization, which activates a voltage-gated outward K^+ channels to repolarize the membrane. Subsequently the Na^+/K^+ gradient must be re-established, perhaps by the Na^+/K^+ ATPase. The event that initiates the ion flux is not yet known. However, it has been reported that adhesion of RBL cells to FN enhances antigen-stimulated secretion (16,21), and that this secretion is associated with a K^+ efflux (9). Based on our data, it seems likely that adhesion to FN is associated directly with modulation of K^+ channels. This may help to explain the FN-mediated enhancement of secretion.

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