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LOCAL ANESTHETIC–DIVALENT CATION BINDING CENTER INTERACTION

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Abstract—This communication explicitly considers the possibility that local anesthetics interact with divalent cation binding centers, such as chlortetracycline, quin 2, ethyleneglycol bis (*B*-aminoethyl ether)-*N*-*N*,*N*′,*N*′-tetraacetic acid (EGTA), ethylenediamine tetraacetic acid (EDTA) and ATP. Alterations of local anesthetic fluorescence spectra have been found in the presence of EGTA, EDTA and ATP. On the other hand, the fluorescence of chlortetracycline is enhanced and that of quin 2 is quenched by local anesthetics. The spectrofluorometric evidence presented in this paper clearly indicates that local anesthetics and these divalent cation chelators interact in solution. The fluorescence alterations observed do not derive from parallel changes of their respective absorption spectra, thus, they appear to be due to quantum yield changes. On the basis of the spectral perturbations observed, it is likely that local anesthetics interact with M^{2+} binding centers via their electron defective aromatic ring. From the association constants obtained in this study, we make an estimation of the free energy of this interaction ranging from −2.8 to −4.0 kcal/mole in the following experimental conditions: pH 7.4 at an ionic strength of 0.1 at 25°. The relevance of these results to define the physical–chemical characteristics of the local anesthetic receptor site is briefly discussed. It is suggested that local anesthetics can bind strongly to Ca^{2+} and Mg^{2+} binding centers, provided that a hydrophobic region is located nearby.

The molecular mechanisms by which the anesthetics exert their actions have not been fully elucidated yet (see Refs 1 and 2 for recent reviews on this topic). Regarding the local anesthetics it is well established that their anesthetic potency can be correlated to the blockade of the voltage-gated Na^+ channel of axonal membranes [3]. Nevertheless, the molecular mechanism of this blockade is still a matter of discussion. In addition, some local anesthetics like lidocaine and procainamide are well-known cardiac antiarrythmic agents [4]. The hypothesis of anesthesia as a result of non-specific structural perturbation of the lipid matrix of biological membranes, e.g. fluidity changes, excess volume or membrane thickness alteration, have not been convincingly supported to date by experimental data [1, 2]. Among the functional perturbations induced by anesthetics in membranes, alterations of ionic fluxes, particularly K^+ and H^+ fluxes, have been claimed to be involved in the phenomenon of anesthesia [5, 6]. Recently, Greenberg and Tsong [7] have shown that membrane proteins likely provide the binding sites for local anesthetics in axonal membranes, thus strongly supporting that the perturbations induced by anesthetics in functions mediated by membrane proteins are a consequence of their direct interaction with, at least, some of them. Furthermore, Greenberg and Tsong [8] have reported experimental data showing the presence of a receptor protein for local anesthetics in axonal membranes, a protein of 18 kDa, different to the Na^+ channel. However, the basic physical–chemical characteristics and putative biological functions of the local anesthetics receptor site remain to be established.

Stemming from a different set of approaches, however, another picture is emerging. This is that many of the biochemical effects of local anesthetics are expressed on calcium-dependent processes [9]. In particular, the interaction of various local anesthetics with phospholipids shows a good correlation with their ability to block the excitation of nerve membranes and it seems to be antagonized by Ca^{2+}, as is their blocking action on nerve membranes [10]. Feinstein has suggested that these compounds can exert their actions by displacing Ca^{2+} from the anionic sites of phospholipids (mostly provided by phosphatidylserine in nerve cell membranes), this being associated to the raise of the threshold for the action potential induced by these drugs [11]. Blaustein and Goldman [12] and Blaustein [13] gave additional experimental support to this hypothesis. On the other hand, Ca^{2+} is known to mediate a wide variety of processes related to cellular excitability, even to be able to induce action potentials in the nerve cell soma in sodium-free solutions [14]. At the cellular level, it has been shown that calcium and procaine compete with one another with respect to their actions on the membrane conductance of lobster axon [15], that the effects of lidocaine on the gametic fusion of Chlamydomonas are reverted by addition of Ca^{2+} [16] and that the effects of local anesthetics on protein turnover in skeletal muscle are mediated by an increase of cytosolic free calcium ion [17]. At a molecular level, it has been shown by different groups of investigators the existence of an apparent competition between local anesthetics and Ca^{2+} for calmodulin binding [18], that they antagonize some of calmodulin actions [9], and that the affinity of sarcoplasmic reticulum Ca^{2+}-ATPase for Ca^{2+}, inferred from the Ca^{2+} concentration depen-

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dence of the Ca^{2+}-ATPase activity, is lowered in the presence of local anesthetics [19]. It has also been shown that a good correlation exists between anesthetic potency and antagonism of calmodulin by local anesthetics [18] or inhibition of $(Ca^{2+} + Mg^{2+})$-ATPase activities and Ca^{2+} transport alterations of rat brain synaptosomes and sarcoplasmic reticulum [20, 21]. The possibility that, in general, local anesthetics interact with Ca^{2+} binding sites is considered explicitly in this study. Spectrofluorometric studies aiming to check this hypothesis were carried out and these are reported in this paper. As will be shown below, we have obtained experimental evidence of the interaction between local anesthetics (dibucaine, lidocaine, procaine and tetracaine) and divalent cation chelators.

MATERIALS AND METHODS

Tris, *N*-2-hydroxyethyl-piperazine-*N'*2-ethanesulfonic acid (Hepes), imidazole (grade III), ethyleneglycol bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), EDTA, ATP, bovine heart calmodulin, chlortetracycline, quin 2, dibucaine, procaine and tetracaine were purchased from Sigma (St. Louis, MO). Hog brain calmodulin was from Boehringer Mannheim (F.R.G.). All other chemicals used were of the highest analytical quality available. Tetracaine solutions were freshly prepared immediately before use. Fluorescence measurements were carried out at room temperature (20–22°) with an Aminco–Bowman and a Hitachi–Perkin–Elmer (model 650-40) spectrophotofluorimeter. Atomic absorption measurements were performed with a Perkin–Elmer (model 360) atomic absorption instrument in order to determine the level of Ca^{2+} in the buffers used, and this was found to be in the range of 5–10 μM.

Correction for light absorption in fluorescence measurements. Some of the studies reported in this paper have required fluorescence measurements of anesthetic solutions, with UV excitation wavelengths in the 240–260 nm range where other compounds of the solution also absorb, resulting in absorbances larger than 0.1 O.D. Thus, appropriate excitation wavelength shifts to longer wavelengths have been introduced to minimize the absorbance of the solution as much as possible. Nevertheless, in some cases the absorbance at the excitation wavelength has been as high as 0.3 O.D. Therefore, the intensity of fluorescence measured needs to be corrected for inner-filter effects of the solutions. This has been done following an empirical approach developed by Birdsall *et al.* [22]. Considering that the inner-filter effect to be corrected corresponds to a maximum absorbance of 0.3 O.D., we have used equation (6) of Ref. 22, that is:

$$C = \frac{1 - 10^{-A}}{2.303 \cdot A} \tag{1}$$

where C is the correction factor which is related to the absorbance, A. This factor allows the corrected fluorescence intensity (F_{corr}) to be obtained from the observed fluorescence (F_{obs}): $F_{obs} = F_{corr} \times C$. As shown by Birdsall *et al.* [22] this simplified empirical equation gives values of C within 1% (for $A \leq 0.2$) or within 2% (for $A \leq 0.3$) of those given by more complex empirical expressions.

Analysis of fluorescence data. The dependence of the intensity of fluorescence of a solution of a given molecule, A, upon the concentration of another molecule, B, that forms a complex AB having different fluorescence quantum yield can be described by the following equations

$$A + B \overset{K_d}{\rightleftharpoons} AB;\ K_d = \frac{|A| \cdot |B|}{|AB|} \tag{2}$$

$$F = F_A \cdot \frac{|A|}{A_T} + F_{AB} \cdot \frac{|AB|}{A_T} \tag{3}$$

where F_A and F_{AB} are the relative fluorescence intensities of A and AB forms and A_T is the total concentration of A. Combining Eqns (2) and (3) it can be derived that

$$\frac{F_A - F_{AB}}{F - F_{AB}} = 1 + \frac{|B|}{K_d} \tag{4}$$

Equation (4) allows us to obtain the value of K_d from the slope of the linear regression plot of $(F_A - F_{AB})/(F - F_{AB})$ vs free B concentration. Since total B concentration, B_T, has been much higher than total A concentration in all the experiments carried out in this study, it can be safely assumed that $|B| \approx B_T$.

RESULTS

Effect of local anesthetics upon the fluorescence of chlortetracycline

To test the possibility of interaction between local anesthetics and divalent cation binding centers, we have measured the effect of several local anesthetics (procaine, tetracaine and lidocaine) upon the fluorescence of the divalent cation indicator chlortetracycline. The fluorescence of chlortetracycline is known to be largely enhanced upon interaction with Mg^{2+} or Ca^{2+} [23]. The titration of chlortetracycline with these local anesthetics results in an increase of the intensity of fluorescence of this compound (Fig. 1). We have observed that when total local anesthetic concentrations above 2 mM are added to the chlortetracycline solutions, the fluorescence intensity in aqueous solution does not stabilize rapidly, but only after several minutes. This is likely due to interaction of chlortetracycline with micelles of the local anesthetics, transiently formed upon dilution of the concentrated local anesthetic solution, since this effect has not been observed in ethanol. Thus, we have plotted in Fig. 1 the steady-state intensity of fluorescence at each local anesthetic concentration. Because it has been shown that local anesthetics are lipophilic drugs that favorably partition in the lipid bilayer [24], where they locate close to the glycerol backbone of phospholipids [25, 26], we have also studied the effect of these drugs on chlortetracycline fluorescence in ethanol. The results obtained are also presented in Fig. 1. It can be readily seen that the fluorescence of chlortetracycline increases nearly to the same extent in ethanol and in aqueous solution in the presence of these drugs.

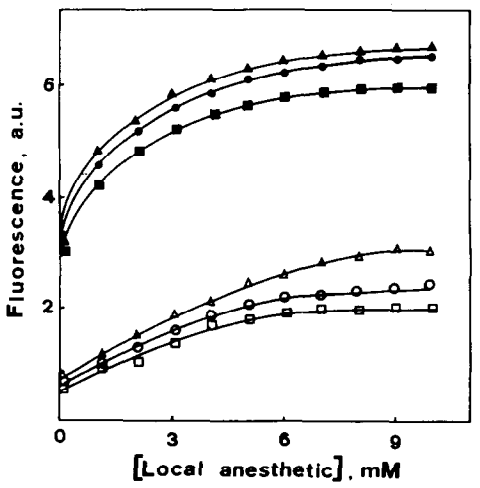

Fig. 1. Dependence of the fluorescence of chlortetracycline (λ_{em} = 520 nm; λ_{exc} = 390 nm) upon local anesthetic concentration in ethanol (filled symbols) and in aqueous solution (open symbols); a.u. stands for arbitrary units. Buffer: 10 mM Tes (pH = 7.0). Different symbols correspond to procaine (▲, △), tetracaine (●, ○) and lidocaine (■, □).

Table 1. Apparent dissociation constants (K_d) of the local anesthetics from divalent cation chelators. These constants have been obtained from the fluorescence data as indicated in Materials and Methods

	K_d (mM)*
EGTA · dibucaine	8.8 ± 0.4†
EGTA · procaine	8.8 ± 0.5†
EDTA · dibucaine	3.4 ± 0.2†
ATP · dibucaine	1.2 ± 0.1†
Chlortetracycline · tetracaine	5.0 ± 0.2‡
	1.2 ± 0.1§
Chlortetracycline · procaine	4.2 ± 0.2‡
	1.3 ± 0.1§
Chlortetracycline · Ca^{2+}	2.6‖
Chlortetracycline · Mg^{2+}	1.4‖
Quin 2 · tetracaine	0.7 ± 0.1§
Quin 2 · procaine	0.6 ± 0.1§

* The data have been fitted with correlation coefficients higher than 0.98.

† Buffer: 14 mM imidazole and 0.1 M KCl (pH = 8.0).

‡ Buffer: 10 mM Tes (pH = 7.0). This pH was used in order to make a direct comparison of these constants with bibliographic dissociation constants of chlortetracycline with Mg^{2+} and Ca^{2+}.

§ Dissociation constants obtained in 3 mM imidazole/HCl buffer.

‖ Ref. 23.

The data of Fig. 1 have been treated as indicated in Materials and Methods and the obtained dissociation constants (K_d) are listed in Table 1. The K_d values of chlortetracycline-Mg^{2+} and chlortetracycline-Ca^{2+} are included in this table for comparison.

The question of whether local anesthetics can revert the interaction of M^{2+} with chlortetracycline (where M^{2+} can be Ca^{2+} or Mg^{2+}) has also been tested. Addition of local anesthetic to chlortetracycline in the presence of millimolar Ca^{2+} or Mg^{2+} concentrations produces an increase of the fluorescence intensity. This is illustrated in Fig. 2 for the case of lidocaine. This effect is lower at higher M^{2+} concentrations, and it reaches a steady level at concentrations larger than *ca.* 25 mM. For the simplest case of reversion of the M^{2+}–chlortetracycline equilibrium by local anesthetics (LA) we can write:

$$C + M^{2+} \overset{K_1}{\rightleftharpoons} C \cdot M^{2+}$$

$$C + LA \overset{K_2}{\rightleftharpoons} C \cdot LA$$

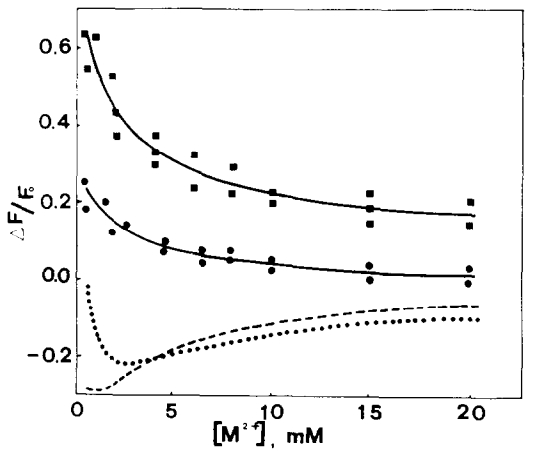

Fig. 2. Effect of lidocaine (5 mM) on the fluorescence of chlortetracycline in ethanol in the presence of the concentrations of Ca^{2+} (■) or Mg^{2+} (●) indicated in the abscissae, where M^{2+} stands for both ions. The relative increase of fluorescence upon addition of 5 mM lidocaine is plotted in the Y axis. The solid lines drawn through the experimental points correspond to the best fit of the data to the equilibrium situation defined by the following chemical equations:

$$M^{2+} + C \overset{K_1}{\rightleftharpoons} C \cdot M^{2+}$$

$$C + LA \overset{K_2}{\rightleftharpoons} C \cdot LA$$

$$C \cdot M^{2+} + LA \overset{K_3}{\rightleftharpoons} LA \cdot C \cdot M^{2+}$$

where all the symbols have the meaning stated in the text. K_1, K_2 and K_3 being the respective association constants. K_1, the association constant of M^{2+}-chlortetracycline has been taken as: 384.6 and 714.3/M for Ca^{2+} and Mg^{2+}, respectively [23]. The best fits of the data have been achieved with $K_3 = 2 \cdot K_2$ (for Ca^{2+}) and $K_3 = 1.5 \cdot K_2$ (for Mg^{2+}). Fixing $K_3 = K_2$ in both cases gave us much poorer fits, thus, indicating a tighter binding of lidocaine to $C \cdot M^{2+}$ than to free chlortetracycline. In addition, the solid lines represent the best fit obtained by setting the following fluorescence levels for the ternary complexes: $F_{(Lido \cdot C \cdot Ca)} = 1.15 \times F_{C \cdot Ca}$ and $F_{(Lido \cdot C \cdot Mg)} = 1.05 \times F_{C \cdot Mg}$. When the ternary complexes contribution to the fluorescence is disregarded the pattern obtained do not satisfactorily fit the experimental data, as shown by dotted and broken lines which correspond to Ca^{2+} and Mg^{2+}, respectively.

where C means chlortetracycline and K_1 and K_2 are the association constants of M^{2+}–chlortetracycline ($C \cdot M^{2+}$) and of local anesthetic–chlortetracycline ($C \cdot LA$). Provided that the chlortetracycline concentration is much lower than total M^{2+} (M_T^{2+}) and local anesthetic concentration (LA_T), the fluorescence intensity, F, can then be related to M^{2+} and LA concentrations by the following expression

$$F = F_C \cdot \left(\frac{1}{1 + K_1 \cdot |M^{2+}|_T + K_2 \cdot |LA|_T}\right) + F_{C \cdot M} \cdot \left(\frac{K_1 \cdot |M^{2+}|_T}{1 + K_1 \cdot |M^{2+}|_T + K_2 \cdot |LA|_T}\right) + F_{C \cdot LA} \cdot \left(\frac{K_2 \cdot |LA|_T}{1 + K_1 \cdot |M_T^{2+}| + K_2 \cdot |LA|_T}\right)$$

where F_C, $F_{C \cdot M}$ and $F_{C \cdot LA}$ are the relative fluorescence intensities of free chlortetracycline, $C \cdot M$ and $C \cdot LA$. These values have been determined from direct titrations of the chlortetracycline fluorescence with M^{2+} and lidocaine and found to be (in arbitrary units): 1, 21.7 ± 0.6, 11.6 ± 0.5 and 2.7 ± 0.2 for F_C, $F_{C \cdot Mg}$, $F_{C \cdot Ca}$ and $F_{C \cdot Lido}$, respectively. Calculations of the expected perturbations of the intensity of fluorescence of chlortetracycline by lidocaine in the presence of several concentrations of Mg^{2+} and of Ca^{2+} are presented as dotted and broken lines in Fig. 2. It is evident from these results that the simple kinetic scheme presented above cannot account for the effects of lidocaine on the fluorescence of Ca^{2+}–chlortetracycline solutions. Therefore, the formation of significant amounts of ternary complexes M^{2+}–chlortetracycline–lidocaine is strongly suggested by these data. Because the effect of lidocaine reaches a steady value at high concentration of Ca^{2+} and/or Mg^{2+} we have fitted the experimental data with the assumption that the ternary complex shows this steady fluorescence level with respect to that of $C \cdot M^{2+}$. The association constants obtained from the best fit of the experimental data are given in the legend of Fig. 2.

Effect of local anesthetics upon the fluorescence of quin 2

Quin 2 is a chelator specific of Ca^{2+}, with a high selectivity over Mg^{2+} [27]. Thus, to test further the possibility of local anesthetic interaction with Ca^{2+} binding centers we have titrated the fluorescence of quin 2 with these drugs. For the reasons indicated above, 3 mM imidazole/HCl (pH 7.0) the titrations have been carried out in 3 mM. We have found that in this buffer Ca^{2+} increases 3–4-fold the intensity of fluorescence of quin 2 with an apparent $K_{0.5}$ of 30 μM. The results obtained with tetracaine and procaine are shown in Fig. 3. Lidocaine was found without effect up to a concentration of 20 mM and dibucaine could not be tested because of its strong fluorescence band centered at *ca.* 410 nm. From these data we have calculated the values of K_d listed in Table 1. On the other hand, it is to be noted that the effect of anesthetics upon the fluorescence of quin 2 were fully reverted by millimolar Ca^{2+} concentrations, and that the increase of the intensity of quin 2 fluorescence by 50 μM Ca^{2+} is fully reverted by millimolar concentrations of procaine and tetracaine.

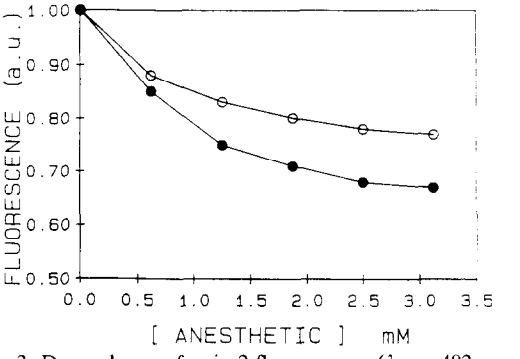

Fig. 3. Dependence of quin 2 fluorescence (λ_{em} = 492 nm; λ_{exc} = 339 nm) upon local anesthetic concentration in buffer, pH 7.0. (a.u. stands for arbitrary units). The symbols correspond to the local anesthetics procaine (○) and tetracaine (●).

Effects of EDTA and ATP on the fluorescence of local anesthetics

These results suggested that we check the possibility that another divalent cation chelating structure interacts with the above mentioned local anesthetics. We first chose EDTA and ATP. Figure 4 shows that both EDTA and ATP significantly quench the fluorescence of dibucaine, thus strongly suggesting that they interact with this anesthetic. This is a real fluorescence quenching because the absorption spectra of dibucaine is not significantly altered by these EDTA or ATP concentrations. Figure 4B also shows that the effects of ATP on dibucaine fluorescence are completely reverted by addition of Ca^{2+}. A complete reversion by Ca^{2+} of the effects of up to 1.9 mM EDTA on dibucaine fluorescence has been noticed as well. Reversion of the effects of higher EDTA concentrations on the fluorescence of dibucaine could not be tested without a significant pH change (larger than 0.1 pH units). Because of the high absorbance of millimolar ATP solutions in the UV wavelength range, the effects of ATP on procaine and tetracaine fluorescence cannot be checked.

In addition, EDTA solutions also exhibit a high absorbance in the 240–250 nm wavelength range, thus, masking any effects on this wavelength range of procaine and tetracaine excitation spectra. Upon correction of fluorescence for inner-filter effects (see Materials and Methods) only slight alterations by millimolar concentrations of EDTA ($K_{0.5}$ = 8–9 mM) of the major excitation bands of procaine (5% quenching) and tetracaine (≤5% increase and 5 nm shift to shorter wavelength) were observed.

Effect of EGTA upon the fluorescence properties of local anesthetics

This latter point has been further studied using EGTA, which shows a much weaker absorption than EDTA and ATP in the wavelength range of 240–250 nm. Figure 5 reproduces the emission and excitation spectra of dibucaine at various EGTA concentrations. Because photolytic decomposition of dibucaine in these experimental conditions takes

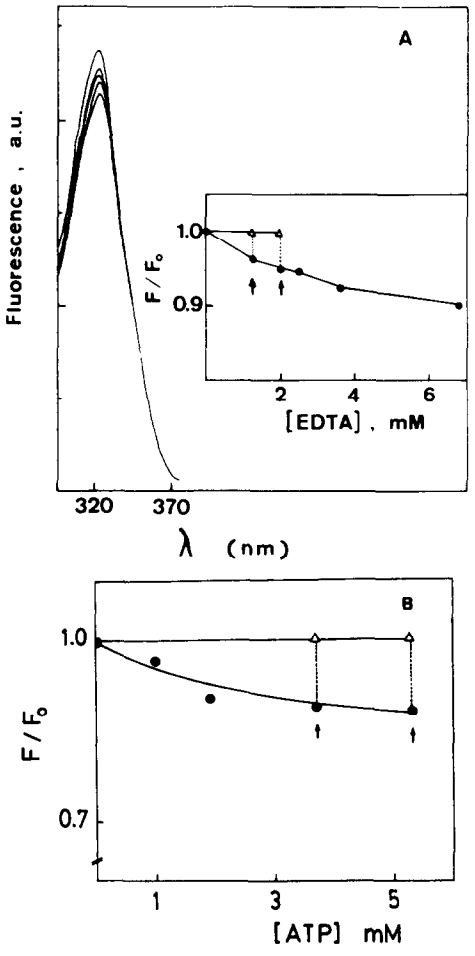

Fig. 4. Dependence of dibucaine (7.7 μM) fluorescence (λ_{exc} = 325 nm; λ_{em} = 412 nm) upon the concentration of EDTA (A) and of ATP (B). A, titration with EDTA: excitation spectra. From top to bottom, the EDTA concentrations are: 0, 1.25, 2.5, 3.75 and 6.9 mM. Inset: fluorescence intensity versus EDTA concentrations (●). Buffer: 14 mM imidazole/0.1 M KCl (pH = 8.0). B, titration with ATP, no Ca^{2+} added (●). Buffer: 50 mM Tes/0.1 M KCl (pH 7.35). In separate experiments 5.5. mM $CaCl_2$ was added at the EDTA (A) or ATP (B) concentrations indicated by arrows and the new dibucaine fluorescence intensity attained is indicated by (△). The dotted lines between the symbols ● and △ indicate that both fluorescence intensity measurements were carried out with the same sample before and after the addition of Cl_2Ca (a.u. means arbitrary units).

place with a half-time of more than 2 hr ($t_{1/2}$ = 135 min, results not shown) photolytic perturbation of the spectra can be neglected. It can be observed that the excitation (A) and emission (B) maxima of the spectra of dibucaine are not shifted by EGTA. The most significant change induced by EGTA is the quenching of the intensity of the fluorescence of dibucaine, more appreciably when excited at 241–242 nm. This quenching cannot be attributed to alterations of the absorption spectra of dibucaine because the effects of EGTA upon the dibucaine absorption spectrum above 245 nm are negligible. The same spectral changes were observed in different buffers (Tris, Hepes) and it was also found that these changes are independent of the ionic strength (I), in the range I = 0.05–1. The fluorescence signals of the buffers were recorded in these experimental conditions and were found to be negligible when compared to the fluorescence of the local anesthetic (results not shown). Because of the high absorbance of EGTA solutions at 240 nm, the observed changes of the intensity of the fluorescence when exciting at 242 nm need to be corrected for the inner filter effect of the solution. This was done using equation 6 of Ref. 22 (see Materials and Methods). This correction is accurate within 2% for $A \leq 0.3$ O.D. [22], i.e. up to 10–11 mM EGTA in our experimental conditions. Because higher errors are expected above this absorbance, i.e. for EGTA, concentrations up to 20 mM the absorbance at 242 nm reaches about 0.5 O.D., we have plotted the corrected data (see Fig. 6) taken from the fluorescence readings using an excitation wavelength slightly larger (245 nm), where the maximum absorbance level at 20 mM EGTA is approximately 0.3 O.D. The possibility that the effects observed upon addition of EGTA were, at least partially, due to the parallel alteration of free Ca^{2+} levels was discarded by running experiments in which the total Ca^{2+} concentration was changed from 80 to 1600 μM, so that the free Ca^{2+} concentration at a fixed EGTA concentration varied up to 20-fold using the Ca^{2+}–EGTA equilibrium constant given in Ref. 29. In all these experiments, the pH of the solution was carefully controlled because of the well-known release of H^+ upon Ca^{2+}-EGTA complexation [30]. In the experimental conditions used in this study, the extent of quenching of dibucaine fluorescence by different EGTA concentrations was found to be independent of total Ca^{2+} concentration in the range 80–1600 μM (see Fig. 6). It is to be noted that due to the high value of $K_{0.5}$ (EGTA-dibucaine), any effect of these changes of Ca^{2+} levels on the dependence of the fluorescence of dibucaine upon EGTA concentration is likely to be within experimental errors. Partial reversal of the quenching of dibucaine fluorescence by EGTA has been observed at higher total Ca^{2+} concentrations. However, these results have to be considered with caution because in these latter conditions the pH change was higher than 0.1 units, and as Fig. 6 shows, the dependence of the fluorescence of dibucaine upon the concentration of EGTA is strongly dependent on a pH change between pH 7.5 and 8.0. Similar results were obtained with procaine, which also show weak alterations of its fluorescence properties in the presence of millimolar concentrations of EGTA (see also Fig. 6).

DISCUSSION

The results presented in this paper show that some local anesthetics can interact with divalent cation binding centers, i.e. chlortetracycline, quin 2, EDTA, ATP and EGTA. In addition, Ca^{2+} completely reverts the changes of dibucaine fluorescence induced by ATP or EDTA (Fig. 4), millimolar Ca^{2+} reverts the effects of tetracaine and procaine on the fluorescence of quin 2 and high Ca^{2+} or Mg^{2+} strongly

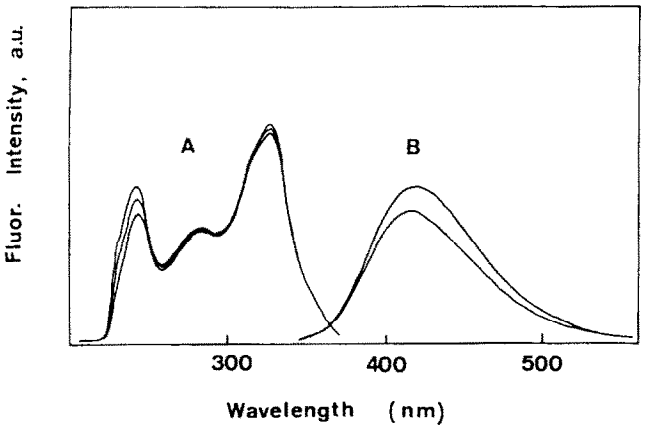

Fig. 5. Dependence of dibucaine (7.7 μM) fluorescence upon the concentration of EGTA. A, uncorrected excitation spectra (λ_{em} = 412 nm). B, uncorrected emission spectra (λ_{exc} = 242 nm). Buffer: 11 mM imidazole/0.1 M KCl (pH = 8.0) and varying EGTA concentrations. From top to bottom, the EGTA concentration in the cuvette was: (A) 0, 3.3 and 6.6 mM; (B) 0 and 6.6 mM.

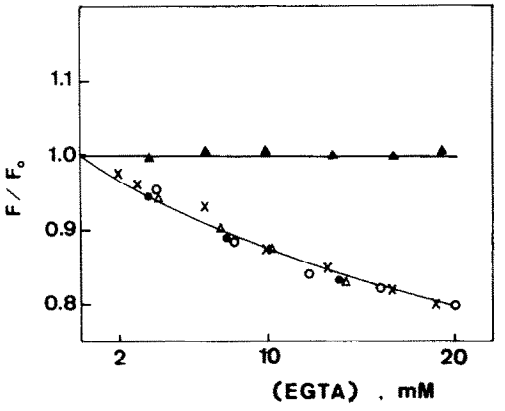

Fig. 6. Dependence of local anesthetic fluorescence upon the EGTA concentration in imidazole buffer (11 mM imidazole/0.1 M KCl), pH 8.0 (×, ○, ●, △) and pH 7.4 (▲). Other conditions were: (×) Procaine (8 μM); λ_{exc} = 242 nm; λ_{em} = 351 nm; (○, ●, △, ▲) Dibucaine (7–10 μM); λ_{exc} = 245 nm; λ_{em} = 412 nm. Total Ca^{2+} concentration: (×, ○, ▲) 10–15 μM; (●) 400 μM and (△) 1600 μM. The data shown are corrected both for dilution and inner filter effects (a.u. means arbitrary units).

decrease the effects of lidocaine and procaine on chlortetracycline fluorescence (Fig. 2). Therefore, it appears that local anesthetics, Ca^{2+} or Mg^{2+} bind to ATP, EDTA, quin 2 and chlortetracycline. From a methodological point of view, the quenching of the fluorescence of quin 2 by the local anesthetics tetracaine and procaine imposes severe restrictions on the use of this Ca^{2+} indicator to quantify free calcium within cells or subcellular organelles in the presence of these drugs. It is to be noted too, that Chan and Wang [24] have presented experimental data suggesting that there is an important electrostatic contribution to the interaction between these local anesthetic and membrane proteins. The comparison of the dissociation constants of dibucaine with EGTA, EDTA and ATP and those of procaine and tetracaine with EGTA, quin 2 and chlortetracycline suggest that these anesthetics interact with Mg^{2+} binding sites stronger than with Ca^{2+} binding centers. The free energy of the interaction between local anesthetics and the divalent cation chelators referred to above can be calculated from the dissociation constants given in Table 1 and ranges from −2.8 to −4.0 kcal/mole at pH 7.4. It might be worthy to note here that the enthalpy of interaction of dibucaine and tetracaine with cytochrome *c* oxidase has been reported to be in this range [31].

Although binding to these chelators is weaker than to their respective receptor(s) in the axonal membrane [7], the ratio between the binding constants to M^{2+}-binding center (K_d) and to axonal receptor(s) (LD_{50}) becomes closer to unity for the local anesthetics of lower (lipid–water) partition coefficient, i.e. procaine and lidocaine. For dibucaine, the more hydrophobic local anesthetic studied here, the ratio of K_d (EDTA)/LD_{50} is *ca.* 250, a value which is larger than the (egg lecithin–water) partition coefficient of this anesthetic, *ca.* 9–10 [25]. Because of the hydrophobicity of these drugs, their binding constants to divalent cation binding centers close to a hydrophobic region should be remarkably enhanced since both energetic terms will then co-operate to promote a more efficient binding. It is to be recalled that this situation is likely to occur *in vivo*, because local anesthetics partition within the lipid matrix of biological membranes, where they locate closer to the lipid–water interphase [32, 26]. The simplest case of such a type of divalent cation binding centers are the polar headgroups of some lipids, e.g. phosphatidylserine or phosphatidylinositols which are known to efficiently bind Ca^{2+} [33]. Furthermore, Browning and Akutsu [34] have shown by extensive NMR studies that local anesthetics and divalent cations have the same effects on the headgroups conformation of phosphatidylcholine and phosphatidylethanolamine. The occurrence of a hydrophobic domain (lipid-binding domain) close to

a Ca^{2+} binding domain is also a feature of calmodulin [35], which has already been shown to be modulated by these local anesthetics [18], and of some Ca^{2+}-regulated membrane binding proteins, such as lipocortin, p36, protein kinase C, endonexin, calelectrin and protein II [36]. The effect of lidocaine and procaine on chlortetracycline fluorescence in the presence of various Ca^{2+} and Mg^{2+} concentrations strongly suggests that ternary complexes between local anesthetic, M^{2+} and chlortetracycline are formed. The results presented in this paper do not allow us to exclude the possibility of ternary complexes of local anesthetics, Ca^{2+} and EGTA. In this regard, it seems worthy to recall here that the formation of ternary complexes (Ca^{2+} · EGTA · Ca^{2+} binding sites) has been suggested to account for the effects of Ca^{2+}–EGTA on the catalytic activity of Ca^{2+} +Mg^{2+}-ATPase [37] and active Ca^{2+} transport [38]. Because of the large pH changes that the addition of high concentrations of Ca^{2+} induce on EGTA solutions, and the strong dependence of the interaction between dibucaine and EGTA upon pH changes between 7.5 and 8.0, another experimental approach must be sought to answer this question.

Therefore, it appears that in an aqueous environment these local anesthetics can bind to Mg^{2+} and to Ca^{2+} binding centers. Moreover, these fluorescence data also indicate that the interaction of these local anesthetics with M^{2+} binding centers involves their aniline-like aromatic ring, for the wavelength of the excitation bands altered are within the wavelength range of the characteristic absorption bands of aniline, acetanilide and N-substituted anilines [28]. The local anesthetics studied here are amines having aromatic groups. Amine groups, on the other hand, have been recently shown to electrostatically interact with aromatic aminoacids in protein crystals [39]. Quantum mechanics calculations have shown that this interaction arises from the asymmetric, partly polarized electron density profile of π-molecular orbitals of aromatic rings. The center of a π-electronic ring has excess electronic charge ($\delta(-)$) thus showing a tendency to interact with positively charged groups, e.g. amino groups at neutral pH. On the other hand, interactions with negatively charged molecules is also feasible, e.g. carboxylates, because of the compensatory defective electron density ($\delta(+)$) of the outer edge of the aromatic ring [39]. Both types of interactions are likely to contribute to local anesthetic binding to Ca^{2+} binding sites of EDTA, EGTA, ATP, chlortetracycline, quin 2 and of proteins, where these are rich in glutamate and aspartate residues [40]. With regard to the molecular basis of quenching of local anesthetic fluorescence by EDTA, ATP or EGTA, considering that there is not a significant change of the absorption spectrum of dibucaine by these quenchers (results not shown), it is likely that this quenching is due to a charge transfer reaction, as the quenching of the fluorescence of electron-donor amines by esters [41, 42].

The results presented in this paper suggest the possibility that local anesthetics can alter the homeostasis of Ca^{2+}, either as a consequence of their possible interaction with Ca^{2+} binding loci or to a close regulatory hydrophobic domain of proteins involved in metabolic regulation or Ca^{2+} transport, or by releasing membrane-bound Ca^{2+} pools, such as the Ca^{2+} bound to lipids (like phosphatidylserine and phosphatidylinositols). Although these drugs appear to bind more efficiently to Mg^{2+} chelating structures (see above), the fact that physiological Mg^{2+} levels are high and that the pharmacological doses for some of these anesthetics are far below the physiological concentration of Mg^{2+} binding centers (nucleotides and proteins that bind Mg^{2+}) makes it unlikely that the anesthetic action could be due to perturbation of Mg^{2+}-dependent functions *in vivo*. Nevertheless, this is to be kept in mind when rationalizing the actions of local anesthetics, such as procaine, that exert their biological effects in the millimolar range [7].

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REFERENCES

1. Franks NP and Lieb WR, Molecular mechanisms of general anesthesia. *Nature* **300**: 487–493, 1982.
2. Dodson BA and Moss J, Molecular mechanisms of action of general anesthetics. *Mol Cell Biochem* **64**: 97–103, 1984.
3. Strichartz G, Molecular mechanisms of nerve block by local anesthetics. *Anesthesiology* **45**: 421–441, 1976.
4. Sasyniuk BI and Ogilvie R, Antiarrythmic drugs: electrophysiological and pharmacokinetic considerations. *Ann Rev Pharmacol* **15**: 131–155, 1975.
5. Barchfeld GL and Deamer DW, The effect of general anesthetics on the proton and potassium permeabilities of liposomes. *Biochim Biophys Acta* **819**: 161–169, 1985.
6. Nicoll RA and Madison DV, General anesthetics hyperpolarize neurons in the vertebrate central nervous system. *Science* **217**: 1055–1057, 1982.
7. Greenberg M and Tsong TY, Binding of quinacrine, a fluorescent local anesthetic probe, to mammalian axonal membranes. *J Biol Chem* **257**: 8964–8971, 1982.
8. Greenberg M and Tsong TY, Detergent solubilization and affinity purification of a local anesthetic binding protein from mammalian axonal membranes. *J Biol Chem* **259**: 13241–13245, 1984.
9. Volpi M, Sha'afi RI, Epstein PM, Andrenyak DM and Feinstein MB, Local anesthetics, mepacrine and propanolol are antagonists of calmodulin. *Proc Natl Acad Sci USA* **78**: 795–799, 1981.
10. Papahadjopoulos D, Studies on the mechanism of action of local anesthetics with phospholipid model membranes. *Biochim Biophys Acta* **265**: 169–186, 1972.
11. Feinstein MB, Reaction of local anesthetics with phospholipids. Possible chemical basis for anesthesia. *J Gen Physiol* **48**: 357–374, 1964.
12. Blaustein MP and Goldman DE, Action of anionic and cationic nerve blocking agents. Experiment and interpretation. *Science* **153**: 429–432, 1966.
13. Blaustein MP, Phospholipids as ion exchangers: implications for a possible role in biological membrane excitability and anesthesia. *Biochim Biophys Acta* **135**: 653–668, 1976.
14. Kostyuk PG, Calcium channels in the neuronal membrane. *Biochim Biophys Acta* **650**: 128–150, 1981.
15. Blaustein MP and Goldman DE, Competitive action of calcium and procaine on lobster axon. *J Gen Physiol* **49**: 1043–1063, 1966.
16. Snell WJ, Buchanan M and Clausell A, Lidocaine reversibly inhibits fertilization in Chlamydomonas: a possible role for calcium in sexual signalling. *J Cell Biol* **94**: 607–612, 1982.

17. Lewis SEM, Anderson P and Goldspink D, The effects of calcium on protein turnover in skeletal muscles of the rat. *Biochem J* **204**: 257–264, 1982.
18. Tanaka T and Hidaka H, Interaction of local anesthetics with calmodulin. *Biochem Biophys Res Comm* **101**: 447–453, 1981.
19. Suko J, Winkler F, Scharinger B and Hellman G, Aspects of the mechanism of action of local anesthetics on the sarcoplasmic reticulum of skeletal muscle. *Biochim Biophys Acta* **443**: 571–586, 1976.
20. Garcia-Martin E and Gutiérrez-Merino C, Local anesthetics inhibit the Ca^{2+}, Mg^{2+}-ATPase activity of rat brain synaptosomes. *J Neurochem* **47**: 668–672, 1986.
21. Escudero B and Gutiérrez-Merino C, Effects of local anesthetics on the passive permeability of sarcoplasmic reticulum vesicles to Ca^{2+} and Mg^{2+}. *Biochim Biophys Acta* **902**: 374–384, 1987.
22. Birdsall B, King RW, Wheeler MR, Lewis Ch A Jr, Goode SR, Dunlap RB and Roberts GCK, Correction for light absorption in fluorescence studies of protein ligand interaction. *Anal Biochem* **132**: 353–361, 1983.
23. Millman MS, Caswell AH and Haynes DH, Kinetics of chlortetracycline permeation in fragmented, ATPase-rich sarcoplasmic reticulum. *Membrane Biochem* **3**: 291–315, 1980.
24. Chan MS and Wang HH, Local anesthetics can interact electrostatically with membrane proteins. *Biochim Biophys Acta* **770**: 55–64, 1984.
25. Ohki S, Adsorption of local anesthetics on phospholipid membranes. *Biochim Biophys Acta* **777**: 56–66, 1984.
26. Kuroda Y and Fujiwara Y, Locations and dynamical perturbations for lipids of cationic forms of procaine, tetracaine, and dibucaine in small unilamellar phosphatidylcholine vesicles as studied by nuclear Overhauser effects in ^1H nuclear magnetic resonance spectroscopy. *Biochim Biophys Acta* **903**: 395–410, 1987.
27. Bryant DTW, Quin 2: the dissociation constants of its Ca^{2+} and Mg^{2+} complexes and its use in a fluorimetric method for determining the dissociation of Ca^{2+}-protein complexes. *Biochem J* **226**: 613–616, 1985.
28. Weast R (Ed.), *Handbook of Chemistry and Physics*, 59th Edn. CRC Press Inc., West Palm Beach, FL, 1978–1979.
29. Harafuji H and Ogawa Y, Re-examination of the apparent binding constant of ethyleneglycol bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid with Ca^{2+} around neutral pH. *J Biochem* **87**: 1305–1312, 1980.
30. Bennett N and Dupont Y, Use of Ca^{2+}-EGTA buffers for transposing Ca^{2+} efflux into pH changes. *FEBS Lett* **98**: 229–232, 1979.
31. Vanderkooi G and Chazotte B, Cytochrome *c* oxidase inhibition by anesthetics: thermodynamic analysis. *Proc Natl Acad Sci USA*, **79**: 3749–3753, 1982.
32. Kelusky EC and Smith ICP, Characterization of the binding of the local anesthetics procaine and tetracaine to model membranes of phosphatidylethanolamine: a deuterium nuclear magnetic resonance study. *Biochem* **22**: 6011–6017, 1983.
33. Lee AG, Lipid phase transitions and phase diagrams. II. Mixtures involving lipids. *Biochim Biophys Acta* **472**: 285–344, 1977.
34. Browning JL and Akutsu H, Local anesthetics and divalent cations have the same effect on the headgroups of phosphatidylethanolamine. *Biochim Biophys Acta* **684**: 172–178, 1982.
35. La Porte DC, Wierman BM and Storm DR, Calcium-induced exposure of a hydrophobic surface on calmodulin. *Biochem* **19**: 3814–3819, 1980.
36. Geislow MJ, Common domain structure of Ca^{2+} and lipid-binding proteins. *FEBS Lett* **203**: 99–103, 1986.
37. Berman MC, Stimulation of calcium transport of sarcoplasmic reticulum vesicles by the calcium complex of ethyleneglycol bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid. *J Biol Chem* **257**: 1953–1957, 1982.
38. Gill DL, Grollman EF and Kohn LD, Calcium transport mechanisms in membrane vesicles from guinea pig brain synaptosomes. *J Biol Chem* **256**: 184–192, 1981.
39. Burley SK and Pestko GA, Amino-aromatic interactions in proteins. *FEBS Lett* **203**: 139–143, 1986.
40. Herzberg O and James MNG, Common structural framework of the two Ca^{2+}/Mg^{2+} binding loops of troponin C and other Ca^{2+}-binding proteins. *Biochem* **24**: 5298–5302, 1985.
41. Costa SMB and Prieto MJ, Charge and energy transfer processes in excited aminoesthers. *J Photochem* **23**: 343–354, 1983.
42. Lakowicz JR, in: *Principles of Fluorescence Spectroscopy*, Chapters 5 and 9. Plenum Press, New York, 1983.