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20. Lind and Hem (*12*) have estimated that waters in the *p*H range of Mount Moosilauke spring water (*p*H 4.6) can maintain approximately 0.85 ppm of aluminum in solution without the benefit of organic complexation. The average aluminum concentration of 0.75 ppm in fir zone spring water is within the limit of that calculated aluminum solubility value.
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Calcium Transport Across the Plasma Membrane: Stimulation by Calmodulin

Abstract. *Active transport of calcium into inside-out vesicles of red blood cell membranes was stimulated equally by (i) the purified protein activator of calcium-activated, magnesium-dependent adenosinetriphosphatase isolated from red cell hemolyzates and (ii) calmodulin, a protein activator of cyclic nucleotide phosphodiesterase isolated from bovine brain. The results provide further evidence for the identity of red blood cell activator and calmodulin and show that this cytoplasmic protein may participate in the regulation of plasma membrane calcium transport.*

The plasma membrane of the human red blood cell (RBC) contains an active Ca^{2+} transport system that uses adenosine triphosphate (ATP) as an energy source (*1, 2*). Membranes isolated from human RBC's contain a Ca^{2+}-activated, Mg^{2+}-dependent adenosinetriphosphatase activity that is believed to be an expression of this transport process (*3*). In isolated RBC membranes this activity can be enhanced by the addition of a small acidic protein found in RBC hemolyzates (*4, 5*). This protein, referred to here as RBC-activator, shares many of the properties of a protein recently named calmodulin (*6*). Calmodulin, also known as modulator protein (*7*) and Ca^{2+}-dependent regulator (CDR) (*6*), has a molecular weight of approximately 17,000 and has been shown to activate adenosine 3′,5′-monophosphate (cyclic AMP) phosphodiesterase (*8*) and adenylate cyclase (9). Jarrett and Penniston (*10*) reported similar amino acid compositions and electrophoretic mobilities of human RBC activator and beef brain calmodulin.

Calmodulin was recently shown to mimic RBC activator in stimulation of (Ca^{2+} + Mg^{2+})-adenosinetriphosphatase of isolated RBC membranes (*11, 12*), and preliminary reports indicated that both crude hemolyzate (*13*) and purified RBC activator and calmodulin (*14*) were capable of stimulating active transport of Ca^{2+} into inside-out (IO) vesicles of RBC membrane.

Red blood cell activator and calmodulin are part of a family of Ca^{2+}-binding proteins that includes the Ca^{2+}-binding regulatory protein of skeletal muscle, troponin C (TnC). In some systems TnC and calmodulin substitute for one another (*15, 16*). Troponin C was found to be about 1000 times less potent than calmodulin in stimulating the (Ca^{2+} + Mg^{2+})-adenosinetriphosphatase activity of RBC membranes (*11*). Considering the similarities between RBC activator and calmodulin, we compared these two proteins along with TnC in their capacities to stimulate Ca^{2+} transport.

The IO vesicles were made by the methods of Steck and Kant (*17*) with certain modifications (*18*). The vesicle preparation contained 45 to 55 percent IO vesicles, as determined by measuring acetylcholinesterase (AchE) activity in the presence and absence of Triton X-100 (*17*). Data are expressed in terms of the IO membrane protein. We did not separate IO vesicles from right-side-out and broken membranes. The dextran gradient normally used for this (*17*) caused significant loss of Ca^{2+} uptake activity.

Calcium uptake experiments were performed as described in the legend of Fig. 1 except that ouabain was not always present. Uptake was the same with or without ouabain. Some experiments were performed with calmodulin or TnC rather than RBC activator. Calcium uptake was approximately a linear function of time for only the first 6 minutes. Samples were taken every 2 minutes and transport rates were determined by linear regression of the four data points.

Adenosinetriphosphatase assay conditions were the same as transport conditions except that in all cases 0.1 m*M* ouabain was present as well as 1 μM ionophore A23187. Ouabain-insensitive activities were assayed for by measuring the concentration of inorganic phosphate (P_i) 5 minutes after the addition of ATP and again 90 minutes later; P_i was measured by an automated method of Fiske and Subbarow (*19*).

The RBC activator protein was purified from RBC hemolyzates by a method that will be described in detail elsewhere (*20*). Vanaman *et al.* (*21*) found a molecular weight for calmodulin of 16,723 based on amino acid analysis, and this value was assumed in our calculations.

Concentrations of RBC activator, calmodulin, and TnC were determined by the Bradford protein assay (*22*), using bovine serum albumin as the standard. Ionophore A23187 was from Eli Lilly & Co., $^{45}CaCl_2$ from ICN Chemical and Radioisotope Division, and ATP from Boehringer Mannheim.

Uptake of Ca^{2+} into IO vesicles can be stimulated by addition of purified Ca^{2+}-binding proteins. Figure 1 shows data from a single experiment with RBC activator. Maximum activation of uptake was achieved with an RBC activator concentration of approximately 0.39 μg/ml (23 n*M*). The results show that Ca^{2+} is taken up into IO vesicles in an ATP-de-

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pendent fashion and that RBC activator increases the rate of uptake. It should be noted that these results demonstrate uptake into IO vesicles of RBC membranes. The transport process being monitored normally functions to extrude Ca^{2+} from the RBC and to maintain very low intracellular Ca^{2+} concentrations (*3*).

Table 1 lists data from three experiments on separate preparations, all conducted 1 day after the initial blood drawing and vesicle preparation (day 1). Vesicles on day 1 took up Ca^{2+} at a mean rate of 6.8 nmole per milligram of IO vesicle protein per minute in the absence of added RBC activator. This was taken as the basal uptake rate. The mean Ca^{2+} uptake rate in the presence of 0.58 μg of RBC activator per milliliter (defined as the activated uptake rate) was 14.4 nmole/mg-min. An estimate of IO vesicular volume was made. Assuming that in the presence of 1.5 μM A23187 the free concentration of Ca^{2+} is equal inside and outside, the vesicular volume can be calculated from the difference between ionophore-treated vesicles and the background binding of Ca^{2+} in the absence of ATP and ionophore. The number of $^{45}Ca^{2+}$ counts remaining with the vesicles after treatment with ionophore was taken as the sum of Ca^{2+} inside plus Ca^{2+} bound. The mean vesicular volume was 17.4 μl per milligram of IO vesicle protein. Therefore, vesicles apparently concentrate Ca^{2+} inside to a total of approximately 5 mM during the 6 minutes shown in Fig. 1 (that is, when fully activated). This uptake represents loss of approximately 12 percent of the total extravesicular Ca^{2+} under these conditions.

Calmodulin from bovine brain also stimulated Ca^{2+} uptake into IO vesicles. Figure 2 is a semilogarithmic plot of the activation of Ca^{2+} uptake (normalized to the maximum uptake in the presence of RBC activator in each preparation) as a function of the concentration of added protein. We could find no consistent difference between RBC activator and calmodulin in terms of their potency and efficacy in stimulation of Ca^{2+} uptake. Assuming equal molecular weights of 16,723 for RBC activator and calmodulin (*21*), the apparent dissociation constant, K_d, was approximately 4.4 nM at 25°C. Troponin C mimicked the stimulatory effects of these proteins (Fig. 2) but was much less potent, as anticipated from its low potency in stimulating (Ca^{2+} + Mg^{2+})-adenosinetriphosphatase of isolated membranes (*11*).

Under these conditions, the ouabain-insensitive adenosinetriphosphatase activity of RBC membranes consists mainly of (Ca^{2+} + Mg^{2+})-adenosinetriphosphatase activity and includes a small component of activator-insensitive Mg^{2+}-adenosinetriphosphatase activity (*4*). The magnitudes of the mean basal and maximally activated ouabain-insensitive activities were 8.3 ± 0.2 and 17.5 ± 0.8 (± standard deviation, N = 2) nmole of P_i per milligram of vesicle protein per minute, respectively, for 2-day-old vesicles. The apparent K_d of RBC activator for stimulation of this ouabain-insensitive activity was 3.6 nM at 25°C (*23*). Thus, the concentration of RBC activator that produced half-maximal stimulation of adenosinetriphosphatase activity of IO vesicles was about the same as that required for half-maximal stimulation of transport.

In activation of the plasma membrane Ca^{2+} pump, Ca^{2+}, ATP (*1*), and RBC activator protein (*24*) interact with sites on the cytoplasmic face of the membrane. The IO vesicles are a convenient preparation in which one can control the concentration of such membrane-impermeant substances at the cytoplasmic face. It should be emphasized that the Ca^{2+} uptake rates reported here are comparable to RBC transport rates (*25*) and are considerably greater than previously reported IO vesicle rates (*14, 26, 27*). The present rates were obtained by omitting an ultracentrifugation step on a dextran gradient. This step may be employed to obtain fractions enriched in IO vesicles (*17*) but we found, as did Quist and Roufogalis (*28*), that exposure to dextran results in severely depressed adenosinetriphosphatase and Ca^{2+} uptake activities (*14, 27*). The same authors (*28*) found that a concentrated soluble protein fraction from RBC's stimulated the (Ca^{2+} + Mg^{2+})-adenosinetriphospha-

Table 1. Calcium uptake into inside-out vesicles of RBC membrane. Data are from three separate vesicle preparations. All experiments were conducted on day 1. Uptake rates are expressed as nanomoles per milligram of IO vesicle protein per minute and are corrected for non-IO vesicle protein. Activated uptake rates were determined in the presence of 0.58 μg of RBC activator per milliliter. The Ca^{2+} ionophore accessible volume is expressed as microliters per milligram of IO vesicle protein; S.D., standard deviation.

Experiment	Uptake rate (nmole/mg-min)		Activation (%)	Ca^{2+} ionophore accessible volume (μl/mg)	Calculated intravesicular Ca^{2+} at 6 minutes (mM)
	Basal	Activated			
1	6.3	14.2	125		
2	7.0	15.2	117	20.3	4.5
3	7.2	14.6	103	14.3	6.1
Mean ± S.D.	6.8 ± 0.2	14.4 ± 0.2	115 ± 11	17.4 ± 4.4	5.3 ± 1.3

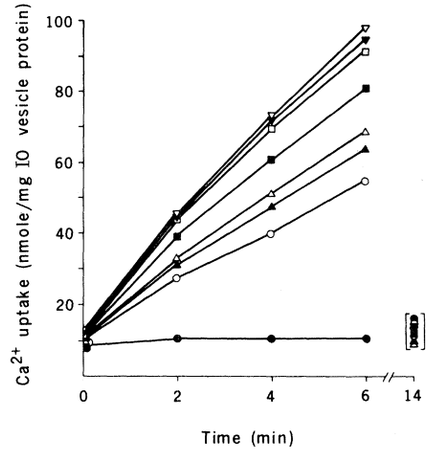

Fig. 1. Time-dependent uptake of Ca^{2+} by RBC membrane vesicles in the presence of various concentrations of RBC activator. The IO vesicles, prepared as described in (*18*), were incubated (0.19 mg of protein per milliliter final volume) for 30 minutes at 25°C in a constantly stirred solution containing (in millimoles per liter) 9 histidine-imidazole (*p*H 7.1), 0.6 tris-glycylglycine (*p*H 7.1), 40 NaCl, 7.5 KCl, 3 $MgCl_2$, 0.15 $CaCl_2$ (Ca^{2+} specific activity, 1.29 × 10^6 cpm/μmole), 0.1 ouabain, and various concentrations of RBC activator (in micrograms per milliliter): (●) no ATP and no RBC activator, (○) no RBC activator, (▲) 0.024, (△) 0.048, (■) 0.096, (□) 0.192, (▼) 0.384, and (▽) 0.576. Reaction was initiated by addition of 3 mM ATP. Samples of 150 μl were taken 8 seconds after addition of ATP and every 2 minutes thereafter and were quenched in 1.5 ml of cold 40 mM tris-glycylglycine buffer (*p*H 7.1) that contained 0.1 mM $MgCl_2$. Vesicles were trapped on 0.45-μm microporous filters (Amicon); the filters were washed once with the same buffer and were dissolved and counted for $^{45}Ca^{2+}$ in Aquasol (New England Nuclear). These results demonstrate lack of Ca^{2+} uptake in the absence of ATP, basal uptake (uptake in the presence of ATP but no added RBC activator), and increased Ca^{2+} uptake in the presence of increasing amounts of RBC activator. After the 6-minute sample was taken, 1.5 μM ionophore A23187 was added to each reaction and samples were taken at 14 minutes. As shown by the points on the right, addition of ionophore caused loss of the accumulated Ca^{2+} in all samples to the level of the ATP-free control, demonstrating that the uptake of Ca^{2+} in the presence of ATP occurred against an electrochemical gradient.

tase activity of IO vesicles of RBC membrane (not dextran treated). However, they did not find an increase in Ca^{2+} uptake rate above the basal rate. By contrast, we found an approximate doubling of the Ca^{2+} transport rate with purified RBC activator or calmodulin. We have no ready explanation for this difference.

The vesicular preparation employed in our experiments is significantly contaminated with leaky "bags" of membrane and probably some sealed right-side-out vesicles (*17*). The presence of these contaminating species does not influence the qualitative nature of the findings. Presumably, only sealed IO vesicles are capable of taking up and concentrating Ca^{2+} and these can be estimated by measuring membrane sidedness (*17*). Leaky membranes exhibit (Ca^{2+} + Mg^{2+})-adenosinetriphosphatase activity but would not exhibit net pumping. Thus, this preparation does not lend itself to obtaining meaningful estimates of pump stoichiometry, an area of some controversy (*29*).

We found no consistent difference between RBC activator and calmodulin in the ability to stimulate Ca^{2+} transport or adenosinetriphosphatase and conclude that they are functionally, if not absolutely, identical. Wang and Desai (*30*) isolated a protein from bovine brain that binds calmodulin and thereby antagonizes its activation of phosphodiesterase. It was shown that this protein, called modulator binding protein, also antagonizes adenosinetriphosphatase and transport stimulation by RBC activator, further demonstrating the equivalence of RBC activator and calmodulin (*27*).

It seems clear that the uptake of Ca^{2+} into IO vesicles is a demonstration of active plasma membrane Ca^{2+} transport. It is also apparent that RBC activator or calmodulin is capable of stimulating the rate of Ca^{2+} transport across the RBC plasma membrane. The stimulation of Ca^{2+} transport is mediated by stimulation of a membrane-bound (Ca^{2+} + Mg^{2+})-adenosinetriphosphatase activity but the mechanism is not defined. This effect is not simply one produced by all Ca^{2+}-binding proteins. Neither TnC (Fig. 2) nor parvalbumin (*11*) is very potent in this effect. Red blood cell activator (calmodulin) is a readily soluble protein, not an intrinsic membrane-bound protein. Thus, the possibility that certain soluble proteins may interact with the cytoplasmic face of the plasma membrane to modulate active transport processes is established. This is worth emphasizing because active transport processes in mammalian cells have been thought to be associated with intrinsic,

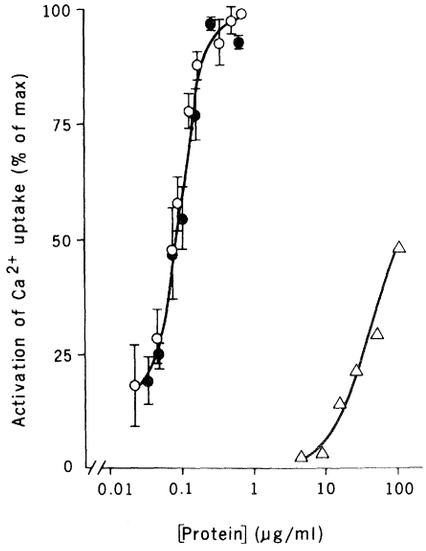

Fig. 2. Stimulation of Ca^{2+} uptake into IO vesicles by RBC activator, calmodulin, and TnC: semilogarithmic plot of protein-stimulated Ca^{2+} uptake against protein concentration. Experiments were performed as described in Fig. 1. Vesicles were incubated at 25°C with various concentrations of (○) RBC activator (N = 4), (●) calmodulin (N = 3), or (△) TnC (N = 1). The ordinate is the percentage of maximum Ca^{2+} uptake stimulation obtained with RBC activator in each preparation. Each point represents the mean ± standard deviation.

membrane-bound proteins. Whether stimulation of Ca^{2+} transport by soluble Ca^{2+}-binding proteins extends to other membranes such as mitochondria or sarcoplasmic reticulum (*31*) is an important question that remains to be answered.

The available evidence implicates calmodulin in the modulation of a number of Ca^{2+}-dependent cellular functions, including regulation of the mitotic apparatus (*32*) as well as activation of adenylate cyclase (*9*), phosphodiesterase (*8*), myosin light chain kinase (*33*), and the plasma membrane Ca^{2+} pump. Not incidentally, activation of the plasma membrane Ca^{2+} pump would serve to terminate the other Ca^{2+}-dependent functions.

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Rank Order of Sarcoma Susceptibility Among Mouse Strains Reverses with Low Concentrations of Carcinogen

Abstract. *Ten mouse strains in which aryl hydrocarbon hydroxylase can be induced, or F_1 hybrids of these strains, were ranked according to their sarcoma susceptibility when exposed to a high concentration (5 percent) of the chemical carcinogen 3-methylcholanthrene. This rank order was reversed when the concentration of 3-methylcholanthrene was reduced to 0.05 percent.*

Tumor induction with chemical carcinogens is known to differ among various inbred mouse strains (*1*). An important determinant in tumor susceptibility is the inducibility of the enzyme aryl hydrocarbon carboxylase (AHH; locus gene symbol *Ah*). Mice that carry the dominant Ah^b allele respond to the injection of aromatic hydrocarbons (including 3-methylcholanthrene) by producing increased concentrations of AHH and a high incidence of solid tumors. In contrast, mouse strains homozygous for the recessive allele Ah^d are not AHH inducible and do not easily produce solid tumors (*2*).

In addition to the large differences in tumor inducibility due to the *Ah* locus, there are smaller differences in tumor inducibility among the AHH-inducible mouse strains. It has been thought that these small differences may have an immunological basis because the AHH system itself does not account for all findings (*3*).

While producing tumors with two different concentrations of 3-methylcholanthrene (MCA) in six inbred mouse strains and four F_1 hybrids (all AHH inducible), we discovered a paradoxical result that we discuss in this report: the mouse strains most susceptible to tumor induction with a high concentration of MCA were the least susceptible at a low concentration. Conversely, the mouse strains least susceptible to tumors with a high concentration of MCA were the most susceptible at low concentrations.

Four- to five-week-old mice (Animal Resources, Jackson Laboratory) were used throughout the study. The inbred mice were females of the strains C3H/HeJ, CBA/J, BALB/cJ, BALB/cByJ, A/J, and males and females of the C57BL/6J strain. The following F_1 hybrid mice also were used: (C57BL/6J × C3H/HeJ)F_1, (C3H/HeJ × C57BL/6J)F_1, and (C57BL/6J × BALB/cByJ)F_1 females and (BALB/cByJ × DBA/2J)F_1 males.

The tumors were induced according to the method of Bartlett (*4*). Briefly, Millipore filter strips were saturated with either 5 or 0.05 percent MCA in paraffin. Disks (6 mm in diameter) were punched from these strips with a ticket punch and kept refrigerated in the dark until used (within 1 month). The disks used in experiment 1 were made at the Institute for Cancer Research, Philadelphia, and shipped to the Jackson Laboratory. The disks in other experiments were made and used at the Jackson Laboratory. The mice were anesthetized with Nembutal alcohol and the MCA disks were inserted dorsally into the subcutaneous space through a small incision in the midline. The incision was closed with a wound clip. The mice were examined weekly by palpation for the presence of tumors. The date was recorded when a tumor had reached a diameter of 5 mm. If the tumor subsequently killed the mouse, that date was used in the calculations as the end of the latency period.

All tumors, and all tumor-free mice, at the end of each experiment were examined during autopsy for the presence of the MCA disk. If the disk was missing, the mouse was discarded from the experiment because it was impossible to know how long the mouse had been exposed to the MCA. Mice of strains C3H/HeJ, C57BL/6J, and A/J rejected from 32 to 50 percent of the 5 percent MCA disks. However, since the two strains that were most different in tumor inducibility, C3H/HeJ and C57BL/6J, were equal in this respect, the sloughing of the MCA disks did not appear to have been a factor in the results. In one of the experiments, designated EL, the mice were not examined for the presence of the MCA disk at the end of the experiment. The tumor-free mice in experiments 1 and 2 were observed for 365 days; in experiments 3 and EL for 245 days. We used these dates for the calculations of the tumor-free days for the mice that did not develop tumors. We used tumor-free days rather than tumor latency in order to be able to use all of the data. The trend of the data was the same if we calculated tumor latency using only the tumorous mice. The Mann-Whitney U test was used for the statistical analysis of the data.

All of the mice, with the exception of some of the (BALB/cByJ × DBA/2J)F_1 hybrids and two C3H/HeJ mice in experiment EL, developed solid tumors when 5 percent MCA disks were used for tumor induction (Tables 1 and 2). Although the average tumor-free time varied considerably among the strains with 5 percent MCA, both the tumor incidences and the observed tumor-free time varied when the induction was done with the 0.05 percent MCA.

The results of experiment 1 are given in Table 1 and Fig. 1. The three inbred strains and the four F_1 hybrids are listed in ascending order of tumor-free days with 5 percent MCA, that is, from the most susceptible to MCA (C3H/HeJ) to the least susceptible (C57BL/6J). The most susceptible strain, C3H/HeJ, became the least susceptible with the low concentration of 0.05 percent MCA; with the low concentration their tumor incidence was only 31 percent with an average of over 300 tumor-free days. In contrast, the strain least susceptible to the high concentration of MCA, C57BL/6J had, with the lower concentration, an 80 percent incidence and less than 250 days tumor-free. The BALB/cBy strain was intermediate between these two ex-

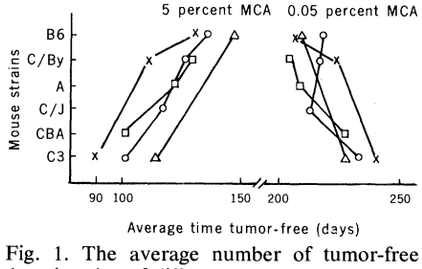

Fig. 1. The average number of tumor-free days in mice of different genotypes. For purposes of comparison, the data plotted are based on an observation period of 245 days for each experiment. Abbreviations of the mouse strains are the same as in Table 2. Symbols: X, experiment 1; ○, experiment 2; □, experiment 3; △, experiment EL.

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