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Further Studies on Effects of Some Local Anesthetics on Calcium Binding to Lipid-extracted RBC Membrane Fragments

—Effect of Modification of Carboxyl Group with Carbodiimide—

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ABSTRACT

The interaction of calcium and local anesthetics was investigated with the lipid extracted human RBC membrane fragments treated with carbodiimide in order to titrate carboxyl groups. A water soluble carbodiimide [1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide methotoluene-p-sulfonate], referred to as a carbodiimide reagent, and glycine methylester were used for this purpose.

About 76% of carboxyl groups of the fragments were modified at a concentration of 0.05 M carbodiimide reagent. The interaction of calcium and local anesthetics such as procaine and lidocaine with these fragments still showed typical competition. However, when the calcium binding was decreased to 8% at a higher concentration of carbodiimide reagent (0.08 M), the local anesthetics still inhibited the calcium binding, but were not competitive in nature. In other words, if concentrations of the carbodiimide reagent were raised, the degree of inhibition by the local anesthetics was gradually decreased and was not competitive in nature. Finally, no inhibition was demonstrated when the concentration of the reagent was 0.1 to 0.4 M.

The above findings, seem to suggest that local anesthetics such as procaine and lidocaine interact with carboxyl groups, in addition to phosphodiester groups of phospholipids as previously reported, and inhibited competitively calcium binding to carboxyl groups of the membrane fragments.

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INTRODUCTION

Local anesthetics have been shown to inhibit calcium binding to many biological and artificial membranes such as isolated sarcolemma (Madeira and Carvalho, 1972), microsomes (Bondani and Karler, 1970), submitochondrial particles (Scarpa and Azzi, 1968), lobster axon (Blaustein and Goldman, 1966) and RBC membrane fragments (Kwant and Seeman, 1969; Kim et al., 1973). Several investigators also showed that local anesthetics inhibited Ca^{++} binding not only to phospholipids, extracted from muscle and nerve (Blaustein, 1967; Feinstein and Paimre, 1964) but also to a phospholipid model membrane (Feinstein and Paimre, 1966; Papahadjopoulos, 1970). This laboratory reported previously that the local anesthetics inhibited competitively the Ca^{++} binding to the lipid-extracted RBC membrane, filter paper and egg albumin preparation. Ca^{++} binding sites of lipid-extracted RBC membrane were speculated to be carboxyl groups of sialic acid, aspartic acid and glutamic acid of the membrane protein (Forstner and Manery 1971).

In this experiment, the carboxyl groups of

lipid-extracted RBC membrane fragments were titrated with soluble carbodiimide reagent, then the effect of local anesthetics on Ca^{++} binding to the membrane fragment, was investigated to see whether competitive inhibition by local anesthetics of Ca^{++} binding was changed.

METHOD

a) Modification of carboxyl groups of RBC membrane fragments

Hemoglobin-free RBC membrane fragments were prepared by the method of Dodge et al. (1963). The fragments were washed twice with 10 vol. of 0.01 M acetic acid, adjusted to pH 4.6 with NaOH, and divided into control and experimental groups. To modify the carboxyl groups, carbodiimide reagent [1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide methotoluene-p-sulfonate] was added to the experimental group, to give a final concentration of 0~0.4 M, along with glycine methyl-ester to a final concentration of 1.33 M, and the control group contained only 1.33 M glycine methyl ester without carbodiimide reagent. The solutions were adjusted to pH 3.0 by addition of 1N HCl and incubated in 22°C water bath for 3 hr. During incubation, the pH was maintained at pH 3.0 for 30~60 min by the addition of 1N HCl when necessary. No further additions of HCl were necessary after 30~60 min, indicating that the reaction was complete.

After overnight storage at 4°C, the contents of the reaction vessels were adjusted to pH 2.0 with 1N HCl and left for 20 min, to hydrolyze potential phosphoamide bonds that might have formed between the membrane phospholipids and glycine methyl ester. The fragments were then separated by centrifugation at 20,000g for 20 min, and washed three times in 10 vol. of 0.01 M Tris buffer, pH 7.4, to remove any excess of reagents. The protein concentration of each sample was determined by the method of Lowry et al. (1951).

An aliquot of each control and carbodiimide treated sample (50μl) was transferred to a cover glass, and dried in a desiccator in a cold room as described in a previous paper (Kim et al., 1973).

b) Ca^{++} binding study

The lipids of membrane fragments dried on the cover glasses were extracted and subsequent Ca^{++} binding study was conducted as described in a previous paper (Kim et al., 1973).

c) Chemicals

$Ca^{45}Cl_2$ was obtained from the radiochemical center of Amersham, Bucking-hamshire, England, a water soluble carbodiimide [1-cycloheyxl-3-(2-morpholinoethyl)carbodiimide methotoluene-p-sulfonate] and glycine methyl-ester hydrochloride (crystalline) from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A., procaine-HCl and lidocaine-HCl from HOEI Pharmaceutical Co., Japan and chloroform and methanol from Shimakju Chemical Co., Japan.

RESULTS

a) Effects of local anesthetics(procaine and lidocaine) on Ca^{++} binding to lipid-extracted RBC membrane fragments.

The amounts of calcium bound to the membrane fragments were calculated from c p m of the cover glasses remaining after the 5th wash and from the specific activities of the medium in which the cover glasses had been incubated. The results of effect of local anesthetics on Ca^{++} binding to membrane frag-

Table I. Effect of Procaine on Ca^{++} Binding to Lipid-extracted RBC Membrane Fragments

$[Ca^{++}]$ mM	Control (mean±S.E.)×10^{-8}moles	With 1 mM procaine (mean±S.E.)×10^{-8}moles	With 5 mM procaine (mean±S.E.)×10^{-8}moles	With 10 mM procaine (mean±S.E.)×10^{-8}moles
0.5	4.86±0.03	4.65±0.07	4.06±0.12	3.28±0.10
1.0	7.68±0.04	7.48±0.12	6.53±0.11	5.56±0.09
5.0	14.52±0.11	15.25±0.23	13.82±0.16	12.91±0.21
10.0	16.63±0.15	16.46±0.14	15.95±0.26	15.12±0.24

Table II. Effect of Lidocaine on Ca^{++} Binding to Lipid-extracted RBC Membrane Fragments

$[Ca^{++}]$ mM	Control (mean±S.E.)×10^{-8}moles	With 1 mM lidocaine (mean±S.E.)×10^{-8}moles	With 5 mM lidocaine (mean±S.E.)×10^{-8}moles	With 10 mM lidocaine (mean±S.E.)×10^{-8}moles
0.5	4.86±0.03	4.60±0.06	4.01±0.02	3.56±0.20
1.0	7.68±0.04	7.45±0.08	6.95±0.07	6.10±0.12
5.0	14.51±0.11	15.03±0.29	14.21±0.18	14.17±0.90
10.0	16.63±0.15	16.02±0.23	15.93±0.32	14.39±0.11

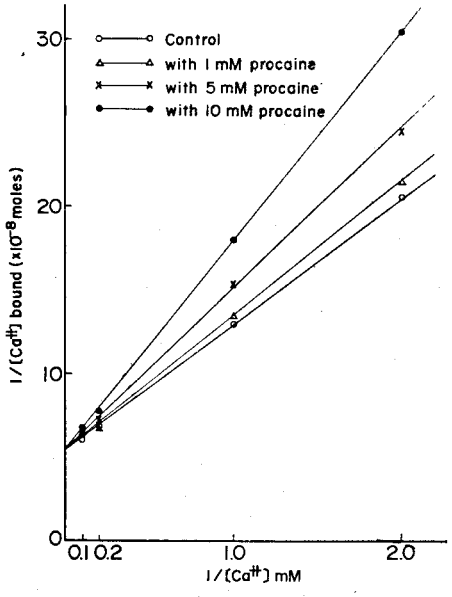

Fig. 1. A Lineweaver-Burk plot of the results shown in Table I. Procaine inhibited competitively Ca^{++} binding to the lipid-extracted RBC membrane fragments.

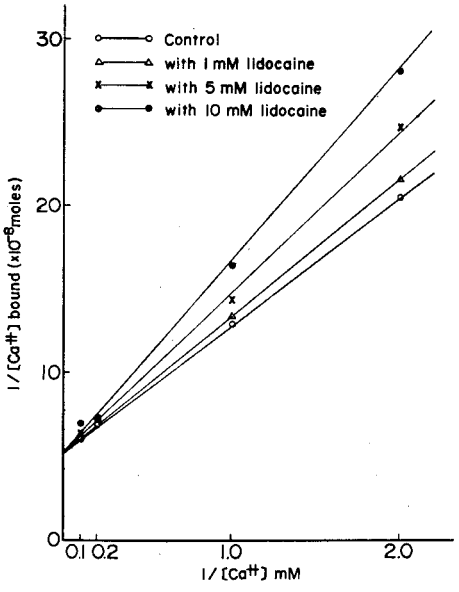

Fig. 2. A Lineweaver-Burk plot of the results shown in Table II. Lidocaine inhibited competitively Ca^{++} binding to the lipid-extracted RBC membrane fragments.

ments are shown in Tables I and II.

Lineweaver-Burk plots of these results are shown in Figs. 1 and 2. As can be seen in these figures, the degree of inhibition of Ca^{++} binding was "dose dependent" on local anesthetics and calcium binding was inhibited competitively by the local anesthetics.

These results are in accordance with previous results (Kim et al., 1973), and indicate that the local anesthetics competitively inhibit Ca^{++} binding to the binding sites of membrane proteins.

The most likely Ca^{++} binding sites associated with the membrane protein would be the carboxyl groups of sialic acid, aspartic acid and glutamic acid as suggested by Forstner and Manery (1971).

b) Ca^{++} binding to the RBC membrane fragments treated with carbodiimide reagents.

A water soluble carbodiimide reagent and a glycine methyl ester were used to modify the free carboxyl groups of the membrane as described in the section on methods. As the concentration of carbodiimide reagent was raised to 0.05~0.4 M, the Ca^{++} binding was decreased by about 76~96%, but maximal reduction (96%) was observed when the membrane fragment was treated with concentrations higher than 0.1M of carbodiimide reagent.

None of the carboxyl groups of membrane protein were modified by 0.01M carbodiimide reagent, since the Ca^{++} binding to the protein component of the fragments was not changed at all.

However, Ca^{++} binding to the fragments was decreased to 24% by 0.05 M carbodiimide reagent and to 8% by 0.08 M carbodiimide reagent. By increasing the concentration of carbodiimide reagent above 0.1 M, the Ca^{++} binding to the carboxyl groups of the membrane fragments has been completely abolished. This view differs from the one presented by Forstner and Manery (1971), who reported that Ca^{++} binding was depressed by all concentrations of carbodiimide reagent employed (0.04~0.4 M), i. e. about 21.6% of Ca^{++} binding capacity was reduced when the fragments were treated by 0.1 M carbodiimide reagent, about 78% by 0.2 M and about 96.5% by 0.4 M.

c) Effects of local anesthetics on Ca^{++} binding to carbodiimide treated and lipid-extracted membrane fragments.

To investigate whether local anesthetics still compete with Ca^{++} for binding sites of lipid-extracted fragments which have been treated with carbodiimide reagent, the membrane fragments treated with various concentrations of carbodiimide reagents ranging from 0 to 0.4 M were used for Ca^{++} binding study as described in the section on methods.

The results obtained with the membrane fragments treated with 0.01 M carbodiimide are shown in Table III and Lineweaver-Burk plots are shown in Fig. 3. The results show that Ca^{++} binding capacity and typical competition between local anesthetics and Ca^{++} for binding sites have not been changed. It was found that local anesthetics still competitively inhibited Ca^{++} binding to the fragments even if the Ca^{++} binding capacity was reduced to 24% by 0.05 M carbodiimide treatment (Table IV, Fig. 4). It should be noted that intersection at the ordinate shifted upward after carboxyl titration in this case,

Table Ⅲ. Effect of Procaine on Calcium Binding to 0.01 M Carbodiimide Treated and Lipid-extracted RBC Membrane Fragments

$[Ca^{++}]$ mM	Control (mean±S.E.)$\times 10^{-8}$moles	With 5 mM procaine (mean±S.E.)$\times 10^{-8}$moles	With 10 mM procaine (mean±S.E.)$\times 10^{-8}$moles
0.5	4.86±0.13	4.40±0.07	3.92±0.11
1.0	8.06±0.11	7.00±0.09	6.19±0.10
5.0	14.38±0.11	12.65±0.96	13.21±0.11
10.0	15.62±0.14	14.82±0.31	14.31±0.64

Table Ⅳ. Effect of Procaine on Calcium Binding to 0.05 M Carbodiimide Treated and Lipid-extracted RBC Membrane Fragments

$[Ca^{++}]$ mM	Control (mean±S.E.)×10^{-8}moles	With 5 mM procaine (mean±S.E.)×10^{-8}moles	With 10 mM procaine (mean±S.E.)×10^{-8}moles
0.5	1.14±0.04	0.97±0.05	0.79±0.05
1.0	1.87±0.06	1.55±0.07	1.33±0.04
5.0	3.45±0.15	3.06±0.12	2.92±0.08
10.0	3.85±0.14	3.39±0.16	3.22±0.13

Table Ⅴ. Effect of Procaine on Calcium Binding to 0.08 M Carbodiimide Treated and Lipid-extracted RBC Membrane Fragments

$[Ca^{++}]$ mM	Control (mean±S.E.)×10^{-8}moles	With 5 mM procaine (mean±S.E.)×10^{-8}moles	With 10 mM procaine (mean±S.E.)×10^{-8}moles
0.5	3.77±0.21	3.51±0.20	3.05±0.13
1.0	5.58±0.21	4.73±0.23	4.72±0.55
5.0	12.62±0.86	11.96±0.68	12.20±0.19
10.0	18.31±1.24	19.38±1.74	20.95±1.95

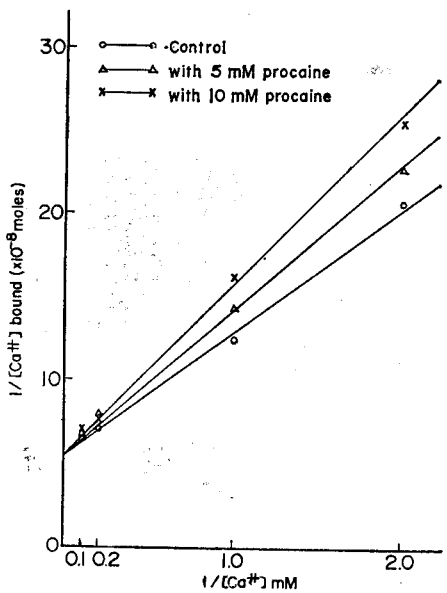

Fig. 3. A Lineweaver-Burk plot of the results shown in Table Ⅲ. Procaine still competitively inhibited Ca^{++} binding to the 0.01M carbodiimide treated and lipid-extracted RBC membrane fragments.

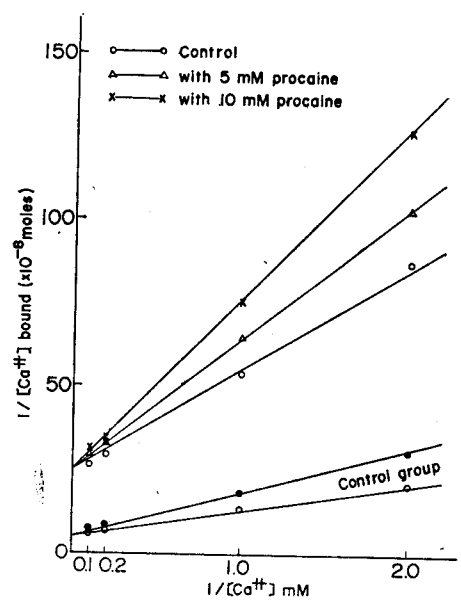

Fig. 4. A Lineweaver-Burk plot of the results shown in Table Ⅳ. Procaine still competitively inhibited Ca^{++} binding to the 0.05M carbodiimide treated and lipid-extracted RBC membrane fragments.

indicating that Ca^{++} binding was reduced by carboxyl titration. However, local anesthetics did not inhibit significantly Ca^{++} binding to the fragments when treated with 0.08 M carbodiimide reagent (Table V, Fig. 5). When the carboxyl groups of fragments were nearly completely modified with higher concentration of carbodiimide reagent 0.1~0.4 M,

Table Ⅵ. Effect of Procaine on Calcium Binding to 0.1 M Carbodiimide Treated and Lipid-extracted RBC Membrane Fragments

$[Ca^{++}]$ mM	Control (mean±S.E.)×10^{-9}moles	With 1 mM procaine (mean±S.E.)×10^{-9}moles	With 5 mM procaine (mean±S.E.)×10^{-9}moles	With 10 mM procaine (mean±S.E.)×10^{-9}moles
0.5	2.25±0.06	2.21±0.07	2.37±0.21	2.34±0.19
1.0	3.24±0.24	3.21±0.09	2.96±0.15	3.14±0.26
5.0	8.34±0.50	6.90±0.56	7.90±0.55	7.27±0.38
10.0	10.98±0.56	9.92±0.45	10.58±0.97	10.65±0.95

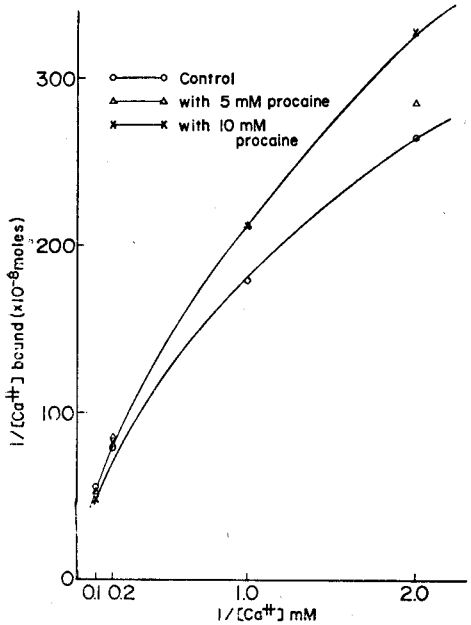

Fig. 5. A Lineweaver-Burk plot of the results shown in Table Ⅴ. Procaine did not inhibit significantly Ca^{++} binding to the 0.08M carbodiimide treated and lipid-extracted RBC membrane fragments.

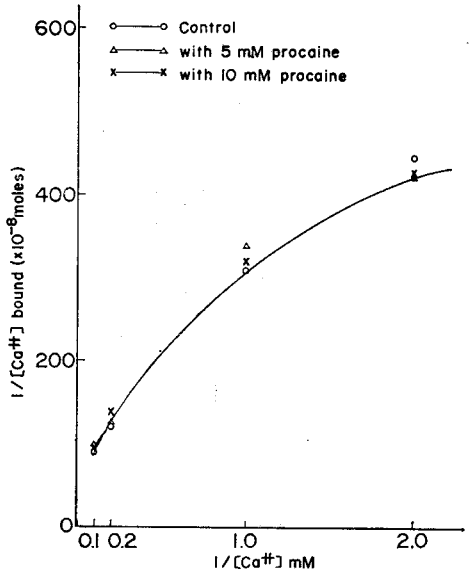

Fig. 6. A Lineweaver-Burk plot of the results shown in Table Ⅵ. A typical competitive inhibition by local anesthetics of Ca^{++} binding to the 0.1M carbodiimide treated and lipid-extracted RBC membrane fragments.

typical competitive inhibition by local anesthetics of Ca^{++} binding to the fragments was not found. Lineweaver-Burk plots no longer showed as linear as can be seen in Table VI and Fig. 6.

DISCUSSION

The Ca^{++} binding sites to the biological membranes were postulated to be phospholipids (Feinstein and Paimre, 1964) and membrane protein (Carvalho, 1966; Forstner and Manery, 1971). Of firmly bound Ca^{++}, 50~80% of Ca^{++} was associated with membrane protein and 20~40% of Ca^{++} with the lipid component (Carvalho, 1966; Duffy and Schwarz, 1973; Forstner and Manery, 1971). Cations compete with each other for binding sites of the biological materials (Carvalho, 1963; 1966).

The competing ions investigated were Ca^{++}, Mg^{++}, Na^{+}, K^{+} and H^{+}. Hydrogen ions have the highest affinity for the apparently non-specific binding sites present in liver microsomes and RBC ghosts (Sanui and Pace 1959; 1962). Many drugs exert effects on Ca^{++} binding by altering the state of bound cellular

calcium. For example, the non-biological complexing agent, ethylenediamine tetraacetate (EDTA) produced a marked increase in Na^{+} binding accompanied by a concomitant decrease in calcium binding.

Adenosine triphosphate (ATP) which is a weaker complexing agent than EDTA, produced quantitatively smaller but qualitatively similar changes in binding (Sanui and Pace, 1967).

Local anesthetics competitively inhibited the Ca^{++} binding at the same binding sites. Of these local anesthetics, chlorpromazine competitively inhibited the Ca^{++} binding to erythrocyte ghosts (Kwant and Seeman, 1969).

Nupercaine and tetracaine, were found to have similar effects even on submitochondrial particles (Scarpa and Azzi, 1968). However, Bondani and Karler(1970) reported a non-competitive type of inhibition by procaine, tetracaine and quinidine on the uptake of Ca^{++} by skeletal muscle microsomes. Madeira and Carvalho (1972) reported that local anesthetics such as quinine, tetracaine, procaine and chlorpromazine inhibited the binding of Ca^{++} to sarcolemma. Quinine, tetracaine and procaine acted as competitive inhibitors while chlorpromazine seemed to be a non-competitive inhibitor.

This laboratory reported that local anesthetics competitively inhibited Ca^{++} binding to the lipid extracted RBC membrane fragments. From the above observation that the lipid extracted membrane still showed a competition between local anesthetics and Ca^{++} for binding sites, it was suggested that carboxyl groups would be one of the sites of competition.

Forstner and Manery (1971) observed that 79% of bound Ca^{++} participated with membrane protein and the Ca^{++} binding sites were suggested to be the carboxyl groups of sialic acid, aspartic acid and glutamic acid. They also observed a decrease in Ca^{++} binding by modification of carboxyl groups with carbodiimide reagent. The carboxyl titration method was also employed by Hoare and Koshland (1967). They virtually reduced the Ca^{++} binding by about 96.5% with 0.4 M carbodiimide reagent. However, it was not known whether local anesthetics still inhibit Ca^{++} binding to the RBC membrane treated with the carbodiimide reagent.

In the present study, it was observed that local anesthetics such as procaine and lidocaine inhibited competitively Ca^{++} binding to the membrane protein, as long as carboxyl groups remained, i.e. the local anesthetics still competitively inhibited Ca^{++} binding to the membrane fragments when 24% of COOH remained (Fig. 4). However, the competitive inhibition by local anesthetics of the Ca^{++} binding disappeared when more than 92% of COOH was modified (Fig. 5, 6).

Therefore it may be suggested that the carboxyl group is one of the major sites for calcium binding and competition between Ca^{++} and local anesthetics. Feinstein and others postulated that Ca^{++} and local anesthetics compete for the binding site of phospholipids in the membrane. However, our results suggest that, besides phospholipids, carboxyl groups are one of the additional sites for Ca^{++} binding and competition between Ca^{++} and local anesthetics in biological membranes.

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