*Biochemical Pharmacology*, Vol. 38, No. 13, pp. 2121–2127, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00


# PARTITION OF $Ca^{2+}$ ANTAGONISTS IN BRAIN PLASMA MEMBRANES

C. M. CARVALHO, C. R. OLIVEIRA, M. P. LIMA, J. E. LEYSEN* and A. P. CARVALHO†
Center for Cell Biology, Department of Zoology, University of Coimbra, 3049 Coimbra Codex, Portugal and * Department of Biochemical Pharmacology, Janssen Research Foundation B-2340 Beerse, Belgium

(*Received* 15 *September* 1988; *accepted* 2 *January* 1989)

**Abstract**—The partition coefficients ($K_p$) of three prototype $Ca^{2+}$ antagonists, nitrendipine, (−)-desmethoxyverapamil and flunarizine were determined in native synaptic plasma membranes (SPM) isolated from sheep brain cortex and in liposomes prepared with the total lipids extracted from the membranes. We found that at 25° and at $5 \times 10^{-6}$ M drug concentration the $K_p$ values of the drugs for native SPM are higher than those obtained for liposomes, and are of the order of $334 \pm 53$, $257 \pm 36$ and $23 \times 10^3$ for nitrendipine, (−)desmethoxyverapamil and flunarizine, respectively, whereas the $K_p$ values in liposomes are $190 \pm 41$, $118 \pm 10$ and $6 \times 10^3$ for the same drugs. These results suggest that the presence of membrane proteins favors the incorporation of the drugs in the membranes. Furthermore, the $K_p$ values of the three $Ca^{2+}$ antagonists studied increase with temperature in native membranes, but not in liposomes. It is concluded that the physical partitioning in membranes of drugs which act on $Ca^{2+}$ channels may play some role in the mechanism of interaction of these drugs with the $Ca^{2+}$ channel proteins.

Several reports now indicate that some $Ca^{2+}$ channel antagonists may interact with their binding sites in the $Ca^{2+}$ channel proteins from the inner surface or from the lipid phase of the plasma membrane [1–6]. Thus, studies using patch-clamp techniques with cardiac cells in culture indicate that dihydropyridine derivatives have access to the $Ca^{2+}$ channel through the lipid phase of the cell membrane since their effect increases with lipophilicity [1]. Also, $Ca^{2+}$ channel blockers with higher lipid solubilities are taken up more extensively into skeletal muscle cells [2]. Furthermore, a recent study shows that phenylalkylamine $Ca^{2+}$ channel blockers need to cross the plasma membrane in order to reach intracellular sites in synaptosomes, where they inhibit $Ca^{2+}$ entry and neurotransmitter release [3]. Other studies using pimozide and other diphenylbutylpiperidine neuroleptics show that these drugs act as very potent $Ca^{2+}$ channel blockers [7–9], and this may be related to their high octanol: water partitioning [10]. There is also indication from biophysical studies that 1,4-dihydropyridine $Ca^{2+}$ channel antagonists interact with biological membranes by a two-stage binding mechanism that involves partitioning of the drugs into the lipid bilayer, with subsequent lateral diffusion to the receptor binding site [4–6].

In most studies, however, the classical partition coefficient determinations of drugs in octanol/water have been utilized as a model for evaluating the lipophilicity of the drugs in biological membranes [1, 2, 10]. We know, however, that biological membranes contain a variety of proteins and lipids which differ very much chemically from octanol. Therefore, it is necessary to determine the partitioning of the drugs in biological membranes, if one wishes to determine the correlation between lipophilicity of the drugs and their pharmacological effect on $Ca^{2+}$ channel blockade.

Therefore, our approach was to determine the partition coefficients ($K_p$) of $Ca^{2+}$ channel antagonists belonging to different chemical groups (dihydropyridines, phenylalkylamines and diphenylpiperazines) in partially purified synaptic plasma membranes (SPM) isolated from sheep brain cortex and in liposomes prepared with the extracted membrane lipids. We found that for nitrendipine and (−)-desmethoxyverapamil the $K_p$ values in native SPM are higher than those determined in octanol/water [2], whereas for flunarizine the $K_p$ values in SPM, although very high, are lower than those reported for octanol/water [10]. Furthermore, the partitioning of the three $Ca^{2+}$ channel antagonists is higher in native SPM than in liposomes, suggesting that the presence of membrane proteins favors the incorporation of the drugs in the membranes. These results were first published in abstract form [11].

## MATERIALS AND METHODS

### *Preparation of biological material*

*Native membranes.* Synaptic plasma membrane vesicles (SPM) were prepared by hypotonic lysis of isolated sheep brain synaptosomes as described previously [12]. The SPM preparation was washed once and resuspended in 50 mM Tris–Cl, pH 7.4, at a protein concentration of about 10 mg/ml, as determined by the biuret method [13]. The final SPM suspension was divided into aliquots which were frozen in liquid nitrogen and kept at −80° for further utilization. All experiments were performed within four weeks after isolation. Sarcoplasmic reticulum was prepared from rabbit skeletal muscle as described previously [14].

*Liposomes.* Multilamellar liposomes [15] were

† Correspondence should be addressed to A. P. Carvalho.

prepared from total lipids extracted from SPM with chloroform/methanol (1:1) as described previously [16]. The final lipid extract was dissolved in chloroform and kept under nitrogen, at −80°, in a sealed tube. Samples of the lipid extract containing about 5 mg of total phospholipid were dried under nitrogen and placed under vacuum at 25° for 3 hr, after which time the residue was hydrated with buffer (50 mM Tris–Cl, pH 7.4), at 25°. The suspension was vortexed and allowed to equilibrate under $N_2$ atmosphere for 12 hr, at 25°. This procedure yields multilamellar liposomes with a mean size of 630 nm as determined by dynamic light scattering [17]. Typically, the final phospholipid concentration after hydration was 398 μM, as determined by measuring the inorganic phosphate, after acid hydrolysis [18], by the method of Bartlett [19].

*Determination of partition coefficients.* The partition coefficients of the $Ca^{2+}$ channel blockers in SPM and in SPM liposomes were determined by using $^3$H-labelled $Ca^{2+}$ channel blockers ([$^3$H]-nitrendipine, ([$^3$H]desmethoxyverapamil and [$^3$H]-flunarizine, and a filtration method was used to separate the membranes from the incubation media [20]. Various concentrations of each drug were taken from 1 mM ethanolic stock solution and diluted in buffer (50 mM Tris–Cl, pH 7.4). The incubation was initiated by adding the membrane suspension in the case of SPM or, in the case of liposomes, the drugs were directly added to the liposome suspension in buffer (50 mM Tris–Cl, pH 7.4). The final concentration in phospholipid in the incubation medium was 398 μM in the cases of [$^3$H]nitrendipine and (−)-[$^3$H]desmethoxyverapamil, or 37 μM in the case of [$^3$H]flunarizine. It should be mentioned that the presence of ethanol (0.5%, final concentration), which was utilized as the solvent for nitrendipine and flunarizine, does not affect liposome size or distribution.

The samples were allowed to equilibrate at various temperatures for 1 hr which, in previous tests, was determined to be sufficient time for maximal incorporation of the drugs in the membranes. Samples of the suspensions (0.5 ml) were filtered through Whatman GF/B filters under vacuum. After filtration, the filters were washed with 10 ml of ice-cold (0–4°) buffer (50 mM Tris–Cl, pH 7.4) supplemented with 0.05% bovine serum albumin in the case of samples containing (−)-[$^3$H]desmethoxyverapamil to decrease the blank binding to the filter [21].

The radioactivity retained in the filter, as well as total radioactivity in the sample, were determined by liquid scintillation spectrometry in a Packard 2000 spectrometer with dpm correction. The degree of retention of the membrane material in the filters was determined in all the experiments by measuring the amount of phospholipid retained in the filters, by first digesting the filters at 180° in the presence of 1 ml $HClO_4$ (70%) for 2 hr [18]. The released inorganic phosphate was determined by the method of Bartlett [19]. The retention of biological material in the GF/B filters was about 90% for SPM and 50% for SPM liposomes. The partition coefficient ($K_p$) was calculated according to the Connor's equation [22], in the following form:

$$p = \frac{K_p(V_l/V_a)}{K_p(V_l/V_a) + 1}$$

where $p$ is the fraction of drug present in the incubation medium which is retained in the membranes, $V_l$ and $V_a$ are the volumes of the lipid ($l$) and aqueous ($a$) phases, respectively. In our experimental conditions $V_a$ was 0.5 ml, $V_l$ was taken as the volume of the lipid compartment of the membranes and was calculated from the phosphate analysis of membrane material retained in the filters, and taking 0.984 μl/mg for mean specific volume of phospholipids.

For the sake of comparison, some determinations of $K_p$ were performed by a centrifugation method [23, 24], specially in the case of [$^3$H]flunarizine, which has a very high partitioning in lipid, as expected from its high $K_p$ in octanol/water [10]. Thus, for the centrifugation method, SPM or liposome suspensions were incubated with the drug at a given concentration and, after 1 hr incubation, the equilibrated membranes were centrifuged for 15 min at 16,000 $g$ in an Eppendorf microfuge, Mod. 5415, at room temperature. Samples containing no membranes or liposomes but only the $^3$H-labelled drug were subjected to the same procedure. Radioactivity in samples of the supernatants was determined by liquid scintillation counting, as described above. The partition coefficient ($K_p$) was directly determined from radioactivity in the supernatants [23]:

$$K_p = \frac{\text{dpm}(m)\ V(b)}{\text{dpm}(b)\ V(m)}$$

where dpm represents the disintegrations per min in membrane ($m$) or buffer ($b$) phases; dpm($m$) was calculated from total dpm minus dpm in supernatant of membrane suspensions. $V(m)$ is the volume of the lipid compartment of the membranes and was calculated on the basis of lipid content (mg/ml) and the specific volume of lipid (0.984 μl/mg). The results obtained for the $K_p$ of [$^3$H]flunarizine in SPM with the centrifugation method did not differ significantly from those of the filtration method (Table 1).

The partition coefficients determined by these methods for the different $Ca^{2+}$ channel blockers studied should be designated "apparent partition coefficients" since they reflect the over-all distribution behaviour of all molecular species present (neutral and charged). The correction which is normally applied to obtain the true partition coefficient of the neutral species in octanol/water [10, 24, 25], taking in consideration the degree of ionization of the substances, is not reasonably applicable in complex biological membranes, since a diversity of charged lipids and proteins is present, and both the neutral and charged species of the drugs are partitioning.

*Drugs and chemicals.* [$^3$H]Nitrendipine (87 Ci/mmol) was purchased from New England Nuclear (F.R.G.), (−)-[$^3$H]Desmethoxyverapamil (83 Ci/mmol) and cold (−)-desmethoxyverapamil were obtained from Amersham International (U.K.), [$^3$H]Flunarizine (7.4 Ci/mmol) and cold flunarizine were a generous gift from Dr J. Leysen, Department

Table 1. Partition coefficients of $Ca^{2+}$ antagonists in synaptic plasma membranes (SPM) and liposomes, at 25° (Data from Fig. 2).

| | Nitrendipine* | (−)-Desmethoxy verapamil* | Flunarizine† | |
|---|---|---|---|---|
| | | | Filtration | Centrifugation |
| Native SPM | 334 ± 53 | 257 ± 36 | $23 \times 10^3$ | $19 \times 10^3$ |
| Liposomes | 190 ± 41 | 118 ± 10 | $6 \times 10^3$ | N.D. |
| Octanol: water | 9.95 ± 0.25[a] | 66.6 ± 05[a] | — | $10^{5.78}$[b] |

* $K_p$ obtained by the filtration method (see Materials and Methods).
† $K_p$ obtained by both filtration and centrifugation methods (see Materials and Methods).
N.D. Not determined.
[a] Taken from Ref. 2.
[b] Taken from Ref. 10.

of Biochemical Pharmacology, Janssen Research Foundation, Beerse, Belgium. Nitrendipine was obtained from Sandoz Laboratories. All other chemicals utilized were of analytical grade.

## RESULTS

### *Partition coefficients* ($K_p$) *of nitrendipine and (−)-desmethoxyverapamil in native membranes and in liposomes at increasing drug concentrations*

The determination of the partition coefficients ($K_p$) by the filtration method was performed at concentrations of the labelled drugs in the incubation medium which did not vary by more than 10% due to drug binding to the membrane. In the case of $Ca^{2+}$ channel blockers belonging to the 1,4-dihydropyridine group (nitrendipine), or to the phenylalkylamine group (desmethoxyverapamil), preliminary tests performed with increasing concentrations of SPM showed that lipid concentrations of about 398 μM bind between 5 and 10% of the drugs at concentrations of the drugs of $5 \times 10^{-6}$ M.

We studied also the effect of increasing drug concentrations on the $K_p$ values for nitrendipine and (−)-desmethoxyverapamil in native SPM, and in sarcoplasmic reticulum (SR), as well as in liposomes prepared from lipids extracted from SPM (Fig. 1). It is observed that the $K_p$ of nitrendipine in SPM is constant at concentrations of the drug up to $3 \times 10^{-5}$ M, and decreases drastically for higher drug concentrations (Fig. 1A). This behaviour has been observed for the partition of other drugs in biomembranes [23], and it simply reflects that the lipid bilayer becomes saturated with the drug for higher drug concentrations, which we have found to be the case in our studies of nitrendipine (results not shown). Therefore, in subsequent experiments, we utilized a drug concentration of $5 \times 10^{-6}$ M, so that we are in the concentration range in which the $K_p$ is constant and the drugs behave as simple solutes. We also refrained from using lower drug concentrations, closer to the $K_D$ values of the drug receptors, to avoid a significant contribution by the receptors to the partition.

The $K_p$ values of [$^3$H]nitrendipine decrease slightly with increasing concentration of the drug in SR membranes (Fig. 1A) over the concentration range studied (1–30 μM), and the $K_p$ of [$^3$H]nitrendipine in SR is only about half of the value obtained in SPM. Therefore, one would expect that saturation of the SR bilayer with drug would be reached at lower concentrations than those found for SPM. However, when we measured the drug bound per nmol of lipid we found that it continues to increase linearly with concentration of drug in the medium. Therefore, the explanation for variation of the $K_p$ value as a function of the free concentration of the drug in the medium is more complex, and it is not readily evident.

In the case of partition of (−)-[$^3$H]desmethoxyverapamil in SPM and liposomes, the $K_p$ values observed (Fig. 1B) decrease gradually in both cases, as was observed for SR in the case of nitrendipine over the concentration range studied (1–30 μM). In subsequent studies a concentration of $5 \times 10^{-6}$ M was utilized for all $Ca^{2+}$ antagonists studied.

### *Effect of temperature on the partition coefficients* ($K_p$) *of* $Ca^{2+}$ *channel blockers in SPM and liposomes*

In order to understand the dependence of the partitioning of the $Ca^{2+}$ channel blockers on the physical state of the membrane components, we determined the effect of temperature on the $K_p$ values for three $Ca^{2+}$ channel blockers belonging to three different chemical groups: nitrendipine (1,4-dihydropyridine), (−)-desmethoxyverapamil (phenylalkylamine) and flunarizine (diphenylpiperazine), in native SPM membranes and in liposomes prepared with the total lipids extracted from native membranes (Fig. 2).

The results presented in Fig. 2A show that the $K_p$ values of [$^3$H]nitrendipine in native SPM increase with temperature from 260 ± 37 at 8° to 464 ± 75 at 37°. However, the $K_p$ values for liposomes prepared from SPM lipids are independent of temperature, and are about 158 ± 10 at 37°, which is less than the mean value obtained for the intact SPM as reported above. The behaviour of (−)-desmethoxyverapamil partitioning in native SPM and in SPM liposomes, as affected by temperature (Fig. 2B), is similar to that observed for nitrendipine (Fig. 2A). Thus, the $K_p$ values of (−)-desmethoxyverapamil are higher in native SPM than in SPM liposomes, and increase with temperature from 162 ± 20 at 8° to 361 ± 40 at 37° in native SPM, whereas they are independent of temperature in liposomes ($K_p$ in liposomes is about 144 ± 14 at 37°, Fig. 2B).

The third type of $Ca^{2+}$ antagonist whose par-

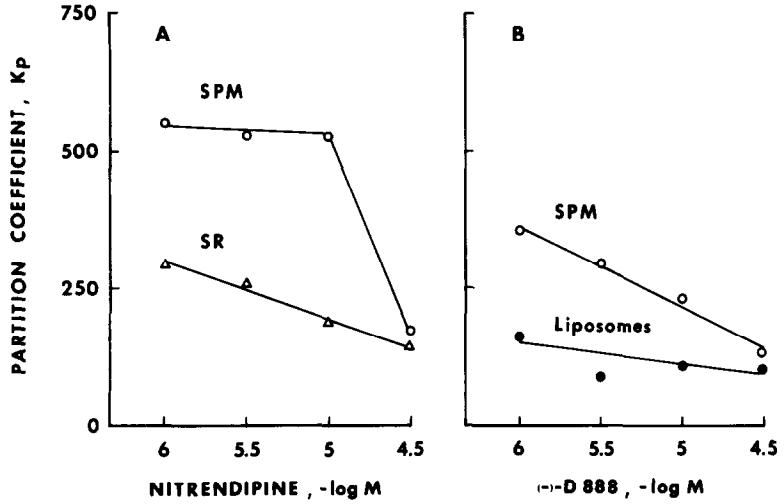


Fig. 1. Concentration dependence of nitrendipine (A) and (−)-desmethoxyverapamil (B) partitioning into liposomes and native membranes. Partition coefficients ($K_p$) for increasing concentrations of the drugs (1–30 $\mu$M) were determined in synaptic plasma membrane vesicles (SPM), sarcoplasmic reticulum vesicles (SR) and in liposomes prepared with total lipid extracted from SPM. SPM or SR membranes and liposomes were equilibrated with [$^3$H]nitrendipine or (−)-[$^3$H]desmethoxyverapamil for 1 hr at 25°. The final concentration in lipid was 398 $\mu$M. The suspensions were filtered and washed and the radioactivity associated with the filter and in the suspension was determined as described in Materials and Methods. The results represent the means of two experiments performed in triplicate. In the abscissa of B, (−)-desmethoxyverapamil was abbreviated as (−)-D888.

titioning in brain membranes was studied was flunarizine, a drug which is very lipophilic, as suggested by its high partition coefficient in octanol/water, which is of the order of $10^{5.78}$ [10]. We found that the $K_p$ for flunarizine in native SPM or in SPM liposomes is, however, much lower than its $K_p$ in octanol/water, as shown in Fig. 2C. Thus, the $K_p$ values for flunarizine in native SPM are between $19 \times 10^3$ at 8° and $28 \times 10^3$ at 37°, and in SPM liposomes the value is even lower, about $6 \times 10^3$, and it is independent of temperature between 8° and 37° (Fig. 2C). The $K_p$ values obtained for the three $Ca^{2+}$ antagonists studied, determined in native SPM and in SPM liposomes at 25°, are summarized in Table 1, in which we also give the partition coefficient values for octanol/water for the three drugs studied.

It is clear that for the three $Ca^{2+}$ antagonists studied the $K_p$ values are higher for native membranes than they are for liposomes prepared from lipids isolated from the membranes, suggesting that even for very lipophilic drugs, such as flunarizine, the membrane proteins increase significantly the incorporation of the drugs into the membrane phase, probably through binding to the proteins themselves or at the lipid–protein interfaces. This behaviour contrasts with the behaviour of other drugs, such as dopamine antagonists, which show higher $K_p$ values in liposomes than in native membranes [26].

*Thermodynamic parameters of the interaction of* $Ca^{2+}$ *channel blockers with brain membranes*

The analysis of the results of Fig. 2 according to the van't Hoff relation (ln equilibrium $K_p$ vs the inverse of the absolute temperature) yielded linear plots, which permitted the determination of the thermodynamic parameters for the transfer of $Ca^{2+}$ channel blockers into the membrane phase (Table 2). The $\Delta G°$ value for the transfer of the drugs from the aqueous phase to the membrane phase was calculated from the equilibrium $K_p$ value at 25°, the $\Delta H°$ value was calculated from the slope of the van't Hoff plots and the value for $\Delta S°$ was derived from the relationship $\Delta G° = \Delta H° - T\Delta S°$.

The results summarized in Table 2 show that for the three $Ca^{2+}$ channel blockers studied, the incorporation of the drugs into SPM liposomes is associated with positive entropy changes ($\Delta S° > 0$), but the enthalpy contribution ($\Delta H°$) associated with the transfer process is essentially zero. In native SPM, positive values for $\Delta S°$ and $\Delta H°$ are obtained. Thus, the incorporation of $Ca^{2+}$ channel blockers both in membranes and in liposomes is entropy driven.

## DISCUSSION

Various research groups, including our own, have demonstrated that $Ca^{2+}$ channel blockers, such as nitrendipine [27, 28], (−)-desmethoxyverapamil [21, 29] and diphenylbutylpiperidine neuroleptics [7–9] bind with high affinity to membranes of excitable tissues, including synaptic plasma membranes [21, 30]. The affinities normally determined are in the nanomolar or subnanomolar range. In these studies, however, the $K_D$ values are determined by Scatchard analysis of saturation binding data, and it is assumed that the free concentrations of the drugs in equilibrium with their binding sites in the $Ca^{2+}$ channel proteins are the aqueous concentrations in the bulk medium. However, for lipophilic drugs, with high partitions into the membrane phase, the actual concentrations attained in the bilayer surrounding their protein receptors may be much dif-

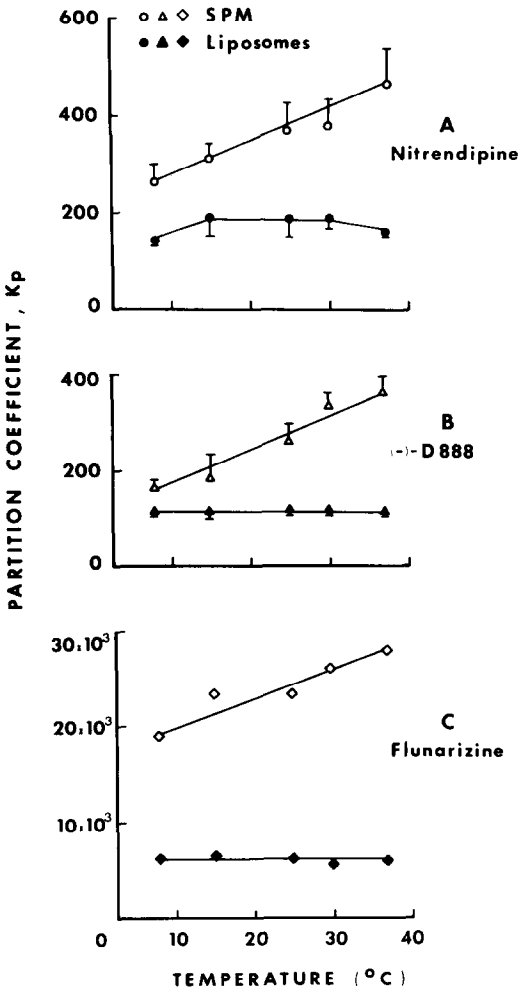


Fig. 2. Partition coefficients of nitrendipine (A), (−)-desmethoxyverapamil (B) and flunarizine (C) in SPM (open symbols) and SPM liposomes (closed symbols) as influenced by temperature. The concentration of the drugs utilized was $5 \times 10^{-6}$ M, and the final concentration of lipid in the suspensions was 398 $\mu$M in (A) and (B) and 37 $\mu$M in (C). Each experimental point represents the mean of three to six independent measurements (vertical bars indicate the range of S.D.). In (B), (−)-D888 was utilized as abbreviation of (−)-desmethoxyverapamil.

ferent from the aqueous concentrations. Thus, if the receptor sites are hydrophobic, they are expected to be in equilibrium with the drug concentration in the membrane phase.

The results of Figs 1 and 2 and Table 1 show that the apparent $K_p$ of the three $Ca^{2+}$ channel blockers studied are lower in liposomes than in native SPM, indicating that the membrane proteins either promote entry of the blockers into the lipid bilayer, or they bind the blockers themselves. Thus, the drugs in the membrane may be present dissolved in the lipid phase, in the lipid–protein interphase or bound to the membrane proteins. However, the increase in the apparent partitioning of the drugs into the membranes induced by the proteins for all $Ca^{2+}$ channel blockers studied must represent non-specific interaction, at least at the relatively high drug concentrations ($5 \times 10^{-6}$ M) we used, which are 5 to 10,000 times higher than the $K_D$ values for the specific binding of the $Ca^{2+}$ antagonists to their $Ca^{2+}$ channel binding sites, which are in the nanomolar or subnanomolar range [21, 27–30].

It is interesting that even for flunarizine, which is the most lipophilic of the drugs studied [10], the $K_p$ is also higher in native SPM than it is in liposomes (Fig. 2C, Table 1), indicating that the presence of proteins in the membrane contribute to the accumulation of the drugs. This non-specific binding to proteins usually is 20–30% of total binding at the nM concentrations which saturate the specific $Ca^{2+}$ antagonist receptors [21], but the concentration in the lipid phase will be determined by the $K_p$ value and, for lipophilic drugs, such as flunarizine, the concentration in the lipid phase may be several hundred-fold higher than it is in aqueous medium (Fig. 2, Table 1). This problem has been discussed by other investigators [31] who have calculated the "membrane" concentrations for various drugs, including 1,4-dihydropyridines, such as nimodipine. These authors conclude that for drugs with high membrane partition coefficients, even at nM aqueous concentration, the "membrane" concentration is $10^4$-fold higher than in the aqueous medium [31].

Some insight into the nature of membrane–drug interaction can be obtained by studying the variation of $K_p$ values with temperature and by determining

Table 2. Thermodynamic parameters for the transfer process of $Ca^{2+}$ antagonists into synaptic plasma membranes and liposomes (data from Fig. 2).

| $Ca^{2+}$ antagonist | Membrane system | $\Delta H°$ (kcal/mol) | $\Delta G°$ (kcal/mol) | $\Delta S°$ (cal/mol, °K) |
|---|---|---|---|---|
| Nitrendipine | SPM | 2.63 | −3.77 | 20.6 |
| | Liposomes | 0.45 | −3.11 | 11.5 |
| (−)-Desmethoxy-verapamil | SPM | 4.38 | −3.62 | 25.8 |
| | Liposomes | −0.02 | −2.91 | 9.3 |
| Flunarizine | SPM | 1.58 | −6.26 | 25.3 |
| | Liposomes | −0.54 | −5.33 | 15.5 |

the thermodynamic parameters ($\Delta G°$, $\Delta H°$ and $\Delta S°$) of the transfer of the drugs from the aqueous phase into the membrane phase. From the results summarized in Table 2 we may conclude that the transfer process is mainly entropy driven ($\Delta S° > 0$), which may indicate that ionic and/or hydrophobic interactions are involved, as has been concluded for the transfer of model compounds into membranes [32, 33].

In general, our results are in agreement with previous results obtained by other investigators [4–6, 31] who have concluded that the 1,4-dihydropyridine $Ca^{2+}$ channel acting drugs partitioning into the lipid bilayer may be a component of the pathway by which these drugs reach specific protein receptors in the membranes, if the specific binding site is buried in the lipid of the membrane on the cytoplasmic side of the plasma membrane. We further extended these studies to other $Ca^{2+}$ channel blockers. The high $K_p$ values obtained for (−)-desmethoxyverapamil in SPM favors the idea that this $Ca^{2+}$ channel blocker may have an intracellular action [2, 3] and, in the case of flunarizine, the high partitioning into the membranes may be responsible for its proposed site of action at the level of intracellular calmodulin modulated processes [34]. We are currently studying the correlation between physical partitioning and $Ca^{2+}$ channel blockade in synaptosomes for other $Ca^{2+}$ channel blocker drugs, specially for those belonging to the diphenylbutylpiperidine group which have been demonstrated recently to act as potent $Ca^{2+}$ channel blockers in muscle [8] and pituitary cells [9].

*Acknowledgements*—This work was supported by INIC, JNICT and the Calouste Gulbenkian Foundation.

## REFERENCES

1. Kockbun S and Reuter H, Dihydropyridine derivatives prolong the open state of Ca channels in cultured cardiac cells. *Proc Natl Acad Sci* **81**: 4824–4827, 1984.
2. Pang DC and Sperelakis N, Uptake of calcium antagonistic drugs into muscles as related to their lipid solubilities. *Biochem Pharmacol* **33**: 821–826, 1984.
3. White EJ and Bradford HF, Participation of intracellular sites in the action of $Ca^{2+}$ channel blockers. *Eur J Pharmacol* **130**: 243–248, 1986.
4. Rhodes DG, Sarmiento JG and Herbette LG, Kinetics of binding of membrane-active drugs to receptor sites. Diffusion-limited rates for a membrane bilayer approach of 1,4-dihydropyridine calcium channel antagonists to their active site. *Molec Pharmacol* **27**: 612–623, 1985.
5. Herbette LG, Chester DW and Rhodes DG, Structural analysis of drug molecules in biological membranes. *Biophys J* **49**: 91–94, 1986.
6. Chester DW, Herbette LG, Mason RP, Joslyn AF, Triggle DJ and Koppel DE, Diffusion of dihydropyridine calcium channel antagonists in cardiac sarcolemmal lipid multibilayers. *Biophys J* **52**: 1021–1030, 1987.
7. Gould RJ, Murphy KMM, Reynolds IJ and Snyder SH, Antischizophrenic drugs of the diphenylbutylpiperidine type act as calcium channel antagonists. *Proc Natl Acad Sci* **80**: 5122–5125, 1983.
8. Galizzi J-P, Fosset M, Romey G, Laduron P and Lazdunski M, Neuroleptics of the diphenylbutylpiperidine series are potent calcium channel inhibitors. *Proc Natl Acad Sci* **83**: 7513–7517, 1986.
9. Enyeart JJ, Chen S-S and Hinkle PM, Pituitary $Ca^{2+}$ channels: blockade by conventional and novel $Ca^{2+}$ antagonists. *Am J Physiol* **253**: C162–C170, 1987.
10. Pauwels PJ, Leysen JE and Laduron PM, [$^3$H]Batrachotoxinin A 20-$\alpha$-benzoate binding to sodium channels in rat brain: characterization and pharmacological significance. *Eur J Pharmacol* **124**: 291–298, 1986.
11. Carvalho CAM, Oliveira CR, Lima MCP and Carvalho AP, Partition of $Ca^{2+}$ channel blockers in brain plasma membranes. *J Neurochem* **48**: (Suppl.): S76, 1987.
12. Coutinho OP, Carvalho AP, and Carvalho CAM, Effect of monovalent cations on $Na^+/Ca^{2+}$ exchange and ATP-dependent $Ca^{2+}$ transport in synaptic plasma membranes. *J Neurochem* **41**: 670–676, 1983.
13. Layne E, Spectrophotometric and turbidimetric method for measuring proteins. In: *Methods in Enzymology* (Eds. Colowick SP and Kaplan NO), pp. 447–451. Academic Press, New York, 1975.
14. Carvalho AP and Leo B, Effects of ATP on the interaction of $Ca^{2+}$, $Mg^{2+}$ and $K^+$ with fragmented sarcoplasmic reticulum isolated from rabbit skeletal muscle. *J Gen Physiol* **50**: 1327–1352, 1967.
15. Bangham AD, Hill MW and Miller NGA, Preparation and use of liposomes as models of biological membranes. In: *Methods in Membrane Biology* (Ed. Korn ED), pp. 1–68. Plenum Press, New York, 1974.
16. Reed CF, Swisher SN, Marinetti GV and Eden EG, Studies of the lipids of erythrocyte. *J Lab Clin Med* **56**: 281–289, 1960.
17. Aurora TS, Li W, Cummins HZ and Haines TH, Preparation and characterization of monodisperse unilamellar phospholipid vesicles with selected diameters from 300 to 600 nm. *Biochim Biophys Acta* **820**: 250–258, 1985.
18. Böttcher, CJF, VanGent CM and Pries C, A rapid and sensitive sub-micro phosphorus determination. *Anal Chim Acta* **24**: 203–204, 1961.
19. Bartlett GR, Phosphorus assay in column chromatography. *J Biol Chem* **234**: 466–468, 1959.
20. Antunes-Madeira MC and Madeira VMC, Partition of parathion in synthethic and native membranes. *Biochim Biophys Acta* **778**: 49–56, 1984.
21. Carvalho CM, Santos SV and Carvalho AP, $\gamma$-aminobutyric acid release from synaptosomes as influenced by $Ca^{2+}$ and $Ca^{2+}$ channel blockers. *Eur J Pharmacol* **131**: 1–12, 1986.
22. Connors KA, *A Textbook of Pharmaceutical Analysis* Wiley, New York, 1967.
23. Luxnat M and Galla H-J, Partition of chlorpromazine into lipid bilayer membranes: the effect of membrane structure and composition. *Biochim Biophys Acta* **856**: 274–282, 1986.
24. Lullman H and Wehling M, The binding of drugs to different polar lipids in vitro. *Biochem Pharmacol* **28**: 3409–3415, 1979.
25. Lullmann H, Timmermans PBMWM and Zieglen A, Accumulation of drugs by resting or beating cardiac tissue. *Eur J Pharmacol* **60**: 277–285, 1979.
26. Oliveira CR, Lima CP, Carvalho CAM and Carvalho AP, Partition coefficients of dopamine antagonists in caudate nucleus microsomal membranes. *J Neurochem* **48**: (Suppl.); S89, 1987.
27. Belleman P, Ferry D, Lübbecke F and Glossmann H, [$^3$H]nitrendipine as tools to directly identify the sites of action of 1,4-dihydropiridine calcium antagonists in guinea pig tissues. *Arzneim Forsch Drug Res* **32**: 361–363, 1982.
28. Gould RJ, Murphy KMM and Snyder S, [$^3$H]Nitrendipine-labeled calcium channels discriminate inorganic calcium agonists and antagonists. *Proc Natl Acad Sci USA* **79**: 3656–3660, 1982.
29. Goll A, Ferry DR, Striessnig J, Schober M and Glossmann H, (−)-[$^3$H]Desmethoxyverapamil, a novel

$Ca^{2+}$ channel probe. *FEBS Lett* **176**: 371–377, 1984.
30. Carvalho CAM, Coutinho OP and Carvalho AP, Effects of $Ca^{2+}$ channel blockers on $Ca^{2+}$ translocation across synaptosomal membranes. *Neurochem* **47**: 1774–1784, 1986.
31. Herbette LG and Katz AM, Molecular model for the binding of 1,4-dihydropyridine calcium channel antagonists to their receptors in the heart: drug "imaging" in membranes and considerations for drug design. In: *Structure and Physiology of the Slow Inward Calcium Channel* (Eds. Venter JC and Triggle D), pp. 89–108, Alan R. Liss, Inc., New York, 1978.
32. Eftink MR and Biltonen RL, Thermodynamics of interacting biological systems. In: *Biological Microcalorimetry* (Ed. Beezer AE), pp. 343–412. Academic Press, London, 1980.
33. Tanford C, *The Hydrophobic Effect.* Wiley, New York, 1973.
34. Lugnier C, Follenius A, Gerard D and Stoclet JC, Bepridil and flunarizine as calmodulin inhibitors. *Eur J Pharmacol* **98**: 157–158, 1984.