stituted analogue. Synthesis of the compounds studied is described[5,6]. For evaluating the efficiency of these compounds against microorganisms, the method of inhibiting the growth directly in the cultivation media was used[7]. Results are presented as minimum inhibitory concentrations (MIC) in μg/ml (table). Basic requirement valid for the effciency of bis-quaternary ammonium salts was confirmed, i.e. presence of long alkyl substituent (C_9-C_{13}) on the quaternary nitrogen atom, with optimum length C_{11}-C_{12}. Further prolongation of alkyl chain reduces the antimicrobial effect of the compounds studied because of their diminished solubility in water (derivatives Nos 1-5). In comparison with bis-quaternary ammonium salts derived from 1,6-hexanediamine[3], no substantial difference in their efficiency was observed.

Substitution in position 2 of the connecting chain by OH-group moderately decreases the inhibiting effect against microorganisms inspite of the fact that the solubility of these compounds in water is greater than that of preceeding compounds.

Bis-quaternary ammonium salts are antimicrobially more effective than mono-quaternary salts[7,8], particularly on gram-negative bacteria *E. coli*. It is a big advantage in view of the possibility of their being used in pharmaceutical and cosmetic preparations.

Antimicrobial efficiency of bis-quaternary ammonium salts derived from 1,3-propanediamine (MIC in μg/ml)

$R_1(CH_3)_2\overset{+}{N}CH_2CH(R_3)CH_2\overset{+}{N}(CH_3)_2R_2\,2Br^-$

No.	$R_1=R_2$	R_3	*S. aureus*	*E. coli*	*C. albicans*
1.	octyl	H	50	400	100
2.	decyl	H	3	60	10
3.	dodecyl	H	3	20	20
4.	tetradecyl	H	40	200	60
5.	hexadecyl	H	> 1000	> 1000	> 1000
6.	octyl	OH	60	900	300
7.	nonyl	OH	20	200	40
8.	decyl	OH	6	50	8
9.	undecyl	OH	5	60	6
10.	dodecyl	OH	5	90	50
11.	tridecyl	OH	8	50	30
12.	tetradecyl	OH	100	600	90
13.	pentadecyl	OH	90	500	300
14.	hexadecyl	OH	90	> 1000	300

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Effects of calcium-EGTA buffers on active calcium transport in inside-out red cell membrane vesicles

B. Sarkadi, A. Schubert and G. Gárdos[1]

National Institute of Haematology and Blood Transfusion, Budapest, and Department of Physics, University of Agricultural Sciences, Gödöllö (Hungary), 5 October 1978

Summary. In inside-out red cell membrane vesicles, the free calcium concentration half-maximally stimulating active calcium uptake is about 2 orders of magnitude smaller in a calcium-EGTA buffer than in media containing unbuffered calcium. In calcium-EGTA buffer, the maximum rate of calcium uptake is determined by the total calcium concentration present. A possible model for explaining these findings is presented.

Sealed inside-out membrane vesicles (IOV) proved useful in studying the characteristics of active calcium transport of human red cells, since these vesicles show a rapid, ATP + Mg^{2+}-dependent calcium accumulation, and the 'active centre' of the calcium transport system is at the external surface of the IOVs[2-6]. The rate of active calcium uptake by inside-out vesicles is increased by the dialysed supernatant of the red cell hemolysate[2,3,5,6], and the K_{Ca} of about 40 μM observed in the control vesicles is decreased to about 15 μM by the supernatant 'activator protein'[6].

In calcium-loaded resealed ghosts it was shown by Schatzmann[7] that the free calcium concentration required for half-maximum activation of Ca^{2+} + Mg^{2+}-ATPase is about 2 orders of magnitude smaller in a calcium-EGTA buffer than in an unbuffered medium. In the present paper we report comparative studies on the kinetics of active calcium uptake by inside-out vesicles in the presence of unbuffered calcium and of Ca^{2+}-EGTA buffers in the incubation media.

Materials and methods. All the chemicals used were of analytical grade. Deionized water and solutions without added calcium contained less than 3 μM of calcium. 100 mM EGTA - ethylene glycol-bis-(2-aminoethylether)-N,N'-tetra-acetic acid - solutions were titrated with 100 mM $CaCl_2$ solutions before the experiments. A23187 calcium ionophore was a gift of R.L. Hamill (Eli Lilly and Co., Indianapolis).

Stability constants of EGTA for calcium and magnesium, and proton dissociation constants were used as by Schatzmann[7]. The about 20-30% differences in the stability constants in the literature were found to cause the same per cent error in the calculated free, ionized calcium concentrations. In media containing unbuffered calcium, free ionized calcium concentrations were not corrected for binding to ATP as the presence of magnesium makes such a calcium binding insignificant[8].

Inside-out vesicles from human red cells were prepared in chelator-free Tris-HCl buffers as described previously[6]. In the calcium uptake experiments, IOVs with a protein concentration of 20-30 μg/ml medium were preincubated at 37 °C in media containing 120 mM KCl, 20 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$ and the indicated concentra-

tions of $CaCl_2$ or calcium-EGTA buffer, supplemented with ^{45}Ca tracer. Active calcium uptake was initiated by the addition of ATP to the medium in a final concentration of 250 µM. Tracer calcium uptake by the vesicles was measured by rapid filtration on Sartorius membrane filters as described previously[3,6]. The rate of calcium uptake was calculated by a linear regression using 3 time-points in a 10-min experimental period. The supernatant activator protein was prepared by dialysing the membrane-free supernatant of the red cell hemolysate against 160 mM KCl for 48 h at 4 °C.

Results and discussion. Active calcium uptake by inside-out red cell membrane vesicles at 37 °C, in the presence of ATP + magnesium has a constant rate at least in the first 10 min (figure 1). During this period intravesicular calcium concentration increases to millimolar levels, and if A23187 calcium ionophore is added to the medium, a rapid leakage of calcium from the vesicles occurs. If excess EGTA, reducing free calcium concentration practically to zero, is added to the medium, there is no further calcium uptake, but no significant leakage of calcium from the vesicles is observed (figure 1). These experiments indicate that excess EGTA stops active calcium uptake, and EGTA or calcium-EGTA cannot cross the membrane.

In the following experiments, the effects of unbuffered calcium concentration and of Ca^{2+}-EGTA buffers on ac-

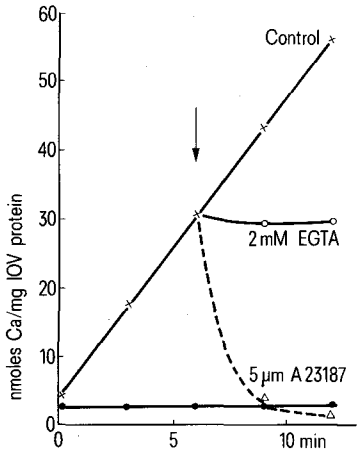

Fig. 1. Active calcium uptake by inside-out vesicles. For the assay conditions see the 'method' section. The incubation medium contained 100 µM $CaCl_2$. Temperature: 37 °C; pH = 7.0. ×——×, Control experiment, 250 µM ATP present; ●——●, vesicles incubated in the absence of ATP. At the time indicated by the arrow 2 mM EGTA (○——○) or 5 µM A23187 calcium ionophore (△——△) was added to the medium.

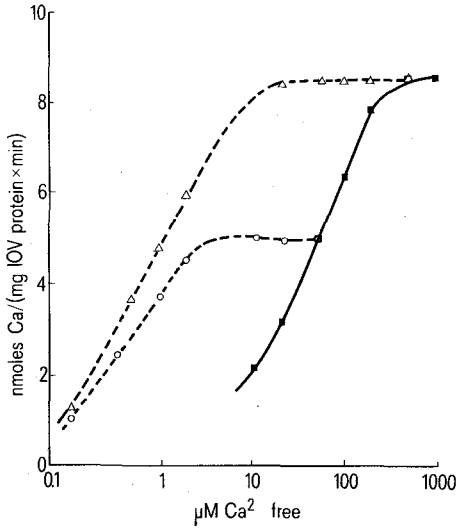

Fig. 2. Active calcium uptake by inside-out vesicles as a function of free ionized calcium concentration in the media. For the assay conditions see the 'method' section. Temperature: 37 °C; pH = 7.0. ■——■, Unbuffered calcium; △——△, ionized calcium concentration adjusted by a calcium-EGTA buffer containing 500 µM total calcium; ○——○, ionized calcium concentration adjusted by a calcium-EGTA buffer containing 50 µM total calcium.

Effects of unbuffered calcium and total calcium concentrations in calcium-EGTA buffers on the rate of active calcium uptake by inside-out vesicles.

Ca_t (µM)	EGTA (µM)	Free Ca^{2+} (µM)	Rate of calcium uptake (nmole calcium/ mg IOV protein × min)
20	-	20	3.2
50	-	50	5.4
50	30	20.7	5.4
100	-	100	7.3
100	80	21.7	7.0
200	-	200	8.0
200	180	23.5	7.9
500	-	500	9.2
500	480	27.9	8.6

For the assay conditions see the 'method' section. The rate of active calcium uptake was calculated by linear regression using at least 3 time-points in a 10-min incubation period. Temperature = 37 °C; pH = 7.0. 1 of 3 similar experiments.

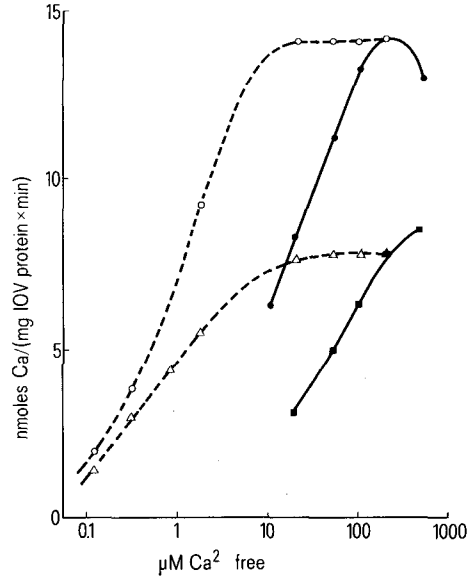

Fig. 3. Active calcium uptake by inside-out vesicles as a function of free ionized calcium concentration in the media - effects of the supernatant activator. For the assay conditions see the 'method' section. The final concentration of the dialysed supernatant in the incubation media was 50 µg protein/ml medium. ■——■, Unbuffered calcium (control vesicles); ●——●, unbuffered calcium (supernatant present); △——△, ionized calcium concentration adjusted by a calcium-EGTA buffer containing 200 µM total calcium (control vesicles); ○——○, ionized calcium concentration adjusted by a calcium-EGTA buffer containing 200 µM total calcium (supernatant present).

tive calcium uptake were studied. Figure 2 shows a typical experiment in which calcium uptake was measured in the same IOV preparation with various free ionized calcium concentrations in the media. The calcium concentration required for half-maximal stimulation of active calcium uptake (K_{Ca}) by using unbuffered calcium is about 40–50 μM, and a saturation of the calcium uptake appears at 300–500 μM calcium. In contrast to this, if the free ionized calcium concentration is adjusted by a calcium-EGTA buffer which contains 500 μM total calcium ($Ca_t = 500$ μM), the value of K_{Ca} obtained is between 0.5 and 0.7 μM, that is about 100 times smaller than in the unbuffered system. At this Ca_t the maximum rate of active calcium uptake equals that observed with unbuffered calcium. If, however, the calcium-EGTA buffer is prepared by using a Ca_t of 50 μM, the K_{Ca} value obtained is about 0.5 μM again, whereas the maximum rate of calcium uptake is smaller than that at 500 μM total calcium, and corresponds to the rate of uptake observed with 50 μM unbuffered calcium in the medium.

In order to analyse the effects of Ca_t in a calcium-EGTA buffer, we determined the rate of active calcium uptake by IOVs at about equal free ionized calcium concentrations (which saturated calcium uptake in calcium-EGTA buffers), but using different total calcium concentrations. As it is shown in the table, the maximum transport rates obtained with calcium-EGTA buffers containing different total calcium levels, are similar to the rates obtained at unbuffered calcium concentrations corresponding to the given Ca_t.

According to the above findings, the rate of active calcium uptake by inside-out vesicles is influenced by both the free ionized and the total calcium concentrations in a calcium-EGTA buffer. Similar data are obtained if the activator protein is added to the medium (figure 3). The maximum rate of active calcium uptake is about doubled by the activator either in the presence of unbuffered calcium or calcium-EGTA buffers containing Ca_t greater than 500 μM. However, if Ca_t in the buffer is smaller than 500 μM, the maximum transport rate depends on the actual value of total calcium (not shown). The values for K_{Ca} in the calcium-EGTA buffers are about 0.5–0.7 μM both in the control and the activator-supplemented vesicles, in contrast to the K_{Ca} of 40 μM and 15 μM obtained with the control and activator-treated vesicles, respectively. The low protein concentration in the experimental systems makes any significant calcium or EGTA binding by the IOVs unlikely, and the high, outwardly directed calcium gradient obtained during calcium uptake suggests a low passive permeability of the vesicle membrane for calcium as well as for EGTA.

The explanation offered by us for the above results is the following: Active calcium transport system in the red cell membrane has at least 2 different binding sites for calcium. One of these sites has high affinity for calcium with a K_{Ca} of about 0.5 μM, whereas the 2nd binding site has a K_{Ca} of 15–50 μM, depending on the presence or absence of the activator protein. Active calcium transport occurs only if both binding sites are occupied by calcium. In order to explain the effects of Ca_t in the calcium-EGTA buffers on the calcium transport rate, we have to suppose that the 2nd binding site accepts calcium also in a chelated form. The calcium-EGTA complex is bound to this site, and in a following step calcium is removed from the chelate and is transported through the membrane. An alternative explanation for the results would be to suppose that the transport enzyme has high affinity for calcium, but higher concentrations of either unbuffered calcium or calcium-EGTA complex are required to alter the conformation of the cell membrane for favouring active calcium transport.

In both explanations it is suggested that the calcium-EGTA complex is recognized by the cell membrane as free calcium. This finding suggests a careful reconsideration of those data in the literature which were obtained by investigating calcium-dependent phenomena in calcium-EGTA buffer systems.

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Studies of the blood of *Ascidia ceratodes*. Total blood cell counts, differential blood cell counts, hematocrit values, seasonal variations, and fluorescent characteristics of blood cells[1]

W.R. Biggs[1] and J.H. Swinehart

Department of Chemistry, University of California, Davis (California 95616, USA), 6 November 1978

Summary. Seasonal, and animal size and weight variations of the blood cells of the vanadium-containing ascidian, *Ascidia ceratodes*, were determined. The fluorescent properties of various cell types were ascertained, and discussed in terms of cell development, phylogenic position of the species, and chemicals in the cells.

Blood cells from ascidians, invertebrates in the phylum Chordata, are unique in many respects[2]. The predominant cell in the blood of vanadium-containing ascidians, the vanadocyte, contains significant levels of vanadium and sulfuric acid. The oxidation state of the vanadium contained in the vanadocytes of the species *Ascidia ceratodes*, the subject of this paper, is plus 3[3].

In preparation for studies of the chemical constituents of the various blood cell types, it was necessary to determine both the normal frequency for each blood cell type and to locate a suitable probe for the study of chemical changes within the cells. To this end total blood cell counts, differential blood cell counts, hematocrit values, seasonal variations in blood cell counts, and the fluorescent characteristics of various blood cells for the solitary ascidian *A. ceratodes* were determined.

Materials and methods. Samples of *A. ceratodes* used in this study were collected from 3 sites in Bodega Bay, California.