Histones and basic polypeptides activate Ca^{2+} /cation influx in various cell types

Alessandra GAMBERUCCI*, Rosella FULCERI*, Paola MARCOLONGO*, William F. PRALONG† and Angelo BENEDETTI*¹

*Institute of General Pathology, University of Siena, 53100-Siena, Italy, and †Institute of Clinical Biochemistry, University of Geneva, School of Medicine, 1211 Geneva 4, Switzerland

Histone H2A (1–10 $\mu\text{g/ml}$) added to Ehrlich ascite cell suspensions promoted: (i) Ca^{2+} influx, but no apparent intracellular Ca^{2+} mobilization; (ii) plasma-membrane depolarization and Na^+ influx in Ca^{2+} -free medium, which were recovered by Ca^{2+} readmission; (iii) influx of other cations such as Ba^{2+} , Mn^{2+} , choline⁺ and *N*-methyl-D-glucamine⁺, but not of propidium⁺, ethidium bromide and Trypan Blue. H2A-induced Ca^{2+} influx and cell depolarization were: (i) blocked by La^{3+} and Gd^{3+} , but not by various inhibitors of receptor-activated Ca^{2+} -

influx pathways/channels; (ii) mimicked by various basic polypeptides, with $M_r > 4000$; (iii) prevented or reversed by polyanions such as polyglutamate or heparin; (iv) present in other cell types, such as Jurkat, PC12 and Friend erythroleukaemia cells, but virtually absent from rat hepatocytes and thymocytes. We conclude that cationic proteins/polypeptides, by interacting in a cell-specific manner with the cell surface, can activate in those cells putative non-selective Ca^{2+} channels and membrane depolarization.

INTRODUCTION

Ca^{2+} ions are crucial regulators of a variety of physiological processes such as muscle contraction, nerve conduction, secretion, cell adhesion and migration, and cell proliferation and differentiation. Dynamic cell Ca^{2+} homeostasis relies on Ca^{2+} ion fluxes among intracellular compartments as well as between cell interior and extracellular milieu, and is therefore regulated, in part, by the opening and closing of plasma-membrane (PM) Ca^{2+} channels. Two major mechanisms are responsible for PM channel gating: one related to membrane potential, the other to ligand–receptor interactions. Voltage-operated PM Ca^{2+} channels are primarily expressed in excitable cells such as neuronal and muscle cells [1], and also in pancreatic β -cells [2]. A great deal of previous experimental work (e.g. [3–5]) indicates that ligand–receptor interactions at the PM may directly or indirectly activate different voltage-independent Ca^{2+} -influx pathways/channels in both excitable and non-excitable cell types. Direct activation of PM Ca^{2+} -influx pathways/channels results from receptor occupancy *per se*, i.e. without any apparent involvement of diffusible second messengers and/or G-proteins. Examples are ionotropic glutamate [6], nicotinic acetylcholine and purinergic $\text{P}_{2\text{U}}$ -type ATP^{4-} receptors [7]. In some instances, inositol trisphosphate (IP_3)-dependent agonists appear to (directly) activate PM Ca^{2+} influx before eliciting diffusible inositol phosphate formation [8,9]. In a variety of instances, ligand–receptor interaction activates PM Ca^{2+} influx indirectly, via release/formation of diffusible messengers such as inositol phosphates [10,11] or Ca^{2+} itself [11,12], and/or because of the emptying of intracellular agonist-sensitive Ca^{2+} stores [13,14]. Signalling events linking (empty) Ca^{2+} stores to PM channel(s) have been reported to involve cytochrome *P*-450 [15], cGMP [16], G-proteins [17,18], direct interactions between endoplasmic

reticulum membranes and PM [19], or a putative intracellular low- M_r messenger named Ca^{2+} -influx factor (CIF) [20]. CIF has been recovered in acidic cell extracts, and appears to activate Ca^{2+} influx even when added extracellularly [20].

Searching for CIF(s) in acidic extracts of Ehrlich ascites tumour cells (ETCs), we observed a probably unrelated phenomenon: high- M_r components of the acidic extracts activated Ca^{2+} influx, but did not mobilize stored Ca^{2+} , when added to ETCs and other cell types [21]. Subsequently, we identified by ion-exchange column chromatography the active components of the acidic ETC extracts as histones (A. Gamberucci, R. Fulceri and A. Benedetti, unpublished work).

Previous studies suggested a hormone-like activity for some histones ([22]; see [23] for additional references), and hypothesized additional functions for histones apart from being nucleosome components [24]. Histones, or proteins resembling them, have been shown to be present on the surface of various cell types [25–30]. Moreover, interactions between extracellular basic proteins/polypeptides and PM surface (negatively charged) components may also take place under a variety of circumstances eventually involving signalling events ([31–34]; see [23] for additional references). We have therefore investigated in detail the effects of histone H2A (H2A) and various other basic proteins/polypeptides on PM Ca^{2+} /cation fluxes in several cell types in suspension, namely ETCs, Jurkat T-lymphocytes, rat thymocytes and hepatocytes, Friend erythroleukaemia and PC12 rat pheochromocytoma cells.

As a main result, we show that extracellularly added submicromolar concentrations of basic proteins can activate, independently of intracellular Ca^{2+} mobilization, a PM Ca^{2+} /cation influx pathway in several (but not all) cell types investigated. The possible biological relevance of the observed phenomena is discussed.

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; PM, plasma membrane; ETC, Ehrlich (ascites) tumour cells; H2A, histone H2A; $[\text{Na}^+]_i$, cytosolic Na^+ concentration; bis-oxonol, bis-(1,3-diethylthiobarbituric acid) trimethine oxonol; SBFI-AM, Na^+ -binding benzofuran isophthalate acetoxymethyl ester; IP_3 , inositol trisphosphate; CIF, Ca^{2+} -influx factor.

¹ To whom correspondence should be addressed (e-mail benedetti@unisi.it).

EXPERIMENTAL

Cells

ETCs (hyperdiploid strain) were transplanted weekly in the peritoneum of 25–30 g male Swiss albino mice and harvested 6–9 days after transplantation [35]. Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Friend erythroleukaemia cells provided by Dr. A. Arcangeli (Institute of General Pathology, Firenze, Italy) were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum. PC12 pheochromocytoma cells provided by Dr. V. Sorrentino (DIBIT, HSR San Raffaele, Milano, Italy) were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% horse serum and 5% fetal calf serum. Thymocytes were prepared from thymic glands of 5–6-week-old male Sprague–Dawley rats as described previously [36]. Hepatocytes were isolated from fed male Sprague–Dawley rats (200–250 g) by collagenase perfusion [37].

Measurements of cytosolic free Ca^{2+} concentration $[\text{Ca}^{2+}]_i$

Cells were loaded with 3 μM fura 2 acetoxymethyl ester according to standard procedures [35]. After loading, the cells were kept at room temperature in a medium containing 120 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1 mM CaCl_2 (1.5 mM for ETCs), 10 mM glucose and 25 mM Hepes (pH 7.4) until used. Just before the experiment, a 1 ml aliquot of the cell suspension (approx. 5×10^6 cells) was rapidly centrifuged and resuspended in 2 ml of fresh medium. Where indicated in the individual experiments, the resuspension medium contained no added CaCl_2 . Fluorescence was measured with a Perkin–Elmer LS-3B fluorimeter (excitation and emission wavelengths 340 and 509 nm respectively) equipped with magnetic stirring and temperature control. Fluorescence mV signal was fed to MacLab[®] hardware (AD Instruments) equipped with a computer and a Chart v3.2.5. software. Continuous traces (mV versus time) were visualized on the monitor and stored for printing and calculations. To avoid leakage of trapped fura 2, the temperature was reduced to 30 °C, or, in some experiments at 37 °C, 200 μM sulfinpyrazone was included in the medium [35]. The fluorescence was calibrated in terms of $[\text{Ca}^{2+}]_i$, as described [35], using a K_d for the Ca^{2+} –fura 2 complex of 185 or 225 nM at 30 and 37 °C respectively [38]. In most experiments, fluorescence was measured with one excitation wavelength only. However, control experiments were also conducted by point measurement of the ratio of fura 2 fluorescence (509 emission) with excitation at 340 and 380 nm; the values obtained by point measurement were fully consistent with those obtained by continuously monitoring fluorescence with excitation fixed at 340 nm as described above. In parallel samples to which no fura 2 acetoxymethyl ester was added, it was verified that all the treatments used caused only minor variations in cell autofluorescence.

Measurement of the influx of Mn^{2+} , Ba^{2+} and propidium⁺

The influx of Mn^{2+} was evaluated in fura-2-loaded cells from the rate of Mn^{2+} quenching of fura 2 fluorescence at the isosbestic point (excitation wavelength 360 nm) [35]. The influx of Ba^{2+} was evaluated in fura-2-loaded cells by measuring the fluorescence of the Ba^{2+} –fura 2 complex (excitation and emission wavelengths 350 and 509 nm respectively). The influx of propidium iodide was evaluated in cell suspensions (2.5×10^6 cells/ml) from the increase in fluorescence (excitation and emission wavelengths 488 and 600 nm respectively) due to the binding of propidium to intracellular (nucleic acid) components.

Measurement of PM potential

PM potential was measured with the potential-sensitive fluorescent dye bis-(1,3-diethylthiobarbituric acid) trimethine oxonol (bis-oxonol) essentially as described [39]. Briefly, a sample of the cell suspension (2.5×10^6 /ml) was incubated in the above medium (including 1.5 mM CaCl_2 or 0.2 mM EGTA, see individual experiments) containing 150 nM bis-oxonol until the fluorescence reached a steady state. After completion of the experiment, the signal was calibrated with 1 μM gramicidin A, which caused complete depolarization. Excitation and emission wavelengths were 540 and 580 nm respectively.

Measurement of cytosolic Na^+ concentration $[\text{Na}^+]_i$

Changes in the $[\text{Na}^+]_i$ were monitored using the fluorescent Na^+ indicator Na^+ -binding benzofuran isophthalate acetoxymethyl ester (SBFI-AM) essentially as described by Carini et al. [40]. The cells were loaded for 30 min with 5 μM SBFI AM and 0.016% Pluronic F-127 by following essentially the same procedure as that described for fura 2 acetoxymethyl ester. Fluorescence was measured with a computer-assisted Perkin–Elmer LS-3B fluorimeter. Excitation wavelength was alternatively positioned at 340 and 380 nm; emission was determined at 509 nm. The fluorescence ratio signal was measured and stored every excitation cycle (3 s).

Materials

Fura 2 acetoxymethyl ester, bis-oxonol and SBFI-AM were from Molecular Probes. Histone H1 and H2A were from Boehringer. Gramicidin A, monensin, verapamil and nifedipine were from Calbiochem. Culture media and sera, poly-L-lysine (at various M_r) and other homopolymers and random copolymers of amino acids, protamine, kemptide, tuftsin, substance P, myelin basic protein, laminin A chain, econazole, heparin, poly-L-glutamic acid (M_r 15000–50000) dibutyl cAMP and cGMP, genistein, okadaic acid, staurosporine and PMA were from Sigma. The inhibitor of *N*-methyl-D-aspartate-receptor/channel, MK-801, was from Research Biochemicals International. Pertussis toxin was a gift from Biocine-Chiron (Siena, Italy). Oxidized ATP was a gift from Professor F. DiVirgilio (Institute of General Pathology, Ferrara, Italy). All other chemicals were of analytical grade.

RESULTS

H2A promotes cation influx in Ehrlich cells

As shown in Figure 1(A), H2A induced a progressive rise in $[\text{Ca}^{2+}]_i$ in ETCs preloaded with the cytosolic free Ca^{2+} indicator fura 2. Both extent and rate of cytosolic $[\text{Ca}^{2+}]_i$ rise were a function of $[\text{H2A}]$ (1–6 $\mu\text{g}/\text{ml}$; i.e. 71 to 429 nM, assuming $M_r = 14000$ for calf thymus H2A). In Ca^{2+} -free medium (Figure 1B), $[\text{Ca}^{2+}]_i$ did not change after H2A addition, which indicates the absence of mobilization of intracellular stored Ca^{2+} ; no change in cytosolic $[\text{Ca}^{2+}]_i$ was present at later times after H2A addition (10 min, not shown in Figure 1B). A rapid rise in $[\text{Ca}^{2+}]_i$, however, was observed when Ca^{2+} was readmitted to the system, which therefore reflected Ca^{2+} influx from the extracellular milieu to the cytosol. The kinetic differences between H2A-induced rises observed in Ca^{2+} -free and Ca^{2+} -supplemented media (Figures 1A and 1B) were probably due to the time required for H2A to maximally activate Ca^{2+} influx. The rate of rise in $[\text{Ca}^{2+}]_i$, in fact, increased by increasing the time interval between H2A addition and Ca^{2+} readmission (0 to 60 s) to ETCs (Figure 1C); it was

Figure 1 Effects of H2A addition on $[Ca^{2+}]_i$ in ETCs

Fura-2-loaded cells were resuspended in medium containing either 1.5 mM Ca^{2+} (A) or no added Ca^{2+} (B and C) as detailed in the Experimental section. (A) $[Ca^{2+}]_i$ increase as a function of $[H2A]$; H2A concentrations are indicated on the right side of each trace. (B) $[Ca^{2+}]_i$ increase after Ca^{2+} readmission as a function of $[H2A]$; EGTA and $CaCl_2$ additions (arrows) were 0.2 and 1.5 mM respectively; H2A concentrations are indicated on the right side of each trace. (C) $[Ca^{2+}]_i$ increase after Ca^{2+} readmission as a function of length of treatment with H2A; conditions were as in (B) except that the time between addition of 3 $\mu\text{g/ml}$ H2A and $CaCl_2$ was varied as indicated by dashed arrows (60", 60 s). The calibrated $[Ca^{2+}]_i$ values are presented on the left side of each trace. These and the following experiments are representative of 3–12 different trials.

maximal at time intervals of 30 s and above. As shown in Figure 2(A), H2A-induced Ca^{2+} influx was reversed by the removal of extracellular free Ca^{2+} (EGTA addition) or by adding excess concentrations (250 $\mu\text{g/ml}$) of the polyanion polyglutamate which presumably binds H2A. Washing ETCs pretreated with H2A (3 $\mu\text{g/ml}$, for 5 min) with the H2A-free buffer resulted in no Ca^{2+} influx on Ca^{2+} readmission (Figure 2B, continuous trace), which indicated that the H2A effect was reversed by the removal of H2A from the system. The washed cells, however, still responded to a second H2A treatment as indicated by the H2A activation of Ca^{2+} influx (Figure 2B, dotted trace).

In the absence of extracellular Ca^{2+} , H2A (3 $\mu\text{g/ml}$) caused PM depolarization, as revealed using the PM potential indicator bis-oxonol, whereas little or no cell depolarization was evident in Ca^{2+} -containing media (Figure 3A). The extent of PM depolarization was in a dose-dependent fashion (H2A from 1 to 6 $\mu\text{g/ml}$; not shown). The H2A-induced loss of membrane potential was almost completely recovered after Ca^{2+} readmission to Ca^{2+} -free systems (Figure 3A). Cell depolarization was probably due to Na^+ influx. Indeed $[Na^+]_i$, measured in ETCs preloaded with the cytosolic Na^+ indicator SBFI, increased after H2A addition; the increase in $[Na^+]_i$ was more marked in the absence of extracellular Ca^{2+} (Figure 3B). The relatively small rise in $[Na^+]_i$ caused by H2A in ETCs in the presence of Ca^{2+} was

Figure 2 Reversal of H2A-induced $[Ca^{2+}]_i$ increase in ETCs

(A) EGTA and polyglutamic acid reverse H2A-induced $[Ca^{2+}]_i$ increase: fura-2-loaded cells resuspended in medium containing 1.5 mM Ca^{2+} were subsequently treated with H2A (3 $\mu\text{g/ml}$) and EGTA (3 mM) or polyglutamic acid (polyglut, 250 $\mu\text{g/ml}$) where indicated by arrows. (B) Washing H2A-treated cells reverses H2A-induced $[Ca^{2+}]_i$ increase: fura-2-loaded cells resuspended in the Ca^{2+} -free medium were treated with H2A (3 $\mu\text{g/ml}$) for 5 min, and washed three times with H2A-free fresh medium, before assaying $[Ca^{2+}]_i$. Additions (arrows) were: 0.2 mM EGTA, 3 $\mu\text{g/ml}$ H2A, and 1.5 mM Ca^{2+} .

presumably not sufficient to cause obvious cell depolarization.

The reversal of H2A-induced cell depolarization by Ca^{2+} may be due to at least two components: a preferential influx of Ca^{2+} instead of Na^+ through the same PM entry pathway, and/or the activation of PM Ca^{2+} -activated K^+ channels. These channels have been reported to be expressed in ETCs [41]. The former component, however, appears to be the major one on the basis of the following results: (i) Ca^{2+} readmission also caused a progressive recovery of the H2A-induced rise in $[Na^+]_i$ (Figure 3D); (ii) the Ca^{2+} -induced recovery of both the rise in $[Na^+]_i$ (Figure 3D) and membrane depolarization (Figure 3C) was blocked by the Na^+/K^+ -ATPase inhibitor ouabain; (iii) various inhibitors of the Ca^{2+} -activated K^+ channels (e.g. apamin, tetraethylammonium chloride, quinine and picrotoxin) were ineffective (results not shown); (iv) several bivalent cations other than Ca^{2+} also reversed the H2A-induced cell depolarization (see below), despite being poor activators (or even inhibitors, e.g. Ba^{2+}) of Ca^{2+} -activated K^+ channels [42].

The variations in $[Na^+]_i$ and PM potential did not appear to quantitatively parallel each other. The small rise in $[Na^+]_i$ caused by H2A in the presence of Ca^{2+} (Figure 3B) was not associated with any obvious PM depolarization, and, on Ca^{2+} addition, the complete recovery of PM potential (Figure 3C) was associated with only a partial recovery of $[Na^+]_i$ (Figure 3D). This could, however, reflect the fact that a relatively high threshold $[Na^+]_i$ is required to give obvious cell depolarization. The possible intracellular heterogeneity in $[Na^+]_i$ variations (not revealed by the present measurement of $[Na^+]_i$) may also underlie the above quantitative discrepancies.

H2A also activated the influx of other uni- or bi-valent cations in addition to Ca^{2+} and Na^+ . As shown in Figure 4, addition of H2A to ETCs resulted in the influx of both Mn^{2+} and Ba^{2+} (as revealed by the quenching of fura 2 or by the increase in fluorescence due to formation of Ba^{2+} -fura 2 complex respectively). Moreover, replacement of extracellular Na^+ with choline⁺ or *N*-methyl-D-glucamine⁺ resulted in an H2A-induced Ca^{2+} -inhibitable membrane depolarization (Figures 5A and 5B).

Figure 3 Effects of H2A on membrane potential (A and C) and $[Na^+]_i$ (B and D) in ETCs

Cells were resuspended in medium containing 1.5 mM Ca^{2+} (dotted traces) or no added Ca^{2+} (continuous traces), and PM potential or $[Na^+]_i$ was determined as detailed in the Experimental section; 0.2 mM EGTA was also added to continuous traces 1 min before H2A addition. (A) H2A-induced PM depolarization in Ca^{2+} -free medium and recovery of PM potential after Ca^{2+} readmission; additions (arrows) were: 3 μ g/ml H2A, 3 mM EGTA and 3 mM $CaCl_2$ to the dotted trace, 1.5 mM $CaCl_2$ to the continuous trace, and 1 μ M gramicidin. (B) H2A-induced $[Na^+]_i$ increases in the presence and absence of extracellular Ca^{2+} ; H2A, EGTA and monensin additions (arrows) were 3 μ g/ml, 3 mM and 2 μ M respectively. (C) Ouabain prevents recovery by Ca^{2+} readmission of H2A-induced PM depolarization; H2A, ouabain, $CaCl_2$ and gramicidin additions (arrows) were 3 μ g/ml, 1 mM, 1.5 mM and 1 μ M respectively. (D) Ouabain prevents recovery by Ca^{2+} readmission of H2A-induced increase in $[Na^+]_i$; additions were as in (C) except that monensin concentration was 2 μ M.

Figure 4 Effect of H2A on the influx of Mn^{2+} (A), Ba^{2+} (B) and propidium $^+$ (C), and recovery of H2A-induced PM depolarization by different bivalent cations (D) in ETCs

Cells were resuspended in medium containing no added Ca^{2+} as detailed in the Experimental section. In (A) the influx of Mn^{2+} (0.1 mM) was evaluated from the rate of quenching of fura 2 fluorescence; H2A concentrations are indicated on the right side of each trace; the vertical bar on the left side represents the total Mn^{2+} -quenchable fura 2 fluorescence. In (B), the influx of Ba^{2+} was evaluated from the increase in fura 2 fluorescence (excitation and emission wavelengths 340 and 509 nm respectively); EGTA and $BaCl_2$ additions (arrows) were 0.2 and 1.5 mM respectively; H2A concentrations are indicated on the right side of each trace. In (C) the influx of propidium iodide (0.1 mM) was evaluated from the increase in fluorescence (excitation and emission wavelengths 488 and 600 nm respectively) due to the binding of propidium to intracellular (nucleic acid) components. H2A addition was 7 μ g/ml. Digitonin (20 μ M, arrow) was added to permeabilize ETCs and thus obtain the maximal fluorescence value. The lower trace represents a control sample without cells, to show no effects of H2A and digitonin on propidium iodide fluorescence. In (D) conditions were as described in Figure 3(A); additions of bivalent cations (Me^{2+} , as chloride salts, arrow) were 1.5 mM; dotted bar on the right represents maximal depolarization (after 1 μ M gramicidin) the extent of which was unaffected by any treatment.

Figure 5 H2A-induced PM depolarization in ETCs in the presence of choline⁺ (A) or *N*-methyl-D-glucamine⁺ (B) as surrogate for extracellular Na⁺

Cells were resuspended in medium containing Ca²⁺ [1.5 mM, dotted traces) or no added Ca²⁺ (continuous traces) in which NaCl was replaced with equimolar concentrations of choline chloride or *N*-methyl-D-glucamine chloride. H2A and EGTA additions (arrows) were 3 µg/ml and 3 mM respectively. Other conditions were as described in the Experimental section and in the legend to Figure 3. Dotted bar on the right represents maximal depolarization (by 1 µM gramicidin).

Furthermore, the bivalent cations Ba²⁺, Mn²⁺ and Sr²⁺ all caused recovery of the loss of membrane potential induced by H2A in cells incubated in Na⁺-containing Ca²⁺-free medium, although at different rates (Figure 4D). Assuming that the rate of bivalent-cation-induced recovery reflects the capability of the cation to compete with Na⁺ for the H2A-activated PM cation-influx pathway, the selectivity of the putative channel appears to be: Ca²⁺, Mn²⁺ >> Ba²⁺, Sr²⁺ >> Na⁺, choline⁺, *N*-methyl-D-glucamine⁺. The exclusion limit for the influx pathway/channel appeared to be lower than 668 M_r, since H2A did not promote any propidium⁺ (iodide) influx (Figure 4C).

In parallel experiments, we verified that the effect of H2A was specific for PM. In fact, H2A (up to concentrations of 100 µg/ml) did not activate Ca²⁺ efflux from the endoplasmic reticulum pool of permeabilized ETCs and liver microsomes preloaded with ⁴⁵Ca²⁺ (in the presence of MgATP), or modify oxygen consumption and ⁴⁵Ca²⁺ accumulation in isolated (liver) mitochondria (results not shown).

In the present study, H2A (and other basic polypeptides, see below) were used at concentrations up to 7 µg/ml. These concentrations did not cause any decrease in the initial cell viability (which was always higher than 92%), as verified by the microscopical observation of cells stained with Trypan Blue or ethidium bromide. The initial cell viability was fully maintained even during prolonged incubations (30 min) in the presence of H2A. Moreover, the fluorimetric assay of propidium iodide influx showed no entry of this compound in cells treated with 7 µM H2A (Figure 4C).

Concentrations of H2A higher than 10 µg/ml were not investigated since they resulted in some efflux from the cells of the indicator fura 2, as shown by the loss of the fluorescence signal in cells treated with H2A before Ca²⁺ was readmitted to the system, and in decreases in PM potential that were only partially recovered by Ca²⁺. At [H2A] > 20 µg/ml, cells become permeable to Trypan Blue, ethidium bromide and propidium iodide, indicating a cell (non-specific) damaging effect due to the high [H2A].

Properties of the H2A-activated PM cation-influx pathway in ETCs

Table 1 shows the effect of various inhibitors and treatments on H2A-activated influx of Ca²⁺ and/or PM depolarization. Both La³⁺ and Gd³⁺ ions inhibited the H2A-induced effects. On the other hand, verapamil, nifedipine and the inhibitor of NMDA receptor/channels, MK-801, did not modify H2A-induced Ca²⁺

Table 1 Effect of various inhibitors and treatments on H2A-induced cation influx in ETCs

Cells were resuspended in medium containing no Ca²⁺ as detailed in the Experimental section and treated with the various compounds before assay of the effect of H2A. Ca²⁺ influx was measured as peak [Ca²⁺]_i increases after Ca²⁺ readmission (1.5 mM) in cells treated with 3 µg/ml H2A for 1 min, as detailed in the legend to Figure 1. PM depolarization by 3 µg/ml H2A was quantified assuming that 1 µM gramicidin caused 100% depolarization, as detailed in the legend to Figure 3. Concentrations and times of treatment for the different compounds were: La³⁺ or Gd³⁺ (in EGTA-free medium), 10 µM, 10 s; verapamil, 100 µM, 1 min; MK-801, 100 µM, 1 min; econazole, 50 µM, 2–5 min; oxidized ATP, 100 µM, 2 h (at 37 °C); dibutyryl cAMP or cGMP, 50 µM, 5 min; okadaic acid, 50 nM, 1 h (at 37 °C); genistein, 100 µM, 45 min (at 37 °C); staurosporine, 400 nM, 5 min; PMA, 500 nM, 5 min; pertussis toxin, 2 µg/ml, 4 h (at 37 °C); arachidonic acid, 5 µM, 5 min. Calf thymus DNA, heparin and polyglutamic acid were included in the assay medium at 40, 50 and 250 µg/ml respectively. ATP depletion was obtained by treating ETCs with 3 µM rotenone, for 5 min at 37 °C, in glucose-free medium [34]. n.d., Not determined.

Treatment/inhibitor	Effect on H2A-induced	
	Ca ²⁺ influx	PM depolarization
La ³⁺	100% inhibition	100% inhibition
Gd ³⁺	100% inhibition	100% inhibition
Verapamil	No	No
Nifedipine	n.d.	No
MK-801	No	No
Econazole	No	n.d.
ATP depletion	No	No
Oxidized ATP	No	n.d.
Dibutyryl cAMP	No	No
Dibutyryl cGMP	No	No
Okadaic acid	50% inhibition	n.d.
Genistein	80% stimulation	50% stimulation
Staurosporine	No	No
PMA	No	No
Pertussis toxin	No	n.d.
Arachidonic acid	n.d.	No
Calf thymus DNA	100% inhibition	100% inhibition
Heparin	100% inhibition	100% inhibition
Polyglutamic acid	100% inhibition	100% inhibition

influx or PM depolarization. As expected because of the lack of H2A mobilization from intracellular Ca²⁺ stores, econazole [15], ATP depletion [35] and genistein [43], all inhibitors of store-dependent Ca²⁺ influx, did not inhibit the activity of H2A. Moreover, the channels responsible for store-dependent Ca²⁺ entry (and/or Ca²⁺-release-activated Ca²⁺ currents), at variance with the H2A-activated cation influx pathway, usually possess a low Ba²⁺ permeability and a high bivalent/univalent selectivity [44] and references therein). Purinergic P_{2x} channels also appeared not to be involved, since cell preincubation with 2',3'-dialdehyde derivative of ATP [45] was without any effect on the H2A-induced cation influxes; this treatment efficiently blocked any Ca²⁺ mobilization and Ca²⁺ influx caused by exogenously added ATP⁴⁻ (up to 1 mM) in ETCs.

Treatment of ETCs with the dibutyryl derivative of cAMP or cGMP did not mimic the H2A effects or modify them (Table 1). However, phosphorylation status (presumably by tyrosine kinases) appeared to down-regulate H2A-induced Ca²⁺ influx, since the inhibitor of tyrosine kinases, genistein, and the inhibitor of phosphatases, okadaic acid, enhanced and reduced the extent of Ca²⁺ influx respectively (Table 1). On the other hand, the inhibitor and activator of protein kinases C, staurosporine and PMA respectively, did not affect H2A-induced Ca²⁺ influx or PM depolarization. Pertussis toxin pretreatment of ETCs, although abolishing [Ca²⁺]_i rises caused by the agonist ATP or bradykinin, did not modify the H2A-induced effects. The possibility that arachidonic acid mediates or affects the activity of H2A was

Table 2 Effect of various basic compounds on cation influx in ETCs

Ca^{2+} influx and PM depolarization were quantified as reported in Table 1. Submaximal concentrations of the different compounds, ranging from 1.5 to 5 $\mu\text{g}/\text{ml}$, were used, and their effects were compared with those determined by the identical concentrations of H2A in each cell preparation. Inactive compounds were tested using concentrations between 10 and 10 $\mu\text{g}/\text{ml}$ (see the text).

Basic compound	$10^{-3} \times M_r$	Ca^{2+} influx	PM depolarization
H2A	14	++	++
H1	23	++	++
Protamine	4.3	+	+
Arg	0.174	No	n.d.
Lys	0.146	No	No
Lys-Lys	0.347	No	No
Lys-Trp-Lys	0.460	No	n.d.
Poly-L-Ala	24	No	n.d.
Poly-L-Lys	1-4	No	No
Poly-L-Lys	4-15	++	n.d.
Poly-L-Lys	15-30	+++	n.d.
Poly-L-Lys	30-70	++++	++
Poly-L-Lys	70-150	++++	n.d.
Poly-L-Lys	150-300	++++	n.d.
Polyornithine	18	+++	+++
Poly-L-Arg	6-8	+++	+++
Poly-L-Arg	45	++++	+++
Poly(Lys,Ala,Glu,Tyr) 5:6:2:1	30-70	+++	+++
Poly (Lys,Ser) 3:1	31	+++	n.d.
Poly (Arg, Ser) 3:1	22	+++	n.d.
Kemptide	0.77	No	No
Tuftsia	0.5	No	No
Substance P	1.3	No	No
Myelin basic protein	14	+	+
Laminin A chain	2	No	No

Figure 6 Influx of Ca^{2+} induced by polylysines at different M_r values in ETCs

Fura-2-loaded cells were resuspended in medium containing no added Ca^{2+} as detailed in the Experimental section. EGTA, polylysine and CaCl_2 additions (arrows) were 0.2 mM, 1 $\mu\text{g}/\text{ml}$ and 1.5 mM respectively. M_r of added polylysine is indicated on the right side of each trace. The calibrated $[\text{Ca}^{2+}]_i$ values are presented on the left side of each trace.

unlikely as this fatty acid neither depolarized ETCs nor modified H2A-induced PM depolarization.

As expected since polyglutamic acid reversed H2A-induced Ca^{2+} influx (Figure 2A), other compounds capable of binding basic peptides, such as DNA or heparin, completely prevented H2A-induced Ca^{2+} influx and PM depolarization (Table 1).

Basic proteins/polypeptides besides H2A promote cation influx in ETCs

Data summarized in Table 2 indicate that, in addition to H2A,

Table 3 Effect of H2A on cation influx in various cell types

Ca^{2+} influx and PM depolarization were quantified as reported in Table 1. H2A concentrations ranging from 3 to 10 $\mu\text{g}/\text{ml}$ were used, and their effects in each cell type were compared.

Cell type	Ca^{2+} influx	PM depolarization
ETCs	+++	+++
Jurkat T-lymphocytes	+++	+++
Friend erythroleukaemia cells	+	+
PC12 pheochromocytoma cells	++	++
Rat thymocytes	±	±
Rat hepatocytes	No	No

various other basic proteins/polypeptides activate PM cation influx in ETCs. However, basic amino acids or polypeptides with relatively low M_r (lower than approx. 4000) were inactive. It should be noted that the low- M_r basic compounds investigated were inactive even at high concentrations (30–100 $\mu\text{g}/\text{ml}$). No activity was observed with control (non-basic) polypeptides such as polyalanine. On the other hand, all of the basic proteins/polypeptides with a relatively high M_r (> 4000) qualitatively mimicked the effects of H2A. In particular, they promoted Ca^{2+} influx without inducing any Ca^{2+} mobilization (e.g. see Figure 6) and PM depolarization (in Ca^{2+} -free medium), which was recovered by Ca^{2+} readmission. The extent of both Ca^{2+} influx and PM depolarization by the various high- M_r compounds was apparently higher or lower than in the case with H2A (when used at the same concentration on a w/v basis; see Table 2). By using synthetic polylysines with different M_r (1000/4000 to 300 000), it was observed that the extent of Ca^{2+} influx roughly increased by increasing the M_r of the basic polypeptide (Figure 6). It appears therefore that active proteins/polypeptides should not only contain a relatively high number of basic amino acid residues (from approx. 20 to 100 % of the amino acid content), but also have a relatively high molecular dimension.

Effect of H2A on cation influx in various cell types

Several cell types/lines other than ETCs were also investigated. To this end, cell suspensions were treated with H2A according to the experimental protocols for evaluating Ca^{2+} influx and PM depolarization. Data are summarized in Table 3, and some representative experiments are shown in Figure 7. Jurkat T-lymphocytes, Friend erythroleukaemia and PC12 cells all shared with Ehrlich cells the pattern of response to H2A. The extents of the two responses (i.e. Ca^{2+} influx and Ca^{2+} -inhibitable PM depolarization), however, were different depending on the cell type. Jurkat T-lymphocytes (Figure 7) behaved similarly to ETCs, whereas Friend erythroleukaemia and PC12 pheochromocytoma cells responded to somewhat lower extents than ETCs with the same doses of H2A. On the other hand, isolated hepatocytes and thymocytes gave no (hepatocytes) or little (thymocytes, Figure 7) response even at $[\text{H2A}]$ higher (up to 10 $\mu\text{g}/\text{ml}$) than the current one (3 $\mu\text{g}/\text{ml}$).

DISCUSSION

The present data indicate that extracellularly added basic proteins/polypeptides can activate a PM cation-influx pathway in various cell types. The activation leads primarily to Ca^{2+} influx, provided that extracellular Ca^{2+} is present at physiological concentrations. The influx of Ca^{2+} was indirectly assessed by

Figure 7 Effects of H2A on Ca^{2+} influx (left traces) and PM potential (right traces) in Jurkat T-lymphocytes, and rat thymocytes

Cells were resuspended in medium containing no added Ca^{2+} as detailed in the Experimental section; in PM potential experiments, 0.2 mM EGTA was added to suspensions 1 min before H2A addition. H2A additions (arrows) were 3 or 10 $\mu\text{g/ml}$, in Jurkat T-lymphocytes and rat thymocytes respectively. Other additions were as described in the legends to Figures 1 and 3.

variation of $[\text{Ca}^{2+}]_i$. However, the fact that H2A also promoted the influx of Ba^{2+} and Mn^{2+} ions makes it unlikely that the H2A-induced $[\text{Ca}^{2+}]_i$ increase is primarily due to inhibition of the PM Ca^{2+} pumps. In fact, the influx of these Ca^{2+} -surrogate ions is (mainly) unidirectional, since they are very poorly extruded from the cytosol [46]. The observed Ca^{2+} /cation influx probably reflects the activity of a specific influx pathway(s)/channel(s), and not a non-specific increase in PM permeability, at least at the relatively low concentrations of the basic proteins/polypeptides used in the present study. The following lines of evidence strongly support this conclusion. (i) H2A promoted the influx of various cations, but with a M_r lower than at least 668, since propidium iodide influx was not activated. Moreover ethidium bromide and Trypan Blue did not enter the cells. (ii) The different cations appeared to enter according to different selectivity, e.g. the influx of Ca^{2+} reduced that of Na^+ . (iii) La^{3+} and Gd^{3+} blocked $\text{Ca}^{2+}/\text{Na}^+$ influx; these cations, although non-specific channel blockers, should hardly act on non-specific alterations of PM permeability. We indeed observed that the increased permeability to propidium iodide and Trypan Blue, caused by high concentrations of H2A ($> 20 \mu\text{g/ml}$), was not inhibited by La^{3+} . (iv) The effects of H2A were fully reversed by treatments designed to remove the H2A bound to PM (addition of the polyanion polyglutamate or washing of cells). (v) H2A did not promote Ca^{2+} permeability of any membranes other than the PM, i.e. microsomal or mitochondrial membranes. (vi) The H2A effects did not occur in all cell types investigated. (vii) H2A-activated cation influx appeared to be modulated by the cell phosphorylation status (Table 1).

It should be also mentioned that the observed dose-response effect in $[\text{Ca}^{2+}]_i$ (Figure 1) to basic polypeptides could be, at least in part, explained by the concentration-dependent recruitment of more cells. Most of the present results were obtained in ETC populations; however, owing to the technical difficulties of making ETC cells adhere to coverslides [47], we tested this in fura 2 experiments in single PC 12 cells (which behaved similarly to ETCs; Table 3). The results indicate that increasing H2A concentrations (1 to 5 $\mu\text{g/ml}$) result in both a larger number of responsive cells (from approx. 60% to 90%) and a higher mean increase in $[\text{Ca}^{2+}]_i$ (A. Gamberucci, W.-F. Pralong and A. Benedetti, unpublished work).

Previous studies showed that polybasic peptides (e.g. polylysines) have various effects on intact cells, such as the

impairment of (tumour) cell viability [31] and the activation of endocytosis [48]. We have also observed that relatively high [H2A] may cause a non-specific increase in PM permeability (see the Results section). To our knowledge, however, the activation of PM Ca^{2+} /cation channels by low (non-toxic) doses of histones and basic polypeptides has not previously been described. The possible relationships between the observed increase in PM Ca^{2+} /channel influx and the activation of endocytosis were not investigated as these were beyond the scope of the present paper.

A variety of pharmacological evidence (Table 1) and the ion selectivity indicate that several known Ca^{2+} /cation channels (e.g. agonist- or receptor-operated, and voltage- or store-dependent ones), and/or various intracellular messengers (e.g. IP_3 , cAMP, arachidonic acid and CIFs), do not appear to be involved. The possibility that H2A (and the other cationic polypeptides) become inserted into the PM and form channels is highly unlikely because they are hydrophilic charged molecules. A logical explanation is that the basic proteins interact with anionic sites on the PM surface and hence activate a cation-influx pathway/channel. Consistent with this assumption, their effects were prevented/reversed by polyanion addition, and previous data indicate that polylysine can bind to anionic sites, presumably sialic acid residues, on the PM surface (e.g. [31]). The interaction of the basic proteins with the PM surface (anionic residues) may at least result in the direct activation of the putative cation channels or in the formation of some active second messenger(s). In any event, a variety of glycoconjugates on the PM surface could possibly be involved in such an interaction. Almost all mammalian cell surface proteins are glycoproteins [49], and a large variety of them have antennary glycosyl chains usually containing terminal sialic acid residues [50,51]. Several PM glycosphingolipids are also known to contain sialic acid residues [52], and polysialic acid chains are bound to the neuronal cell adhesion molecules in many neuronal and non-neuronal cell types [53].

The observed phenomena are clearly non-specific with respect to the stimulus. A relatively long chain length for the basic protein/polypeptide to be active is, however, required (Table 2 and Figure 6); the interaction with PM (sialosyl) negative charges of basic short-chain peptides ($M_r < 4000$) or amino acids was ineffective. Cell treatment with basic proteins/polypeptides may merely reveal (the activation of) an at present undefined cation-influx pathway, the function of which is unrelated to that

stimulus. Alternatively, it may well underline (Ca^{2+}) signalling events operative under a variety of circumstances in which cells interact with components of cell membrane surface and extracellular matrix, or with soluble molecules, and involving the cation pathway(s)/channel(s) described here. Proteins closely related to or identical with histones have been found on the PM (surface) of various cell types, including endothelial cells [25] cytotoxic T-cells [26], monocytes [28], spermatozoa [29], lymphocytes [30] and human lung carcinoma [27]. In the last study [27], heparin was shown to bind to the basic proteins present in cell membrane preparations. The surface expression of these proteins was often found associated with cell activation [25,26,28,30], or is relevant to egg fertilization [29], which involves cell ionic events. H2A and H2B have a primary structure identical with those of the homeostatic thymus hormones HTH_a and HTH_b, respectively [22,23], and the ovarian gonadotropin-releasing hormone-binding inhibitor was identified as H2A [54].

Polylysine (and polyornithine) is widely used as a non-specific artificial substrate to allow adhesion of cultured cell lines. Cell adhesion to natural extracellular substrates (e.g. collagen, fibronectin or laminin) involves a variety of interactions between specific molecular residues [55–59], which also affect cell differentiation/proliferation and ionic signalling events [55–58]. Natural substrates, however, possess cationic domains that can bind, for example, the anionic proteoglycan heparin through electrostatic interactions [34,60–62]. It is tempting to speculate that interactions between clusters of basic amino acids of the extracellular matrix and cell surface can elicit activation of Ca^{2+} influx. Heterogeneity in the expression of the putative binding sites/cation channels, among cell types and different areas of PM surface, may play a role in rendering the ionic signalling event specific.

In conclusion, it can be proposed that the cation-influx pathway/channels described here may participate in a cell-specific manner in Ca^{2+} -signalling events involved in (and required for) a variety of processes such as cell-to-cell and cell-to-matrix interactions. Further study, however, is required to confirm this hypothesis.

This work was supported in part by grants from Italian Consiglio Nazionale delle Ricerche (target project ACRO), from the FNSRS (grant 32-40511.94 to W.-F.P.) and from the Ministry of University and Research (MURST 60%).

REFERENCES

- McDonald, T. F., Pelzer, S., Trautwein, W. and Pelzer, D. J. (1994) *Physiol. Rev.* **74**, 365–507.
- Newgard, C. B. and McGarry, J. D. (1995) *Annu. Rev. Biochem.* **64**, 689–719.
- Clapham, D. E. (1995) *Cell* **80**, 259–268.
- Felder, C. C., Singer-Lahat, D. and Mathes, C. (1994) *Biochem. Pharmacol.* **48**, 1997–2004.
- Fasolato, C., Innocenti, B. and Pozzan, T. (1994) *Trends Pharmacol. Sci.* **15**, 77–83.
- Sommer, B. and Seeburg, P. H. (1992) *Trends Pharmacol. Sci.* **13**, 291–296.
- Abracchio, M. P. and Bunstock, G. (1994) *Pharmacol. Therapeut.* **64**, 445–475.
- Sage, S. O. and Rink, T. J. (1987) *J. Biol. Chem.* **262**, 16364–16369.
- Cheek, T. R., Murawsky, M. M. and Stauderman, A. (1994) *Biochem. J.* **304**, 469–476.
- Kuno, M. and Gardner, P. (1987) *Nature (London)* **326**, 301–304.
- Lückoff, A. and Clapham, D. (1992) *Nature (London)* **355**, 356–358.
- Tschanner, V., von Prud'homme, B., Baggolini, M. and Reuter, H. (1986) *Nature (London)* **324**, 369–371.
- Takemura, H., Hughes, A. R., Thastrup, O. and Putney, Jr., J. W. (1989) *J. Biol. Chem.* **264**, 12266–12271.
- Hoth, M. and Penner, R. (1992) *Nature (London)* **355**, 353–356.
- Alvarez, J., Montero, M. and Garcia-Sancho, J. (1991) *Biochem. J.* **274**, 193–197.
- Bahson, T. D., Pandol, S. J. and Dionne, V. E. (1993) *J. Biol. Chem.* **268**, 10808–10812.
- Fasolato, C., Hoth, M. and Penner, R. (1993) *J. Biol. Chem.* **268**, 20737–20740.
- Bird, G. St. J. and Putney, Jr., J. W. (1993) *J. Biol. Chem.* **268**, 21486–21488.
- Berridge, M. J. (1995) *Biochem. J.* **312**, 1–11.
- Randriamampita, C. and Tsien, R. Y. (1993) *Nature (London)* **364**, 809–814.
- Gamberucci, A., Fulceri, R., Giunti, R. and Benedetti, A. (1994) Third European Symposium on Calcium Binding Proteins in Normal and Transformed Cells, March 6–9, Zurich, abstract p. 94, Schippt AG, Zurich.
- Reichhart, R., Zeppezauer, M. and Jornvall, H. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4871–4875.
- Louters, L. L., Henriksen, E. J. and Tipton, C. M. (1993) *Biochem. J.* **295**, 549–553.
- Reichhart, R., Jornvall, H., Carlquist, M. and Zeppezauer, M. (1985) *FEBS Lett.* **188**, 63–67.
- Horneland, M., Rekvig, O. P., Jorgensen, L. and Hannestad, K. (1983) *Clin. Exp. Immunol.* **54**, 373–377.
- Ojcius, D. M., Muller, S., Asselkus-Light, C. S., Young, J. D. and Jiang, S. (1991) *Immunol. Lett.* **28**, 101–108.
- Bilours, M. E. and Biswas, C. (1990) *J. Biol. Chem.* **265**, 19697–19703.
- Elmen, W., Holers, V. M., Arend, W. P. and Kotzlin, B. (1992) *J. Immunol.* **148**, 3042–3048.
- Bernardini, G., Dalle Donne, I., Norreri, S., Negri, A. and Milanzi, A. (1992) *J. Exp. Zool.* **263**, 210–214.
- Watson, K., Edwards, R. J., Shaunak, S., Parmelee, D. C., Sarraf, C., Gooderham, N. J. and Davies, D. S. (1995) *Biochem. Pharmacol.* **50**, 299–309.
- Arnold, Jr., L. J., Dagan, A., Gutheil, J. and Kaplan, N. O. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3246–3250.
- Kornguth, S. E., Kalinke, T., Robins, H. I., Cohen, J. D. and Turski, P. (1989) *Cancer Res.* **49**, 6390–6395.
- McEwan, G. T. A., Jepson, M. A., Hirst, B. H. and Simmons, N. L. (1993) *Biochim. Biophys. Acta* **1148**, 51–60.
- Fromm, J. R., Hileman, R. E., Caldwell, E. E. O., Weiler, J. M. and Linhardt, R. J. (1995) *Arch. Biochem. Biophys.* **323**, 279–287.
- Gamberucci, A., Innocenti, B., Fulceri, R., Bänhegyi, G., Giunti, R., Pozzan, T. and Benedetti, A. (1994) *J. Biol. Chem.* **269**, 23597–23602.
- McConkey, D. J., Nicotera, P., Hartzell, P., Bellomo, G., Wyllie, A. H. and Orrenius, S. (1989) *Arch. Biochem. Biophys.* **369**, 365–370.
- Moldéus, P., Hogberg, J. and Orrenius, S. (1978) *Methods Enzymol.* **52**, 60–71.
- Gryniewicz, G., Poenie, M. and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3445.
- Fasolato, C., Pandiella, A., Meldolesi, J. and Pozzan, T. (1988) *J. Biol. Chem.* **263**, 17350–17359.
- Carini, R., Bellomo, G., Benedetti, A., Fulceri, R., Gamberucci, A., Parola, M., Dianzani, M. U. and Albano, E. (1995) *Hepatology* **21**, 1089–1098.
- Christensen, O. and Hoffmann, E. K. (1991) *J. Membr. Biol.* **129**, 13–36.
- Latorre, R., Oberhauser, A., Labarca, P. and Alvarez, O. (1989) *Annu. Rev. Physiol.* **51**, 385–399.
- Vostal, J. G., Jackson, W. L. and Shulman, N. R. (1991) *J. Biol. Chem.* **266**, 16911–16916.
- Putney, Jr., J. W. (1997) *Cell Calcium* **21**, 257–261.
- Murgia, M., Hanau, S., Pizzo, P., Rippa, M. and DiVirgilio, F. (1993) *J. Biol. Chem.* **268**, 8199–8203.
- Broad, L. M., Powis, D. A. and Taylor, C. W. (1996) *Biochem. J.* **316**, 759–764.
- Bogdanov, Jr., A. A., Gordeeva, L. V., Baibakov, B. A., Margolis, L. B. and Torchilin, V. P. (1992) *J. Cell. Physiol.* **147**, 182–190.
- Arnold, Jr., L. J. (1985) *Methods Enzymol.* **112**, 270–285.
- Gahmberg, C. G. and Tolvanen, M. (1996) *Trends Biochem. Sci.* **248**, 308–311.
- Varki, A. (1994) *Methods Enzymol.* **230**, 16–32.
- Reuter, G. and Schauer, R. (1994) *Methods Enzymol.* **230**, 168–199.
- Ledeer, R. W. and Yu, R. K. (1982) *Methods Enzymol.* **83**, 139–191.
- Joliot, A. H., Triller, A., Volovitch, M., Pernelle, C. and Prochiantz, A. (1991) *New Biol.* **3**, 1121–1134.
- Aten, R. F. and Behnam, H. R. (1989) *J. Biol. Chem.* **264**, 11072–11075.
- Jalkanen, M., Jalkanen, S. and Bernfield, M. (1991) in *Receptors for Extracellular Matrix* (McDonald, J. A. and Mecham, R. P., eds.), pp. 1–37, Academic Press, San Diego.
- Rutishauser, U. S. (1991) in *Receptors for Extracellular Matrix* (McDonald, J. A. and Mecham, R. P., eds.), pp. 131–156, Academic Press, San Diego.
- Edelman, G. M. and Crossin, K. L. (1991) *Annu. Rev. Biochem.* **60**, 155–190.
- Jackson, R. L., Busch, S. J. and Cardin, A. D. (1991) *Physiol. Rev.* **71**, 481–539.
- Albini, A., Noonan, D. M., Melchiori, A., Fassina, G. F., Percario, M., Genteman, S., Toffenetti, J. and Chader, G. J. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2257–2261.
- Margalit, H., Fischer, N. and Ben-Sasson, S. A. (1993) *J. Biol. Chem.* **268**, 19228–19231.
- Lubec, G. (1986) *Nature (London)* **323**, 743–744.
- Hynes, R. O. and Yamada, K. M. (1982) *J. Cell Biol.* **95**, 369–387.