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IONOPHORETIC AND INHIBITORY ACTION OF THE ANALGESIC, DIFLUNISAL, ON SARCOPLASMIC RETICULUM

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Abstract—Diflunisal decreased the ATP-dependent transport rate and calcium accumulation by the sarcoplasmic reticulum. Inhibition of calcium transport by diflunisal was pH dependent, and a pK_a of 6.7 to 6.9 was observed for the carboxylic acid group. In sealed sarcoplasmic reticulum vesicles, diflunisal at concentrations below 1 mM increased the rate of Ca^{2+}-dependent hydrolysis of ATP; above 1 mM, the Ca^{2+}-ATPase activity was inhibited. In purified Ca^{2+}-ATPase, diflunisal acted only as an inhibitor. Methylation of the phenolic group of diflunisal eliminated both its analgesic and ionophoretic properties.

Diflunisal (2′,4′-difluoro 4-hydroxy-3-diphenylcarboxylic acid), an analgesic and anti-inflammatory drug, has been proven recently to be an uncoupler of oxidative phosphorylation in mitochondria [1, 2]. Diflunisal also behaves as a calcium ionophore in model systems and induces calcium movements across the mitochondrial membrane [2].

With this in mind, it seemed interesting to investigate its ionophoretic properties on a membrane model such as the sarcoplasmic reticulum (SR*), specialized in the transport and accumulation of calcium in muscle cells. Diflunisal is a salicylic acid derivative [3], simpler than the divalent cation ionophore X537A. It has been reported [4] that modification of the phenolic group of X537A does not change its ionophoretic properties appreciably, considering this, the corresponding methyl ether derivative of diflunisal was synthesized and assayed for both ionophoretic and analgesic properties. The results presented in this report demonstrate that diflunisal was a good inophore for Ca^{2+} and Sr^{2+} in SR vesicles. It is also shown that diflunisal inhibited Ca^{2+}-ATPase activity and that methylation of its phenyl group caused loss of the ionophoretic as well as the analgesic properties of diflunisal.

MATERIALS AND METHODS

SR vesicles were prepared from white skeletal muscle of rabbits by the method of Boland *et al.* [5]. Ca^{2-}ATPase was purified from SR vesicles as reported by Meissner *et al.* [6]. Both SR vesicles and Ca^{2+}-ATPase were stored under liquid N_2 for several weeks. Ca^{2+} uptake was followed in an Aminco double beam spectrophotometer at 650 − 685 nm in the following medium: 80 mM KCl; 20 mM Pipes–Tris, pH 6.1 to 7.4; 5 mM potassium oxalate; 30 μM arsenazo III; 10–50 μM $CaCl_2$; 4 mM $MgCl_2$; 2 mM ATP–Tris; 20 μg/ml of SR proteins; and increasing concentrations of diflunisal indicated in Results. For each pH assayed, the rate of Ca^{2+} transport was optimized, varying the Ca^{2+} added to reach the maximal rate of transport and calibrating the signal with known amounts of Ca^{2+} to calculate the stationary rates of transport. Ca^{2+} binding was measured at 37° in 100 mM KCl; 20 mM Pipes, pH 6.6; 4 mM $MgCl_2$; 50 μM $^{45}CaCl_2$ and DFNS as indicated in Results. SR proteins (100 μg/ml) were preincubated for 2 min, and the reaction was started with 0.2 mM ATP–Tris. At 2 min an aliquot was filtered and measured as described previously [7]. A similar procedure was used with 50 μM $^{90}SrCl_2$ instead of $^{45}CaCl_2$ but filtration was done at 1 min, before Sr^{2+} could be released when ATP is exhausted [8]. Ca^{2+} and Sr^{2+} release was followed in an Aminco double beam spectrophotometer at 37°, in 2 ml of 100 mM KCl; 20 mM Pipes, pH 6.8; 4 mM $MgCl_2$; 50 μM Ca^{2+} or 50 μM Sr^{2+}; 200 μg/ml of SR proteins and 30 μM arsenazo III; reaction was started with 0.2 mM ATP–Tris. Water or DFNS was added in a constant volume, 0.1 ml/ml of reaction mixture at the time indicated in Results when Ca^{2+} or Sr^{2+} binding was stabilized. Ca^{2+}-ATPase was measured at 37° in a medium that contained 100 mM KCl; 20 mM Pipes for pH 6.4 or 6.8, or 20 mM Hepes for pH 7.3; 4 mM $MgCl_2$; 1 mM EGTA; 2 mM ATP–Tris; and 100 μg of SR proteins or 5–10 μg protein of purified ATPase per ml. Ca^{2+} concentration was optimized, as indicated previously, for each pH value to obtain the maximal rate of ATP hydrolysis. Diflunisal was added at the different concentrations indicated in Results. Basal magnesium-dependent ATPase was determined in the same medium but without Ca^{2+} added. When calcium-dependent ATPase in SR ves-

* Abbreviations: SR, sarcoplasmic reticulum; DFNS, diflunisal; DFNS-ME, diflunisal methyl ether; SDS, sodium dodecyl sulfate; Pipes, 1,4-piperazine diethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; EGTA, ethyleneglycolbis(β-aminoethylether)*N*,*N*′-tetraacetic acid; and DMSO, dimethyl sulfoxide.

icles was assayed, a control for maximal hydrolytic activity was performed for each pH value in the presence of a 30 μM concentration of the ionophore X537A. The reaction was stopped by the addition of cold trichloroacetic acid, 5% final concentration. Since diflunisal is insoluble in acid solutions, it was eliminated by centrifugation together with the precipated proteins. The inorganic phosphate was determined in an aliquot of the supernatant fraction by the method of Taussky and Shorr [9]. Proteins were determined by the method of Lowry *et al.* [10], using bovine serum albumin as standard.

DFNS-methyl ether. Methylation of DFNS was performed, with modifications, as described by Vogel [11]. We placed 6.4 g (25.6 mmol) DFNS, 1.28 g (32 mmol) NaOH, and 15 ml DMSO in a round-bottomed flask provided with a reflux condenser. Then, after magnetic stirring for 10 min, 4 ml (43 mmol) dimethyl sulfate was added dropwise and stirred continuously; at the end of this addition, the flask content was heated at 90° during 4 hr until almost all the DFNS was dissolved. The content was cooled and poured on 500 ml of 1% NH_4OH, and the diflunisal methyl ether (DFNS-ME) was extracted several times with ether. DFNS remained in the ammoniacal solution. DFNS-ME was obtained by evaporating the ethereal solution and was recrystallized from a hot ethanolic-water solution. The yield was 47% calculated from DFNS. The DFNS-ME spectrum in ethanol had the same maximum at 312 nm ($E_M = 3.23.10^3\ M^{-1}\cdot cm^{-1}$) and the maximum at 252 of DFNS was lost. The blue fluorescence of DFNS changes to yellow green in DFNS-ME.

Analgesic assay of DFNS and DFNS-ME. Fifteen male adult rats, weighing around 260 g, separated in three groups of five each, with free access to food and water were used in the study. The rats were slightly anesthesized with ether 15 min before the experiment, and a suspension of placebo, DFNS, or DFNS-ME in 0.5 ml of water was introduced orally into the stomach, with a cannula, to each of the groups; the dose administered was always 100 mg/kg body weight for each substance [3]. The animals were maintained in a plastic restrainer cage with the tail protruding. At 1, 2 and 3 hr after administration of the drugs, 5 cm of the tail was dipped into a beaker of water, maintained at 55°, and the latency to curl the tail out of the water was recorded. The experiment was repeated three times, alternating the drug and the group of rats. The experiments were performed with a 1-week interval to allow the elimination of drugs [12, 13].

RESULTS

Ca^{2+} and Sr^{2+} binding and release by SR vesicles. The inhibitory effect of DFNS on the Ca^{2+} and Sr^{2+} accumulation capacity of SR vesicles is clearly seen in Fig. 1A. As observed, the total Ca^{2+} and Sr^{2+} accumulated decreased as the DFNS concentration was raised in the reaction medium. The corresponding Dixon graph (Fig. 1B) shows a biphasic behavior of DFNS, with a break point at 1.0 mM approximately. For two different SR preparations, the $K_{0.5}$ of this inhibitory effect was 1.05 mM with Ca^{2+} and 1.20 mM with Sr^{2+}. As shown in Fig. 2A

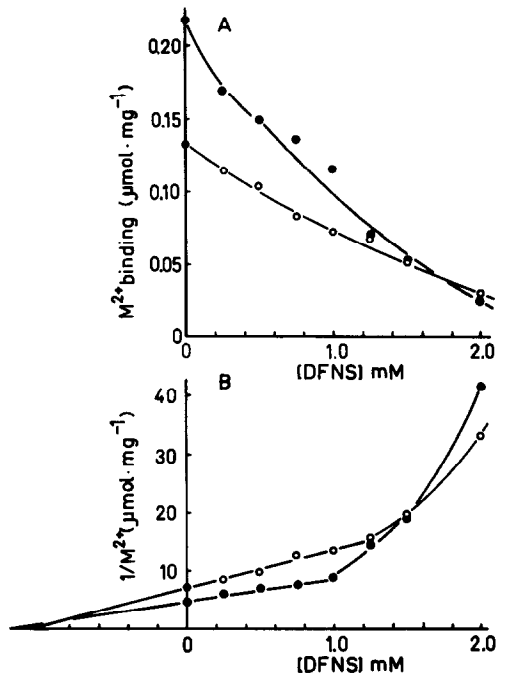

Fig. 1. Ca^{2+} and Sr^{2+} binding by SR vesicles in the presence of DFNS. (A) Ca^{2+} (○) and Sr^{2+} (●) transported by SR vesicles at various DFNS concentrations. (B) Dixon plot of the data.

and 2B, DFNS also induced the release of the actively accumulated Ca^{2+} and Sr^{2+} in the vesicles, as can be expected from a calcium ionophore. To obtain the release rate of the divalent cations without the complication of the ATP-dependent transport, the time necessary to hydrolyze all ATP present was determined and, at this predetermined time, DFNS was added; it is seen that calcium was released in a dose-dependent fashion. As Sr^{2+} is partially released after ATP depletion [8], DFNS was added after a new state of equilibrium had been attained (Fig. 2B). The initial Ca^{2+} or Sr^{2+} release rate was measured, and the results were plotted in Fig. 2C; as observed, an exponential behavior was obtained. These data were replotted in Fig. 2D in double logarithmic form [14]; the calculated slopes were 1.83 for Ca^{2+} and 1.68 for Sr^{2+}, suggesting that more than one (probably two) molecule of DFNS is involved in the release of Ca^{2+} and Sr^{2+}. Also, as observed, Sr^{2+} was released slower than Ca^{2+}, probably due to the partial spontaneous release of Sr^{2+}, which in turn decreased the gradient, and by the lower affinity of DFNS for Sr^{2+}, as judged from the above-mentioned $K_{0.5}$ values.

The possibility of a detergent-like action of DFNS was ruled out by adding DFNS or SDS to an SR vesicle suspension under conditions similar to those used for Ca^{2+} binding; at the same time, measurements of changes in light dispersion at 510 nm were performed [15]. SDS cleared the suspension, and the dispersed light was decreased markedly, whereas water or DFNS additions had no effect (data not shown). Moreover, if SR vesicles were incubated for 10 min with 6 mM DFNS, diluted 10-fold, and then centrifuged and resuspended, they retained their

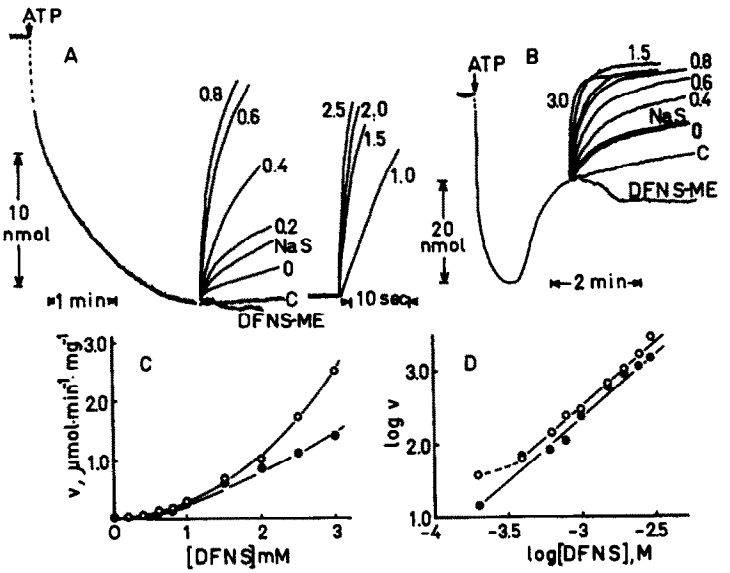

Fig. 2. Ca^{2+} and Sr^{2+} release by SR vesicles on addition of DFNS. (A) Ca^{2+} release; (B) Sr^{2+} release; (C) rate of Ca^{2+} (○) and Sr^{2+} (●) release versus DFNS concentration; and (D) double logarithmic plot of the data in C. Additions in A and B were: water (○), DFNS at the millimolar concentrations indicated; sodium salicylate (NaS), 5 mM final concentration; and 2 mM DFNS-ME in 1% dimethyl sulfoxide, final concentration; C is the control without additions.

capacity to accumulate calcium dependent on ATP hydrolysis, indicating that the effect of DFNS is reversible.

Ca^{2+} *uptake.* Since DFNS has a carboxylic group, it is likely that its properties are pH dependent [2]; therefore, the effect of H^+ concentrations in the medium on the rate of Ca^{2+} transport by SR vesicles was investigated from pH 6.1 to 7.4, since Ca^{2+} uptake decreases drastically at higher pH values [16, 17]. Figure 3A shows that, as the pH increased, lower DFNS concentrations were required to produce 50% inhibition in the rate of Ca^{2+} transported; I_{50} values were determined and plotted in Fig. 3B, and pK_a values of 6.7 to 6.9, from two different

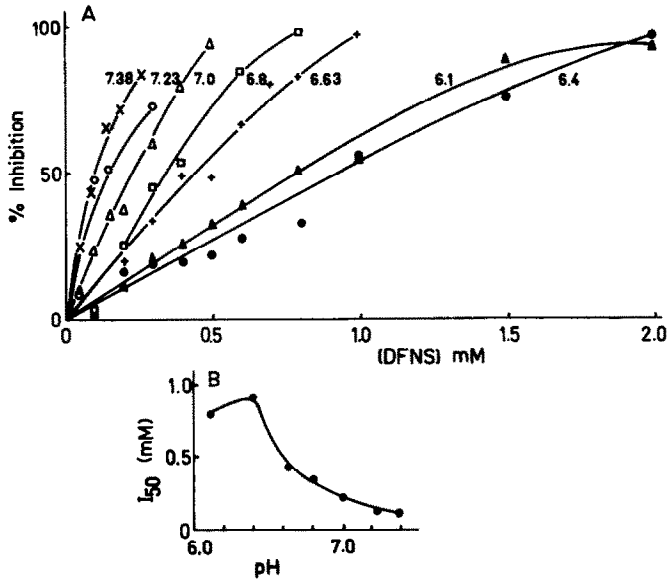

Fig. 3. Effect of DFNS on the rate of Ca^{2+} uptake by SR vesicles at various pH values. Conditions are described in Materials and Methods. SR vesicles were preincubated for 2 min in the presence of DFNS, and the reaction was started with ATP–Tris, pH 6.1 (▲); 6.4 (●); 6.63 (+); 6.8 (□); 7.0 (△); 7.23 (○); and 7.38 (×). (A) Data expressed as percent of inhibition of optimal Ca^{2+} transport rate. (B) I_{50} versus pH.

experiments, were calculated, which agree with the pK_a values reported for the carboxylic ionophore X537A in SR membranes [18]. It was also observed that, when DFNS was added during the stationary course of Ca^{2+} uptake, i.e. when calcium-oxalate precipitates inside the vesicles, the inhibition by DFNS was lower than when the drug was added before the Ca^{2+} transport started (data not shown). This was probably due to the lower Ca^{2+} concentration in the vesicle lumen caused by the calcium-oxalate precipitate [19].

Considering that charge surface and ionic strength modify the properties of SR membranes (i.e. [20–22]), the effect of the monovalent cation, potassium, using choline as control, on DFNS inhibition of Ca^{2+} uptake was explored. As shown in Fig. 4, A and B, the inhibitory effect of 0.8 mM DFNS was maximal in the absence of potassium or choline-chloride. The inhibition decreased to a mimimum at an 80 mM concentration of either of the monovalent cations added; at higher concentrations of K^+, the inhibition by DFNS increased again, whereas at higher concentrations of choline, it remained constant. DFNS inhibition of Ca^{2+} uptake was always higher in the presence of choline. The possibility that the inhibitory activity in Ca^{2+} transport by DFNS was due to a decrease in the free-Ca^{2+} concentration by complexation and not to its ionophoretic effect on the membrane was ruled out by the direct titration with DFNS or EGTA as a control of 1 mM Ca^{2+} at pH 7.5, in the presence of 20 mM Hepes; 0.2 mM murexide; 4 mM $MgCl_2$; 100 mM KCl and 50% ethanol [14] at 507–540 nm. The K_D estimated for the Ca^{2+}-DFNS complex was on the order of 100 mM assuming a 1:1 stoichiometry, and 357 $(mM)^2$ assuming a 2:1 $(DFNS)_2$-Ca^{2+} complex; this signifies that, under the conditions of the experiments, only a negligible amount (3–6%) of the total Ca^{2+} was in the form of the Ca^{2+}-DFNS complex (not shown).

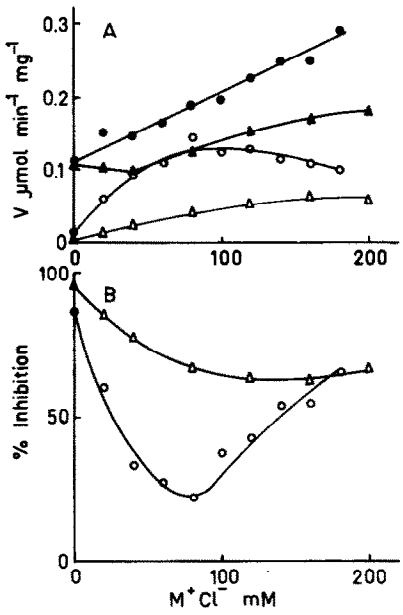

Fig. 4. Effect of monovalent cations in the inhibitory action of DFNS on Ca^{2+} uptake. Conditions were the same as in Fig. 3, except that KCl or choline chloride was varied as indicated. (A) Ca^{2+} uptake rate at various KCl (●, ○) or choline chloride (▲, △) concentrations without (●, ▲) or with 0.8 mM DFNS (○, △). (B) Percent of inhibition of Ca^{2+} transport by 0.8 mM DFNS in the KCl (○) or choline chloride (△) medium.

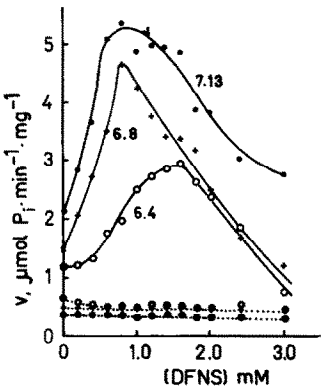

Fig. 5. Effect of DFNS on ATPase activity of SR vesicles at various pH values. Conditions are described in Materials and Methods. Key: pH 6.4 (○), 6.8 (+) and 7.13 (●); (——) Ca^{2+}-ATPase, and (. . . .) basal ATPase.

DFNS effects on basal and Ca^{2+}*-ATPase.* The decrease of the transport rate and accumulation of Ca^{2+} and Sr^{2+} in SR vesicles may have been due to the ionophoretic action of DFNS, but it also can be explained by a direct inhibitory effect of DFNS on ATPase. Therefore, basal and Ca^{2+}-dependent ATPase activities were determined in SR vesicles, as well as purified ATPase at various pH values and at various DFNS concentrations.

As seen in Fig. 5, at all the assayed pH values, an initial increase in the activity of Ca^{2+}-ATPase in SR vesicles occurred. Nevertheless, the maximum level

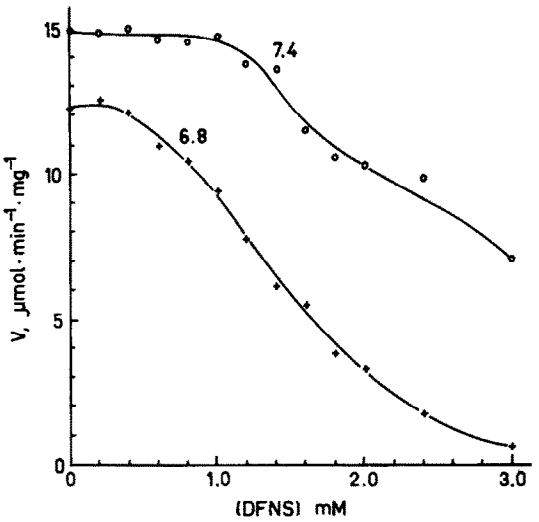

Fig. 6. Inhibition of purified Ca^{2+}-ATPase by DFNS. Conditions are described in Materials and Methods. Purified Ca^{2+}-ATPase was preincubated for 5 min at 37° in the presence of DFNS and the reaction was initiated by addition of ATP. Key: pH 6.8 (+); and 7.4 (○).

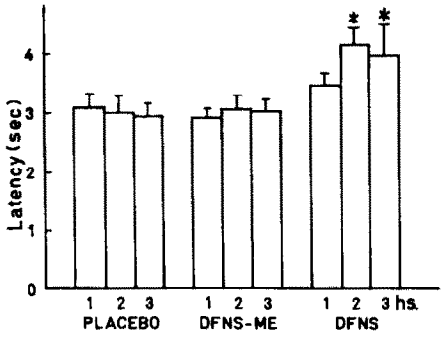

Fig. 7. Analgesia of DFNS and DFNS-ME: Latency time of the tail-flick test in seconds versus time in hours after administration of the drugs. Conditions are described in Materials and Methods. Key: (*) $P < 0.02$.

of activation was 1.3 to 1.8 lower than that obtained with X537A ionophore (data not shown). As the DFNS concentration was increased, the activating effect diminished and an inhibitory effect on ATPase took place. For purified Ca^{2+}-ATPase, Fig. 6 shows that at low DFNS concentrations there was no effect at the analyzed pH values; the inhibitory effect was observed only at DFNS concentrations higher than 0.4 and 1.0 mM and was higher at pH 6.7 than at pH 7.4, suggesting that the inhibitory effect could be higher with the protonated form of DFNS. The basal or Mg^{2+}-dependent ATPase in SR vesicles was always slightly inhibited by DFNS (Fig. 5).

Analgesia of DFNS and DFNS-ME in rats. The data obtained on analgesia with DFNS and DFNS-ME are presented in Fig. 7. The latencies measured for placebo and DFNS-ME were similar. DFNS showed analgesic properties particularly at 2 and 3 hr after the administration of the drug; data were statistically significant at $P < 0.02$.

DISCUSSION

A previous work showed that DFNS is able to release intramitochondrial calcium and that it behaves as an ionophore for this cation on model systems [2]. The experimental results presented here indicate that DFNS acts as an ionophore for Ca^{2+} and Sr^{2+} in muscle sarcoplasmic reticulum, since, as described, DFNS at concentrations below 1 mM inhibited the ATP-dependent transport of Ca^{2+} and Sr^{2+} by SR vesicles, whereas Ca^{2+}-ATPase activity was increased because Ca^{2+} accumulation was impaired and back inhibition was no longer attained [23]; in contrast, DFNS at this low concentration did not modify the activity of purified Ca^{2+}-ATPase in leaky vesicles. As could be expected from an ionophore, the results also demonstrated that DFNS releases the Ca^{2+} and Sr^{2+} previously accumulated in SR vesicles. These results agree, in general, with those reported previously for carboxylic ionophore actions on SR [4, 14, 24, 25].

Nevertheless, DFNS at concentrations higher than 1 mM acted also as an inhibitor of Ca^{2+}-ATPase either in SR vesicles or on the purified enzyme. In this respect, it has been reported recently that high concentrations of ionophore A23187 inhibit selectively SR Ca^{2+}-ATPase, presumably by disruption of the lipid–protein interaction [26]. Also, it has been proposed that the ionophores X537A and A23187 interact with Ca^{2+}-ATPase in the protein–lipid interface [27, 28]. Accordingly, since DFNS is a lipophilic molecule and a carboxylic ionophore, it is suggested that its inhibitory effect on ATPase could be due also to disruption of the lipid–protein interactions.

In regard to the carboxylic group, all the observed effects of DFNS in SR were pH dependent, due to the fact that the pK of this group was shifted from a value of 2.6 in aqueous solution* to near neutrality in SR membrane, which agrees with the pK reported for the carboxylic ionophore X537A in the same membranes [18]. Monovalent cations, other than H^+, seem to compete with Ca^{2+} for DFNS, as has been reported for Na^+ [2] and as shown for K^+ and, to a lesser extent, for choline (Fig. 4). Also, the phenolic group seems to be important for the ionophoretic and analgesic properties of DFNS, since the methylated derivative, DFNS-ME, lost both properties, whereas opposite results have been reported for the ionophore X537A, which retains its ionophoretic properties when the phenolic group is modified [4]. This suggests that both ionophores form different types of complexes with calcium, which is in agreement with the observation that X537A forms 1:1 and 2:1 complexes with Ca^{2+}, depending on the polarity of the medium, and also the "head-to-tail" complex does not directly involve the phenolic group [4, 14, 29], whereas the analgesic, presumably forms $(DFNS)_2$-calcium complexes with both the carboxylic and phenolic groups involved [2, and present work].

As sodium salicylate was a poor ionophore in SR vesicles (Fig. 2, A and B), it suggests that the second benzyl group with two fluorines, present in DFNS, plays an important role because it increases hydrophobicity and, consequently, it must enhance the solubility of DFNS in membrane lipids.

From the results reported here, it would be tempting to suppose that the ionophoretic action of DFNS and its therapeutic properties were closely related. However, in contrast, there is evidence that the ionophore X537A by increasing intracellular Ca^{2+} produces hyperalgesia [30]. On the other hand, DFNS acting as a Ca^{2+} ionophore would induce positive inotropic effects in cardiac muscle; however, such an effect has not been reported. Finally, considering the above, it is possible that, *in vivo*, the ionophoretic properties of DFNS may be poorly manifested or not at all, probably due to the competition of Na^+, Mg^{2+} [2], and K^+ (Fig. 4) with Ca^{2+} and also to the low affinity of DFNS for Ca^{2+} as evidenced by the high value of the K_D of the complex. Thus, it seems clear that there is no correlation between the *in vivo* analgesia of DFNS and the Ca^{2+} ionophoretic properties demonstrated in the present work.

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