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ENHANCEMENT OF (Ca^{2+} + Mg^{2+})-ATPase ACTIVITY OF HUMAN ERYTHROCYTE MEMBRANES BY HEMOLYSIS IN ISOSMOTIC IMIDAZOLE BUFFER

II. DEPENDENCE ON CALCIUM AND A CYTOPLASMIC ACTIVATOR *

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Summary

1. Activity of the (Ca^{2+} + Mg^{2+})-ATPase of erythrocyte membrane may be enhanced by a cytoplasmic protein activator. The presence of Ca^{2+} is necessary for the ionic strength-dependent interaction between the erythrocyte membrane and the activator. This is true no matter the purity of activator (unfractionated hemolysis supernatant or partially purified activator) or the major source of ionic strength (imidazole or NaCl).

2. When the endogenous activator enhances (Ca^{2+} + Mg^{2+})-ATPase activity of the erythrocyte membrane, there is a physical association between activator and membrane. This association is not disrupted by a decrease in ionic strength to 0.005 but is reversed by exposure to 5 mM ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

3. Activator binding necessary for enhancement of (Ca^{2+} + Mg^{2+})-ATPase activity may occur during preparation of membranes or during incubation for assay of ATPase.

Introduction

(Ca^{2+} + Mg^{2+})-ATPase and active Ca^{2+} transport have been studied extensively in the human erythrocyte membrane [1]. Most evidence is consistent

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Abbreviations: I20, I310, ideal milliosmolar imidazole at 20 and 310 imosM, respectively; HI-40, 40 mM-40 mM histidine/imidazole buffer; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

with the interpretation that the $(Ca^{2+} + Mg^{2+})$-ATPase is a biochemical expression of an outwardly-directed active transport system for Ca^{2+}. Suggestions have been made about preparation procedures which would maximize $(Ca^{2+} + Mg^{2+})$-ATPase activity of isolated erythrocyte membranes [2,3]. The $(Ca^{2+} + Mg^{2+})$-ATPase activity of erythrocyte membranes can also be markedly enhanced by addition of membrane-free hemolysate [4] or partially purified endogenous $(Ca^{2+} + Mg^{2+})$-ATPase activator [5] to the incubation medium during ATPase assay. In a companion paper, we report [6] enhanced $(Ca^{2+} + Mg^{2+})$-ATPase activity in membranes prepared from erythrocytes hemolyzed in isosmotic imidazole buffer (310 ideal milliosmolar imidazole, I310) when compared to membranes prepared from erythrocytes hemolyzed in 20 ideal milliosmolar imidazole buffer (I20) or 20 ideal milliosmolar phosphate buffer (P20) (the so-called Dodge method [7]). The effect of isosmotic imidazole hemolysis was shown to be mediated via an ionic strength-dependent interaction between an endogenous $(Ca^{2+} + Mg^{2+})$-ATPase activator [4,5] and the erythrocyte plasma membrane [6]. Results presented in this paper demonstrate physical association of this activator with the membrane in conjunction with high $(Ca^{2+} + Mg^{2+})$-ATPase activity and further show that the association is dependent on the presence of Ca^{2+}.

Materials

Na_2ATP (Sigma grade), EGTA and Tris (Tris(hydroxymethyl)amino-methane, "Trizma base") were purchased from Sigma. Sodium dodecyl sulfate (SDS), bis and acrylamide were purchased from Bio-Rad. Carboxymethyl-Sephadex C_{50} was from Pharmacia. All other chemicals were reagent grade. Glass distilled, deionized water was used for all solutions.

Methods

Membranes were prepared and designated as previously described [6]. If membranes were treated with hemolysate, etc. during preparation, they were subsequently washed at least twice. The protein content of each membrane suspension was measured by the method of Lowry et al. [8].

The ATPase assay contained (in a volume of 1.0 ml) 3 mM ATP (Na_2ATP, neutralized to pH 7), 80 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 18 mM-18 mM histidine-imidazole buffer, pH 7.1, 0.2 ml activator fraction or buffer, and 10^{-4} M ouabain. In appropriate tubes, 10^{-4} M $CaCl_2$ was added for the determination of $(Ca^{2+} + Mg^{2+})$-ATPase. The tubes for Mg^{2+}-ATPase also included the membrane and ATP blanks. All tubes were run in duplicate. Enzymatic hydrolysis of ATP was estimated by measurement of inorganic phosphate released using the method of Fiske and SubbaRow [9]. $(Ca^{2+} + Mg^{2+})$-ATPase was taken as the extra ATP splitting caused by Ca^{2+} in the presence of Mg^{2+} and ouabain.

The endogenous $(Ca^{2+} + Mg^{2+})$-ATPase activator was partially purified according to Luthra et al. [5] using a Carboxymethyl-Sephadex C_{50} column equilibrated and washed with 20 mM Tris/maleate buffer, pH 6.8 at 25°C. The resultant effluent contained a peak of protein material capable of enhancing

$(Ca^{2+} + Mg^{2+})$-ATPase activity of I20 membranes under appropriate conditions. This partially purified material will be referred to as "activator" in experiments described below. An Amicon apparatus with a membrane molecular weight cut-off of 10000 was used to concentrate solutions containing activator. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Fairbanks et al. [10]. SDS-polyacrylamide gel electrophoresis was carried out on both membrane preparations and concentrated activator fractions.

Results

Table I summarizes data for experiments in which buffers were varied during membrane preparation. Membranes from erythrocytes hemolyzed in I310 (I310 membranes) (see ref. 6) had much higher $(Ca^{2+} + Mg^{2+})$ATPase activities than membranes from erythrocytes hemolyzed in I20 (I20 membranes) [6]. Inclusion of 5 mM EGTA in either the hemolysis or washing buffers during the I310 preparation (I310/EGTA membranes) yielded preparations with $(Ca^{2+} + Mg^{2+})$-ATPase activity no greater than that of I20 membranes. Inclusion of 5 mM EGTA during the preparation of I20 membranes (I20/EGTA membranes) did not significantly change activity from that of I20 membranes.

As demonstrated previously [6], it was possible to enhance the $(Ca^{2+} + Mg^{2+})$-ATPase activity of otherwise "low activity" membranes by exposing the membranes, during preparation, to the supernatant of erythrocyte hemolysis. It was important that exposure to the supernatant take place in a relatively high ionic strength such as that afforded by I310 or 0.9% NaCl (S310). Enhancement of I20 membrane activity by the supernatant of hemolysis in the presence of I310 or S310 could be blocked by the inclusion of 5 mM EGTA. Enhancement of $(Ca^{2+} + Mg^{2+})$-ATPase activity of I20 membranes by treat-

TABLE I

$(Ca^{2+} + Mg^{2+})$-ATPase ACTIVITY OF ERYTHROCYTE MEMBRANES: ENHANCEMENT DURING PREPARATION OF MEMBRANES

Membranes prepared in different ways were treated with activator under various conditions and assayed for $(Ca^{2+} + Mg^{2+})$-ATPase activity as described in Methods. $(Ca^{2+} + Mg^{2+})$-ATPase activity expressed as $\mu mol\ PO_4^{3-} \cdot mg^{-1}$ protein $\cdot h^{-1}$, mean ± S.E.

Type of preparation	*n*	$(Ca^{2+} + Mg^{2+})$-ATPase activity
I310	6	2.24 ± 0.21 *
I20	9	0.67 ± 0.08
I310/EGTA	16	0.72 ± 0.04
I20/EGTA	15	0.70 ± 0.03
I20, treated with hemolysis supernatant/I310 or S310	14	2.32 ± 0.18 *
I20, treated with hemolysis supernatant/(I310/EGTA or S310/EGTA)	2	0.82 ± 0.03
I20, treated with activator/I310	8	1.93 ± 0.20 *
I20, treated with activator/(I310/EGTA)	2	0.87 ± 0.19
I20, treated with activator/(I310/Ca/EGTA)	2	1.37 ± 0.22 *
I20/EGTA or I310/EGTA treated with activator/I310	4	0.76 ± 0.16
I20/EGTA or I310/EGTA treated with activator/(I310/Ca)	3	1.70 ± 0.20 *

* Significantly greater than I20 membranes, $P < 0.0005$.

ment with activator/I310 was prevented by 5 mM EGTA (unless 5 mM $CaCl_2$ was also included). Once I20 or I310 membranes were exposed to 5 mM EGTA (I20/EGTA or I310/EGTA membranes), their $(Ca^{2+} + Mg^{2+})$-ATPase activity was not enhanced by treatment during preparation with activator/I310 unless approximately 10^{-5} M Ca^{2+} was also added (e.g., I20/EGTA treated with activator/I310/Ca). Since EGTA is a relatively selective chelating agent for Ca^{2+}, these results (all summarized in Table I) suggest that the presence of Ca^{2+} is essential to promote and/or sustain the activator-mediated membrane modification.

Experiments were designed to test whether the enhancement of $(Ca^{2+} + Mg^{2+})$-ATPase activity observed in I20 membranes upon addition of activator to the incubation medium for ATPase assay [4—6] was due to a persistent effect of the activator on the erythrocyte plasma membrane. Membranes were preincubated in a complete ATPase assay incubation medium (including 10^{-4} M $CaCl_2$) with or without inclusion of activator. Membranes were preincubated for 1 h at 37°C; they were then spun down, washed once with I20 and once with HI-40, and were incubated in the absence of added activator to test for any persistent change in $(Ca^{2+} + Mg^{2+})$-ATPase activity (Table II). Preincubation of I310 membranes yielded membranes with no significant difference ($P > 0.10$) in $(Ca^{2+} + Mg^{2+})$-ATPase activity from that of non-preincubated I310 membranes. The $(Ca^{2+} + Mg^{2+})$-ATPase activity of I310 membranes preincubated with or without activator was not significantly different ($P > 0.50$). By contrast, I20, I20/EGTA, and I310/EGTA membranes showed significant differences ($P < 0.03$) in $(Ca^{2+} + Mg^{2+})$-ATPase activity between those preincubated with activator, and those preincubated without activator, respectively. Membranes preincubated without activator had the same $(Ca^{2+} + Mg^{2+})$-ATPase activity as non-preincubated I20 membranes. The $(Ca^{2+} + Mg^{2+})$-ATPase activity of I20 membranes preincubated with activator was greatly enhanced and was not significantly different ($P > 0.20$) from that of non-preincubated I310

TABLE II

$(Ca^{2+} + Mg^{2+})$-ATPase ACTIVITY: DEPENDENCE ON PREINCUBATION CONDITIONS

Membranes were prepared in various ways, preincubated in different media for 1 h at 37°C, and subsequently assayed for $(Ca^{2+} + Mg^{2+})$-ATPase activity as described in the text and in Methods. $(Ca^{2+} + Mg^{2+})$-ATPase activity expressed as $\mu mol\ PO_4^{3-} \cdot mg^{-1}$ protein $\cdot h^{-1}$, mean ± S.E.

Type of membrane	Preincubation condition	n	$(Ca^{2+} + Mg^{2+})$-ATPase activity
I310	Non-preincubated	4	1.86 ± 0.11 *
I310	+ Activator	3	1.58 ± 0.18 *
I310	— Activator	4	1.72 ± 0.34 *
I20	Non-preincubated	3	0.76 ± 0.17
I310/EGTA	Non-preincubated	3	0.71 ± 0.14
I20/EGTA	Non-preincubated	2	0.63 ± 0.07
I20	— Activator	4	0.76 ± 0.09
I310/EGTA	— Activator	3	0.73 ± 0.09
I20/EGTA	— Activator	2	0.74 ± 0.13
I20	+ Activator	3	1.32 ± 0.30 *
I310/EGTA	+ Activator	2	1.48 ± 0.25 *
I20/EGTA	+ Activator	2	1.86 ± 0.68 *

* Significantly greater than I20 (non-preincubated) ($P < 0.03$).

membranes. These data suggest that membranes prepared with low (Ca^{2+} + Mg^{2+})-ATPase activity (i.e., I20, I310/EGTA, and I20/EGTA membranes) can be persistently modified by activator during incubation at 37°C. Membranes prepared with high (Ca^{2+} + Mg^{2+})-ATPase (i.e., I310 membranes) neither gained nor lost ATPase activity during preincubation in a complete incubation medium, whether activator was present or not.

As part of one experiment, I20, I310, I20/EGTA, and I310/EGTA membranes were preincubated with or without activator in an otherwise normal but $CaCl_2$-free ATPase assay incubation medium. After washing and upon subsequent testing for ATPase in a complete medium, all of the resulting membranes showed low (Ca^{2+} + Mg^{2+})-ATPase activity whether preincubated with (0.69 ± 0.10 μmol $PO_4^{3-} \cdot mg^{-1}$ protein $\cdot h^{-1}$) or without (0.63 ± 0.05 μmol $PO_4^{3-} \cdot mg^{-1}$ protein $\cdot h^{-1}$) activator. These (Ca^{2+} + Mg^{2+})-ATPase activities were not significantly different ($P > 0.30$) from those of non-preincubated I20, I310/EGTA, and I20/EGTA membranes (0.73 ± 0.07 μmol $PO_4^{3-} \cdot mg^{-1}$ protein $\cdot h^{-1}$). It should be emphasized that I310 membranes lost their potential to express high (Ca^{2+} + Mg^{2+})-ATPase activity when preincubated in a Ca^{2+}-free medium. This indicates that during incubation, as well as during preparation (Table I), the effect of activator on the membrane required the presence of Ca^{2+}. As noted previously, activation during preparation is dependent on ionic strength [6]. Since the ionic strength of the incubation medium is approximately 0.14 and since Ca^{2+} is normally present (10^{-4} M), the data strongly suggest that activation during preparation and the activation during incubation have the same basis.

We wanted to examine whether the activator molecule became physically associated and remained with the erythrocyte plasma membrane after erythrocyte hemolysis in I310. It was possible, alternatively, that the activator, under proper conditions of ionic strength and Ca^{2+} concentration, merely changed the membrane in some specific fashion but did not remain associated with it. The

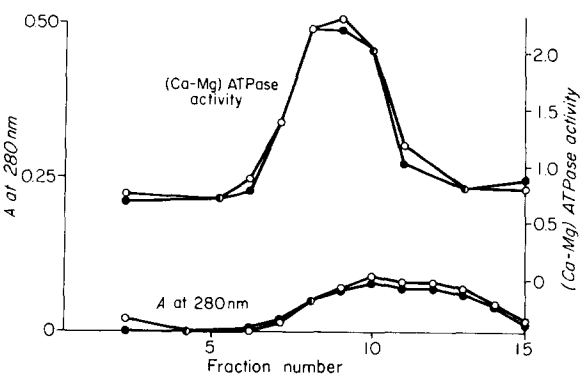

Fig. 1. Comparison of the amounts of (Ca^{2+} + Mg^{2+})-ATPase activator in supernatants of I20 and I310 hemolysis. Supernatants of I20 (○) and I310 (●) hemolysis were diluted 1 : 4 with 20 mM Tris/maleate buffer, pH 6.8 at 5°C, dialyzed against the same buffer for 18 h and were partially purified by a column method [5]. Fractions were analyzed for the presence of protein (*A* at 280 nm) and for their ability to enhance the (Ca^{2+} + Mg^{2+})-ATPase activity of I20 membranes over baseline levels (0.78 ± 0.02 μmol $PO_4^{3-} \cdot mg^{-1}$ protein $\cdot h^{-1}$). Procedures are more completely described in Methods. (Ca^{2+} + Mg^{2+})-ATPase activity is expressed as μmol $PO_4^{3-} \cdot mg^{-1}$ protein $\cdot h^{-1}$.

removal of Ca^{2+} would then allow the membrane to return to its previous condition. Up to this point, our experiments were consistent with either possibility. It was felt more likely that the activator did associate with the membrane, and the following experiments demonstrate that this is indeed the case.

If the I310 and I20 hemolysate supernatants, respectively, (from carefully measured amounts of erythrocytes) were dialyzed and activator fractions were isolated according to Luthra et al. [5], the activator fractions showed nearly the same (Ca^{2+} + Mg^{2+})-ATPase activating ability (Fig. 1). This indicated that: (1) There was approximately the same amount of activator in the membrane-free hemolysate from I20 or I310 hemolyzed cells, and (2) if the activator were associating with the I310 but not I20 membranes, there then must be a great excess of activator within the cell. Close comparison of analogous I20 and I310 fractions from several runs showed a 3—6% greater total activator activity in the I20 hemolysate supernatant. This was not significantly greater ($P > 0.2$) than the activator activity in the I310 hemolysate supernatant. If the activator does associate with the membrane when erythrocytes are hemolyzed in I310, apparently only a small percentage of the total available activator is needed.

Samples of concentrated activator fraction, I310, I20, I20-activated and I310/EGTA membranes were run on SDS-polyacrylamide gel electrophoresis. The gel from the concentrated activator fraction had many bands. Much of the protein of the concentrated activator fraction is apparently not activator protein. Visible protein bands on the gels from the different types of membranes showed no clear-cut differences. It was readily apparent that SDS-polyacrylamide gel electrophoresis gives no definite answer on the presence of the specific protein activator molecule on the I310 membrane. Since a specific activator protein could not be demonstrated in situ, (i.e., in the membrane preparations), we attempted to show that it could be functionally removed from I310, but not I20, membranes. 10-ml samples of erythrocytes were thus hemolyzed in I310 or I20

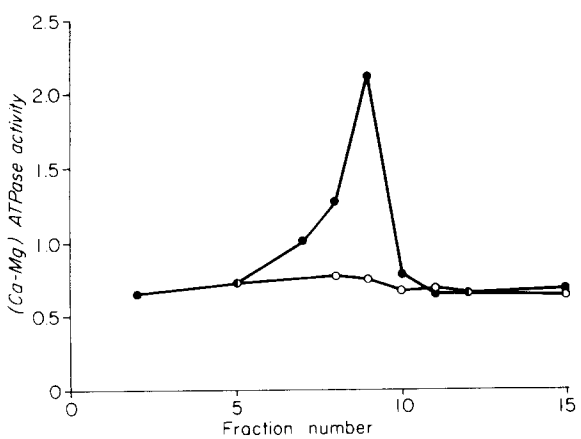

Fig. 2. Removal by EGTA of (Ca^{2+} + Mg^{2+})-ATPase activator from I310 membranes. Membranes from erythrocytes hemolyzed in I20 or I310 were washed with 5 mM EGTA in I20. Washes were separately concentrated approximately 10-fold using an Amicon apparatus with a membrane molecular weight cut-off of 10 000. The concentrated washes from the I20 membranes (○) and from the I310 membranes (●) were partially purified by a column method [5]. Fractions were assayed for their ability to enhance the (Ca^{2+} + Mg^{2+})-ATPase activity of I20 membranes over baseline levels (0.68 ± 0.01 μmol $PO_4^{3-} \cdot mg^{-1}$ protein $\cdot h^{-1}$). Procedures are more completely described in Methods. (Ca^{2+} + Mg^{2+})-ATPase activity is expressed as μmol $PO_4^{3-} \cdot mg^{-1}$ protein $\cdot h^{-1}$.

and washed several times in I20. The I310 or I20 membranes, respectively, were then washed with 5 mM EGTA in 120 ml of I20. The EGTA washes were concentrated about 10-fold in an Amicon apparatus. These concentrated washes were dialyzed, and the column method [5] was used to obtain activator fractions (Fig. 2). $(Ca^{2+} + Mg^{2+})$-ATPase activator appeared in fractions from the EGTA wash of I310, but not I20, membranes. If one compares the amount of activator activity in the EGTA wash of I310 membranes (Fig. 2, i.e., amount of activator actually associated with the activated membrane) with the amount in the supernatant of I20 hemolysis (Fig. 1, i.e., total amount of activator in the erythrocyte), one can estimate the percentage of total activator associated with the activated membrane. This calculation indicates that about 1% of the endogenous activator of the erythrocyte is enough to fully activate the $(Ca^{2+} + Mg^{2+})$-ATPase of the membrane when that activator is membrane-associated.

Discussion

The observation that $(Ca^{2+} + Mg^{2+})$-ATPase activator associates with low activity membranes while enhancing their activity and that removal of activator from high activity membranes is correlated with a significant decrease in $(Ca^{2+} + Mg^{2+})$-ATPase activity is consistent with the suggestion that the cytoplasmic endogenous activator binds to the membrane to modify ATPase activity. A stoichiometric interaction seems likely, although an enzymatic modification has not been rigorously excluded. In any event, the activator physically associates with the membrane and the association is dependent upon calcium. The degree of association/dissociation of this endogenous activator with the membrane either during preparation or incubation could help account for the range of $(Ca^{2+} + Mg^{2+})$-ATPase activities reported in the literature [1].

Further work similar to that on the Ca^{2+}-dependent adenylate cyclase activator [see references in 11] may demonstrate the molecular mechanism(s) of the interactions of Ca^{2+}, the activator, and membrane sites. For example, at this time we do not know if Ca^{2+} interacts with the activator molecule, the plasma membrane, or both. It is clear that erythrocyte membranes may act as a source of sufficient Ca^{2+} for the activator association process. I20 membranes exposed to activator in I310 become activated, but if the membranes have previously been exposed to EGTA, the activation does not occur (Table I). It should be noted that EGTA-treated membranes can be activated during incubation (when Ca^{2+} is present in the medium); compare Table I and Table II. Thus, the necessary and sufficient "pool" of Ca^{2+} for the association can apparently be depleted and replenished. The concentration of Ca^{2+} necessary to promote activator/membrane association has not been quantified. It is anticipated that the apparent $K_{\text{dissociation}}$ for Ca^{2+} in this process will be in the physiological intracellular range because a mixture of 5 mM EGTA and 5 mM $CaCl_2$ (which provides approximately 10^{-5} M Ca^{2+}) was able to support significant, although not maximal, interaction of activator with membranes (Table I).

We assume that activation of $(Ca^{2+} + Mg^{2+})$-ATPase by the cytoplasmic activator would result in increased calcium transport in appropriate test systems. Preliminary evidence supporting this assumption has been presented [12]. Based on this assumption, and on the observations that: (1) The activator

has a marked and reasonably specific effect on $(Ca^{2+} + Mg^{2+})$-ATPase activity [4,5,13] and (2) activator interaction with the membrane is reversible and dependent on Ca^{2+}, we wish to put forward a speculative model for control of the Ca^{2+} pump in the erythrocyte membrane. We suggest that intracellular free Ca^{2+} regulates binding of activator to the membrane and thereby regulates $(Ca^{2+} + Mg^{2+})$-ATPase. Because the activator is present in excess of the amount necessary to fully activate the erythrocyte membrane $(Ca^{2+} + Mg^{2+})$-ATPase, the concentration of intracellular ionized calcium could determine the degree of the activator-membrane association and, thus, Ca^{2+} transport from the erythrocyte. Whether or not the above conjecture will be confirmed remains to be seen. To our knowledge, a cytoplasmic activator of plasma membrane Ca^{2+}-transport has not been suggested in other cells. If present in other cell types, the activator could have great significance.

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References

1 Schatzmann, H.J. (1975) in Current Topics in Membranes and Transport (Bronner, F. and Kleinzeller, A., eds.), Vol. 6, pp. 126—168, Academic Press, New York
2 Scharff, O. (1972) Scand. J. Clin. Lab. Invest. 30, 313—320
3 Wolf, H.U. (1970) Biochim. Biophys. Acta 219, 521—524
4 Bond, G.H. and Clough, D.L. (1973) Biochim. Biophys. Acta 323, 592—599
5 Luthra, M.G., Hildenbrandt, G.R. and Hanahan, D.J. (1976) Biochim. Biophys. Acta 419, 164—179
6 Farrance, M.L. and Vincenzi, F.F (1977) Biochim. Biophys. Acta 471, 49—58
7 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119—130
8 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
9 Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375—400
10 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2617
11 Lynch, T.J., Tallant, E.A. and Cheung, W.Y. (1976) Biochem. Biophys. Res. Commun. 68, 616—625
12 Macintyre, J.D. and Green, J.W. (1977) Fed. Proc. 36, abst., 5
13 Vincenzi, F.F. and Farrance, M.L. (1975) Pharmacologist 17, abst., 199