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# Variable, voltage-dependent, blocking effects of nitrendipine, verapamil, diltiazem, cinnarizine and cadmium on adrenomedullary secretion

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**1** Catecholamine release from cat adrenal glands perfused at a high rate (4 ml $min^{-1}$) at 37°C with modified Krebs solutions lacking Ca and containing 1.2 mM K (hyperpolarizing solution) or 118 mM K (depolarizing solution) was triggered by 10-s pulses of Ca (0.5 mM) in the presence of 118 mM K. Hyperpolarized glands released 1280 ± 135 ng per pulse and depolarized glands 831 ± 98 ng per pulse ($n = 29$).

**2** While the dihydropyridine Ca channel blocker nitrendipine inhibited secretion in hyperpolarized glands with an $IC_{50}$ of 214 nM, in depolarizing conditions the drug was much more potent ($IC_{50} = 0.99$ nM). In contrast, the inorganic Ca channel blocker cadmium inhibited secretion with the same potency both in hyperpolarized or depolarized glands.

**3** Cinnarizine, diltiazem and verapamil exhibited intermediate degrees of voltage-dependence in blocking secretion. The $IC_{50}$ ratios between hyperpolarized and depolarized glands were 215, 36, 19, 8 and 0.76 respectively for nitrendipine, cinnarizine, diltiazem, verapamil and cadmium. Because the experimental design (strong depolarization in the absence of Ca) favours the highest opening probability of Ca channels, it seems that these drugs bind preferentially to their receptors when these channels are in their open state.

**4** Variable voltage-dependent effects of the five Ca channel blockers on adrenomedullary catecholamine release suggests different sites and mechanisms of action on, or near L-type Ca channels in chromaffin cells. In addition, these findings might help to explain why these drugs exhibit tissue selectivity and why they act differently in normal polarized as compared to ischaemic depolarized cells.

## Introduction

Since both the early activation and inactivation of Ca uptake into K-depolarized chromaffin cells proceeds in a few seconds, and closely parallels the activation and inactivation of catecholamine release (Artalejo *et al.*, 1986) it seems plausible that voltage-dependent Ca channels located on the plasma membrane constitute an early step in the modulation of cytosolic processes leading to secretion (Artalejo *et al.*, 1988a). These channels were first analysed electrophysiologically by Neher's group (Fenwick *et al.*, 1982) and are likely to be of the L-type (Nowicky *et al.*, 1985) since both Ca uptake and secretion are very sensitive to dihydropyridine (DHP) Ca channel activators such as Bay K 8644, CGP28392 or (+)-Sandoz 202-791 (García *et al.*, 1984; Montiel *et al.*, 1984; Ladona *et al.*, 1987; Fonteriz *et al.*, 1987) and blockers such as (+)-PN200-110, nitrendipine, nifedipine, niludipine or nimodipine (Ceña *et al.*, 1983; Montiel *et al.*, 1984; Ladona *et al.*, 1987; Gandia *et al.*, 1987).

Voltage-dependence of DHPs, phenylalkylamines and benzothiazepines is now fairly well established, chiefly on the basis of electrophysiological data from Ca current kinetic analysis (Lee & Tsien, 1983; Sanguinetti & Kass, 1984; Bean, 1984; Uehara & Hume, 1985; Gurney *et al.*, 1985; Sanguinetti *et al.*, 1986; Rane *et al.*, 1987); functional data to define the importance of such effects are therefore clearly needed. These data may show: (a) the relevance of the voltage-dependence of the major categories of Ca channel blockers; (b) the mechanism of action of the Ca channel blockers and the fundamentals of their

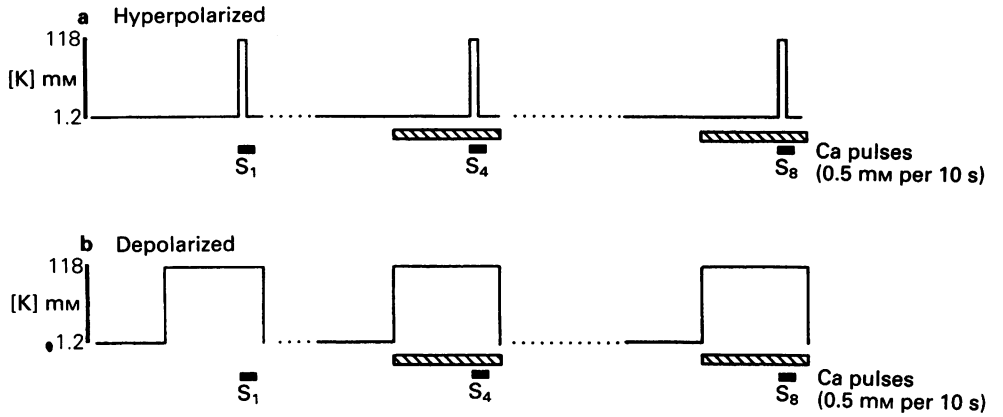


**Figure 1** Scheme representing the protocol used to study the voltage-dependent effects of Ca channels blocking drugs on catecholamine release from hyperpolarized (a) or depolarized (b) perfused cat adrenal glands. Hatched bar indicates blocking drug. See Methods for further detail.

tissue selectivity according to variable membrane resting potential of several excitable cells in physiological or ischaemic (depolarized) conditions; (c) whether the electrophysiological findings have a functional correlate as far as voltage-dependence is concerned; and (d) by means other than radioligand binding studies in isolated membranes, whether different degrees of voltage-dependence in blocking a physiological function suggest different binding sites on the Ca channel complex where the four categories of drugs (nitrendipine, cinnarizine, diltiazem and verapamil) might exert their blocking effects through different mechanisms. In addition, in view of the fact that these drugs are widely used to treat cardiovascular diseases and their therapeutic indications are being extended to many other diseases, knowledge of whether they affect other non-cardiovascular physiological functions (for instance, catecholamine release) at pharmacologically relevant concentrations has clinical implications.

In the light of these observations, it seemed appropriate to perform the experiments presented here demonstrating that the various categories of Ca channel blockers exhibit different degrees of voltage-dependence in inhibiting catecholamine release from perfused cat adrenal glands.

## Methods

Cat adrenal glands were isolated and prepared for retrograde perfusion at a rate of 4 ml $min^{-1}$, at 37°C with Krebs-HEPES solution pH 7.4, continuously bubbled with pure $O_2$ and having the following composition (mM): NaCl 144, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.5, HEPES 10 and glucose 11. Glands were initially perfused with this solution for 60 min to allow their equilibration with the experimental conditions.

### *Experimental design*

After 1 h, two glands from the same cat were stimulated to secrete catecholamines with a four-step pattern of perfusing solutions (see protocols in Figure 1): (1st) both glands were perfused for 20 min with 1.2 K/0 Ca (a Krebs-HEPES solution containing 1.2 mM K and no Ca); (2nd) one gland was perfused for an additional 10 min with this solution (hyperpolarized gland) and the contralateral (depolarized gland) was perfused with 118 K/0 Ca (a Krebs-HEPES solution in which 118 mM NaCl was substituted by 118 mM KCl, and lacking Ca); (3rd) 10 s with 118 K/0.5 Ca (secretion test pulse) to allow a brief Ca entry and secretion in both glands; and (4th) 50 s back to 1.2 K/0 Ca solution (hyperpolarized) or 118 K/0 Ca solution (depolarized) to collect the secreted catecholamines in 10 s periods. These stimulation cycles were repeated 8 times in each pair of glands at 30 min intervals.

Control glands were exposed to vehicle only (ethanol). Drugs were tested by adding increasing concentrations ($10^{-10}$–$10^{-5}$ M) during steps 2, 3 and 4 of each stimulation cycle; they were absent during the first step (20 min in 1.2 K/0 Ca). These protocols are similar to those used when measuring Ca current inactivation by conditioned prepulses (Fenwick *et al.*, 1982): we tried to promote the inhibition of catecholamine release by various Ca channel blockers by pre-exposing the adrenals to them and high K in the absence of Ca (118 K/0 Ca), before initiating release by addition of external Ca (0.5 mM for 10 s), and comparing this release with secretion obtained in hyperpolarized glands.

*Catecholamine assay and quantitation of results*

Catecholamines present in each 10-s collection sample (600 $\mu$l of medium acidified with perchloric acid to 0.05 N) during the last 10 s of the 2nd stimulation step (basal secretion) and steps 3 and 4, were measured fluorometrically according to Shellenberger & Gordon (1971). Data were expressed as ng of total catecholamine release per Ca test pulse and are means $\pm$ their s.e. To calculate the concentrations of drugs blocking the secretory process by 50%, the sigmoid inhibition curves were converted into straight lines by plotting values in the ordinates as log $(y/100 - y)$; the intercepts with the abscissae ($y = 0$) gave the $IC_{50}$ s.

## Results

*Profile of secretory responses evoked by Ca test pulses*

Figure 2 shows the time-course of secretion evoked by 10-s Ca test pulses in hyperpolarized (a) and depolarized (b) adrenal glands. Secretion increased immediately to reach a peak, and then quickly declined to basal resting levels within the 50-s period following the Ca test pulse. The initial secretory Ca tests gave figures of 1280 $\pm$ 135 and 831 $\pm$ 98 ng per pulse ($n = 29$) for hyperpolarized and depolarized adrenals respectively.

To test the effects of increasing concentrations of drugs on secretion in the same gland to which Ca test pulses were sequentially applied, it was of utmost importance that those pulses evoked similar secretory responses throughout the experiment. In Figure 2c, catecholamine releases were normalized as % of the individual secretion obtained in $S_3$ for each experiment. We realised in earlier experiments that secretory responses were fairly stable from the third Ca pulse onwards; therefore, the amounts of catecholamine released in each experiment in $S_3$ were taken as 100% and from $S_4$ to $S_8$ was expressed as % of $S_3$.

*Effects of nitrendipine on catecholamine release evoked by Ca pulses in hyperpolarized and depolarized adrenal glands*

Figure 3a shows that nitrendipine caused a concentration-dependent blockade of Ca-evoked catecholamine release in both, hyperpolarized and depolarized adrenal glands. However, the drug could not fully inhibit secretion even at the high concentrations of 1 $\mu$M if given to glands preperfused with 1.2 K/0 Ca solution. In contrast, 10 nM nitrendipine caused 80% blockade in depolarized glands. The

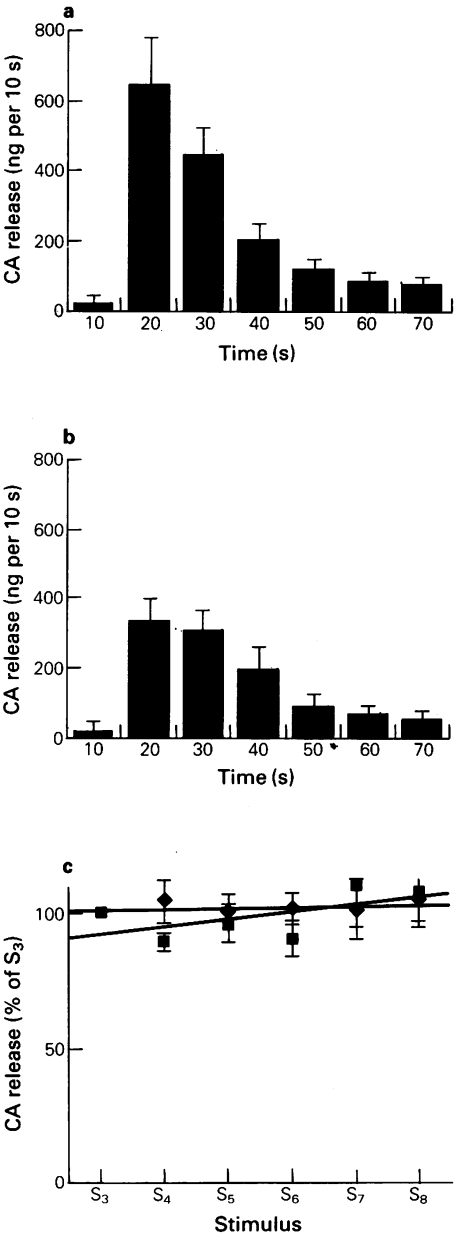


**Figure 2** Time-course of catecholamine release evoked by reintroducing Ca (0.5 mM for 10 s) into glands preperfused with hyperpolarizing (1.2 K/0 Ca) (a) or depolarizing (118 K/0 Ca) (b) solutions. Data are taken from the third test pulse and represent means of 29 paired experiments; s.e. shown by vertical bars. In (c) secretory responses obtained in 5 subsequent Ca pulses ($S_4$ to $S_8$) are expressed as a % of those obtained in $S_3$; data are means $\pm$ s.e. of 12 paired experiments: (■) hyperpolarized; (◆) depolarized.

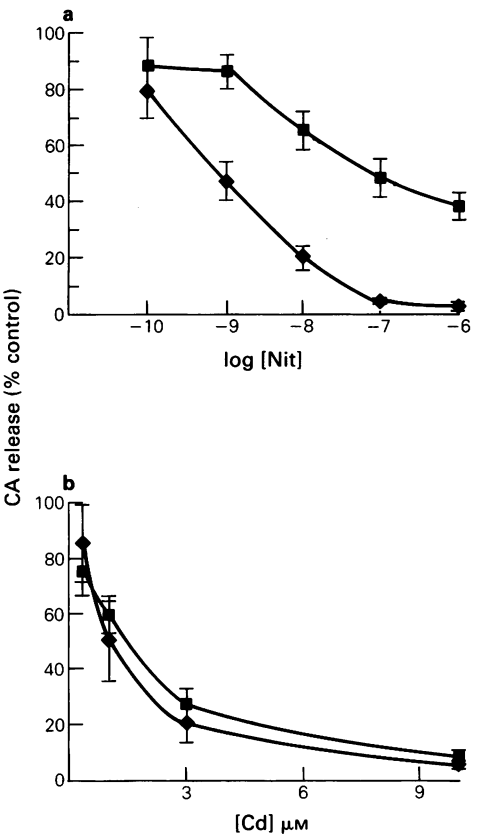


**Figure 3** Concentration-dependent blockade by nitrendipine (a) and Cd (b) of catecholamine release evoked by 10-s Ca pulses given at 30 min intervals to glands pre-perfused with 1.2 K/0 Ca (hyperpolarizing) or 118 K/0 Ca (depolarizing) solutions. Increasing concentrations of nitrendipine (a) or Cd (b) were given 10 min before and during stimulations with 118 K/0.5 Ca (Ca pulses). See Methods for further detail of the experimental protocol. Secretion data in ng were normalized to 100% ($S_3$) and are expressed as % of catecholamine release obtained in $S_3$ (ordinates); they are expressed as means of 10 paired experiments, s.e. shown by vertical bars: (■) hyperpolarized; (◆) depolarized.

$IC_{50}$ in hyperpolarization was 214 nM, as opposed to depolarization which was 0.99 nM.

*Cadmium effects on secretion*

Various inorganic Ca channel blockers inhibit K-evoked catecholamine release in perfused cat adrenal glands; one of the most potent is Cd (Gandia *et al.*, 1987). Therefore, we compared its effects with those of nitrendipine, and determined whether it also exhibited voltage-dependence. In Figure 3b, it can be seen that Cd ions inhibited Ca-evoked secretion equally in both hyperpolarized and depolarized adrenal glands; at 10 μM, secretion was fully inhibited in both types of glands. If anything, the $IC_{50}$ in depolarized glands was slightly greater (1510 nM versus 1150 nM) than in hyperpolarized glands, a result opposite to that obtained with nitrendipine.

*Effects of cinnarizine, diltiazem and verapamil on catecholamine release from hyperpolarized and depolarized glands*

Ca channel blocking drugs belonging to chemical classes different from DHPs, such as cinnarizine, diltiazem and verapamil, also inhibited Ca-evoked secretion in a concentration-dependent manner; however, they behaved quite differently. For instance, little blockade was observed with verapamil and diltiazem at 1 μM in hyperpolarized glands, but cinnarizine caused over 60% inhibition of secretion. Depolarization enhanced the blocking effects of the three chemicals, though it was more pronounced with cinnarizine, followed by diltiazem and last by verapamil.

It is worth comparing the $IC_{50}$ ratios required to block secretion from hyperpolarized or depolarized glands of the five compounds tested (Table 1). This ratio was highest for the DHP (215), followed by piperazine (36), the benzothiazepine (19) and lastly the benzylalkylamine (8); Cd showed no voltage-dependency.

## Discussion

The experiments presented here show that inhibition by various Ca channel blockers of adrenomedullary

**Table 1** Variable voltage-dependent effects of five Ca channel blockers on adrenomedullary secretion

| *Drug* | n | $IC_{50}$ 1.2 K | $IC_{50}$ 118 K | $\frac{IC_{50}\,1.2\,K}{IC_{50}\,118\,K}$ |
|---|---|---|---|---|
| Nitrendipine | 10 | 214 | 0.99 | 215 |
| Cinnarizine | 8 | 189 | 5.23 | 36 |
| Diltiazem | 11 | 2040 | 106 | 19 |
| Verapamil | 7 | 1770 | 217 | 8 |
| Cadmium | 8 | 1150 | 1510 | 0.76 |

Catecholamine release from perfused cat adrenal glands was evoked by a test pulse of 118 mM K, 0.5 mM Ca for 10 s, after pretreatment with hyperpolarizing (1.2 K/0 Ca) or depolarizing (118 K/0 Ca) solutions for 10 min. $IC_{50}$ is the drug concentration that inhibits by 50% the initial control release. Values are expressed in nM; $n$ = number of paired experiments.

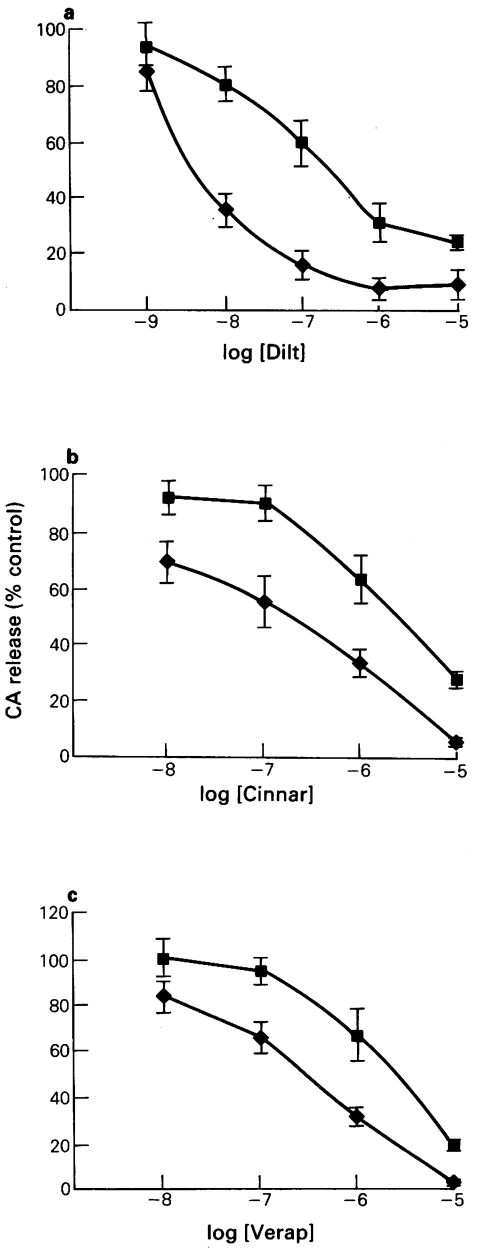


**Figure 4** Effects of diltiazem (Dilt) (a), cinnarizine (Cinnar) (b) and verapamil (Verap) (c) on catecholamine release from cat adrenal glands evoked by test pulses of 118 mM K/0.5 mM Ca for 10 s, after pre-perfusion with hyperpolarizing (1.2 K/0 Ca) (■) or depolarizing (118 K/0 Ca) (◆) solutions for 10 min. Data are normalized to percentages of secretion obtained in $S_3$ in the absence of drug (100%), and represent means of 6–8 paired experiments; vertical bars show s.e.

catecholamine release evoked by brief pulses of Ca is strongly dependent on whether the adrenal glands were exposed to the drugs under hyperpolarizing or depolarizing conditions. Our present studies are based on experimental designs similar to those used by electrophysiologists when measuring Ca current inactivation by conditioned prepulses (Fenwick *et al.*, 1982). There are several new features compared to our previous experiments in which we could not discriminate the voltage-dependent effects of these drugs (Ceña *et al.*, 1983; García *et al.*, 1984; Ladona *et al.*, 1987; Gandia *et al.*, 1987). First, tissues were exposed to drugs under hyperpolarizing (1.2 K/0 Ca) or depolarizing (118 K/0 Ca) conditions with the intention of manipulating the affinities of the drugs for their receptors as a function of the membrane potential. It is now established that the effects of various Ca channel blockers on the heart (Sanguinetti & Kass, 1984; Bean, 1984; Uehara & Hume, 1985; Gurney *et al.*, 1985; Sanguinetti *et al.*, 1986; Rane *et al.*, 1987) and the intact adrenal medulla (Artalejo *et al.*, 1988b) are dependent on membrane potential. Second, secretion was triggered by a brief Ca test pulse applied to adrenals previously perfused with Ca-free solutions containing low (1.2 mM) or high (118 mM) K. Under these conditions, drugs will bind to their receptors with variable affinities, depending on the membrane potential. Thirdly, the duration of the Ca pulses was very short (10 s) in order to decrease the opportunity for drugs to change their equilibrium receptor binding during the pre-test 10-min perfusion period. This is important because the onset of blockade of cardiac Ca currents by DHP is completed in about 10 s (Sanguinetti & Kass, 1984). It is, therefore, clear that in our earlier experiments (Gandia *et al.*, 1987) we were really measuring effects of drugs mainly in depolarized cells since the test secretory pulse lasted over 60 s and the preperfusion period with drugs was performed only under resting (5.9 mM K) conditions. With those earlier protocols we could not determine the possible voltage-dependence of the blocking effects of various drugs. However, in the present studies, we observed that secretory responses in hyperpolarized glands were very resistant to blockade by the four drugs tested, while those obtained in depolarized glands were highly sensitive to the drugs.

The question arises as to whether the observed secretory patterns might be correlated with events taking place in membrane Ca channels; the observation that Cd, a potent inorganic blocker of L-type Ca channels (Nowicky *et al.*, 1985) inhibited both secretory responses, supports this view. Further evidence favouring the correlation between both events comes from the observation that nitrendipine binds with much higher affinity to cat adrenomedullary tissues, and blocks much more efficaciously $^{45}Ca$

uptake into and catecholamine release from such tissues, in pre-depolarized than in pre-hyperpolarized conditions (Artalejo *et al.*, 1988b). It is therefore plausible to explain the present results on the basis of the voltage-dependence of nitrendipine, cinnarizine, diltiazem and verapamil in binding to their receptors on L-type Ca channels.

Voltage-dependent Ca channels are dynamic molecular entities that undergo constant transitions between resting, open or inactivated states (Hess *et al.*, 1984). Since sustained depolarization in the absence of Ca (for instance, 10 min in 118 K/0 Ca) causes little inactivation of chromaffin cell Ca channels (Sala *et al.*, 1986; Artalejo *et al.*, 1987), it seems that nitrendipine, cinnarizine, diltiazem and verapamil preferentially bind to their receptors when Ca channels are in their open state. This conclusion is consistent with the interpretation of the mechanism of action of DHPs based on electrical recordings of Ca currents, showing that the drugs display an increased affinity for the L-type channel as the holding potential of the cell is increased (Uehara & Hume, 1985; Cohen & McCarthy, 1987); however, it differs from other reports suggesting that nitrendipine binds tightly to the inactivated state of the channel (Bean, 1984; Sanguinetti & Kass, 1984).

Radioligand binding studies have generated a model for three separate, but allosterically linked binding sites for 1,4-DHPs (nitrendipine), phenylalkylamines (verapamil) and benzothiazepines (diltiazem) (Triggle & Janis, 1987). However, this model has only limited and controversial functional support in non-secretory tissues (DePover *et al.*, 1983; Spedding, 1983; Yousif & Triggle, 1985). Here, we have shown marked differences in potencies for blockade of secretion in hyperpolarized or depolarized glands; voltage-dependent blocking effects are prominent for DHPs, followed by cinnarizine and diltiazem, small for verapamil and absent for the inorganic Ca channel blocker Cd. Though these findings suggest different sites and mechanisms of action of the five agents, they might also be explained by different polarity of the molecules that limit their access to specific receptors sites in depolarizing or hyperpolarizing conditions through the inner or outer part of the channel.

Whatever the mechanism, different degrees of voltage-dependence might help to explain why verapamil and diltiazem are much more cardioactive compounds than DHPs, or why cinnarizine may have some selectivity for certain vascular beds, as well as their different effects on healthy polarized, or depolarized ischaemic tissues. Our data suggest that at clinically relevant concentrations, DHPs might interfere with adrenomedullary secretion; this could constitute an additional contributory mechanism to the well known vasodilator effects of the drugs that make them very useful in treating hypertensive patients.

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