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Inhibition of [^{3}H]-U69593 binding and the cardiac effects of U50,488H by calcium channel blockers in the rat heart

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1 The calcium channel blockers (CCBs), nifedipine, nicardipine, diltiazem and verapamil, were used to displace the binding of [^{3}H]-U69593 ((5a,7a,8b)-(+)-N-methyl-N-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]dec-8-yl)-benzeneacetamide), a specific κ-opioid agonist, in the rat cardiac sarcolemma. The CCBs competed with the binding of [^{3}H]-U69593 (4 nM) in a dose-dependent manner. The displacing potency of verapamil was 55 times greater than that of nifedipine.

2 The effects of two CCBs, verapamil and nifedipine, on the arrhythmogenic action of κ-receptor stimulation by a specific κ-receptor agonist, U50,488H (*trans*-($\pm$-3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl] cyclohexyl) benzeacetamide methanesulphonate), were also studied in the rat isolated perfused heart. U50,488H 80–800 nmol dose-dependently induced arrhythmias, which were completely abolished by a selective κ-receptor antagonist, nor-BNI (nor-binaltorphimine,17,17′-(dicyclopropylmethyl)-6,6′,7,7′-6,6′-imino-7,7′-binorphinan-3,4′,14, 14′-tetrol), at 100 nmol. The arrhythmogenic effect was also attenuated by both verapamil and nifedipine in a dose-dependent manner. The ED_{50} values for verapamil and nifedipine were 2.75 and 63.7 nmol, respectively. The antiarrhythmic potencies of these two CCBs were correlated to their displacing potencies and inversely related to their well known potencies in inhibiting transmembrane Ca^{2+} influx in the cardiac muscle.

3 Measurement of $[Ca^{2+}]_i$ in the absence of free extracellular Ca^{2+} by a spectrofluorometric method, with fura-2 as Ca^{2+} indicator, showed that U50,488H 5×10^{-5} M slowly increased $[Ca^{2+}]_i$ in single ventricular myocytes and this effect was abolished by pretreatment with nor-BNI (5 μM), or ryanodine (5 μM). Verapamil 1 and 10 μM abolished the effect of U50,488H in 37.5% (3 out of 8) and 100% (12 out of 12) of the cells studied, respectively. On the other hand, nifedipine 10 and 100 μM had no effect at all. Neither verapamil nor nifedipine exerted any significant effect on the caffeine-induced Ca^{2+} transient.

4 The observations suggest that CCBs may inhibit the actions of κ-receptor stimulation at the level of the κ-receptor.

Keywords: Ca^{2+} channel blocker; rat isolated heart; arrhythmias; κ-opioid receptor; receptor binding assay; ventricular myocytes; intracellular calcium

Introduction

It is well established that calcium channel blockers (CCBs) inhibit Ca^{2+} influx and this effect is thought to be responsible for their antiarrhythmic actions (Fleckenstein, 1977; Opie & Thandroyen, 1983). However, at higher concentrations (>1 μM) their actions are not confined to Ca^{2+} influx only. In this higher concentration range, CCBs especially verapamil and its methoxy derivative, D600, can inhibit the binding and modify the effects of stimulation of adrenoceptors (Karliner *et al*., 1982; Nayler *et al*., 1982; Kushida *et al*., 1990), and muscarinic receptors (Cavey *et al*., 1977; Quist & Satumtira, 1987) in the heart. CCBs also inhibit the binding of [^{3}H]-U50,488H (*trans*-($\pm$)-3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl] cyclohexyl) benzeacetamide methanesulphonate), a selective κ-receptor agonist (Niwa *et al*., 1992) and [^{3}H]-naloxone (Fairhurst *et al*., 1980; Simpkins *et al*., 1986), as well as altering the effects of κ-receptor stimulation (El-Sharkawy *et al*., 1991). The observations suggest that CCBs may inhibit the binding of κ-opioid receptors and thus alter the effects of its stimulation.

κ-Opioid receptors have been shown to exist in the heart by receptor binding assay (Ventura *et al*., 1989; Tai *et al*., 1991) and physiological studies (Tai *et al*., 1992; Ventura *et al*., 1992). Accumulating evidence suggests that stimulation of cardiac opioid receptors may contribute to arrhythmias induced by myocardial ischaemia-reperfusion (Lee & Wong, 1986; Machuganska *et al*., 1987; Boachie-Ansah *et al*., 1989; McIntosh *et al*., 1992) and that the κ-receptor is the most likely opioid receptor subtype involved (Sitsapesan & Parratt, 1989; Wong *et al*., 1990). Previous studies have shown that cardiac κ-receptor stimulation increases the formation of inositol (1,4,5)-trisphosphate (IP_3), which mobilizes Ca^{2+} from the intracellular stores, leading to an increase in intracellular free calcium ($[Ca^{2+}]_i$) (Ventura *et al*., 1992; Wong *et al*., 1995). The elevated $[Ca^{2+}]_i$ may be responsible for arrhythmias because (a) addition of sufficient Ca^{2+} to elevate $[Ca^{2+}]_i$ leads to arrhythmias in single isolated myocytes (Thandroyen *et al*., 1991) and (b) IP_3 transduction pathway inhibitors, such as aminoglycoside antibiotics suppress ventricular arrhythmias during ischaemia-reperfusion (Du *et al*., 1995).

In the present study, we determined the effects of CCBs on the binding of a κ-agonist and correlated these effects with the actions of κ-receptor stimulation in the heart. The results support the hypothesis that CCBs inhibit the binding of the κ-receptor, and thus reduce the effects of κ-receptor stimulation. Part of the results has been presented in a preliminary form (Zhang *et al*., 1995).

Methods

Preparation of cardiac sarcolemma

Cardiac sarcolemma was prepared by the hypotonic shock-LiBr treatment as described previously (Ventura *et al*., 1989). Male Sprague Dawley rats (190–210 g) were decapitated with a guillotine. The hearts were quickly removed and placed in ice-cold 10 mM Tris-HCl buffer (pH 7.4). The ventricles were

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washed thoroughly, cut into small pieces, and then homogenized in an Ultra Turrax homogenizer (velocity setting 8 for 15 s) in 20 ml of 10 mM Tris-HCl buffer (pH 7.4), containing 1 mM EDTA. The homogenate was filtered through four layers of cheesecloth and centrifuged at 1000 *g* for 10 min. The sediment was suspended in 20 ml of 10 mM Tris-HCl buffer (pH 7.4), stirred for 30 min and centrifuged at 1000 *g* for 10 min. This procedure was repeated twice more, first by suspending the sediment in 10 mM Tris-HCl buffer (pH 8.0), and then in 10 mM Tris-HCl buffer (pH 7.4). The resulting sediment was extracted for 45 min with 40 ml of 0.4 M lithium bromide containing 10 mM Tris-HCl buffer (pH 7.4) and centrifuged at 1000 *g* for 10 min. The sediment was again washed and stirred for 20 min in 10 mM Tris-HCl buffer (pH 7.4), and then centrifuged at 1000 *g* for 10 min. It was further purified by extracting for 30 min in 40 ml of 0.6 M KCl containing 10 mM Tris-HCl buffer (pH 8.0) and centrifuged at 1000 *g* for 10 min. This sediment was resuspended in 40 ml of 10 mM Tris-HCl buffer (pH 7.4) and again centrifuged at 1000 *g* for 10 min. The final pellet was suspended in 10 mM Tris-HCl buffer (pH 7.5) and frozen at −70°C. All procedures were carried out at 0°C–4°C. Protein was determined according to the method of Lowry *et al.* (1951), with bovine serum albumin (BSA) as a standard.

Receptor binding assay

Receptor binding assays were performed as described previously (Tai *et al.*, 1991). Briefly, the equilibrium binding assays of [^{3}H]-U69593 ((5a,7a,8b)-(+)-N-methyl-N-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]dec-8-yl)-benzeneacetamide) were performed at 25°C for 45 min by incubating 0.5 mg of the sarcolemmal membrane proteins in 1 ml of 50 mM Tris-HCl buffer (pH 7.4). After incubation the mixture was immediately cooled in an ice bath, which terminated the reaction. Bound and free radioligands were separated by rapid filtration in vacuum through Whatman GF/B filters. The filters were washed 3 times rapidly with 4 ml aliquots of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and transferred to scintillation vials. Four millilitres of hydrophilic scintillation cocktail (PPO, POPOP, Toluene, Triton X-100) was added and the vials were allowed to stand over-night. Radioactivity was counted by a model Tri Carb 2000 CA liquid scintillation analyser. Nonspecific binding of [^{3}H]-U69593 was determined in the presence of 20 μM U69593. Specific binding was defined as the difference between the radiolabel bound in the absence and presence of U69593 (20 μM). Displacement experiments of [^{3}H]-U69593 (4 nM) were performed with 9 concentrations of cold ligands. The dissociation constant of inhibitor (K_i) was calculated according to the equation: $K_i = IC_{50}/(1+[L]/K_D)$. For calculation of K_i values, saturation experiments with [^{3}H]-U69593 were carried out in the concentration range 0.25–20 nM. The equilibrium dissociation constant (K_D) and the maximum binding capacity (B_{max}) obtained from Scatchard plot analysis were 7.4 ± 1.2 nM and 139 ± 25 fmol mg^{-1} protein, respectively, which were comparable to those obtained by Ventura *et al.* (1989). All experiments were repeated six times in triplicate and the values represented the means of six determinations. The error of the triplicate determinations was less than 10%. The binding data were analysed by and the values of K_D, B_{max} and IC_{50} obtained from the EBDA/LIGAND computer programme.

Langendorff perfused heart of the rat

Male Sprague Dawley rats weighing 190–210 g were decapitated with a guillotine. The heart was removed immediately and mounted to a Langendorff apparatus. The procedure previously described (Lee & Wong, 1986) was adopted. Briefly, the heart was perfused through the aorta with a Krebs-Ringer solution (pH 7.4) aerated with 95% O_2 and 5% CO_2 under a constant pressure (60 mmHg) and a flow rate of 8–10 ml min^{-1}. The temperature of the heart was maintained at 34°C. The first 10 min of perfusion allowed the heart to stabilize and any heart exhibiting cardiac arrhythmias during this period was discarded. Drugs were injected into the heart by separate cannulae leading directly into the aorta. U50,488H was infused in 2 ml over 10 min, while verapamil, nifedipine and nor-BNI (nor-binaltorphimine,17,17′-(dicyclopropylmethyl)-6,6′,7,7′-6,6′-imino-7,7′-binorphinan-3,4′,14,14′-tetrol) was 1 ml over 5 min. Based on our previous studies (Wong *et al.*, 1990; Xia *et al.*, 1994), the doses of U50,488H were 80, 400 and 800 nmol. The dose of nor-BNI used was 100 nmol based on the dose of another κ-antagonist, MR2266, used in previous studies (Wong *et al.*, 1990; Xia *et al.*, 1994) and by trial and error in our preliminary experiment. For CCBs the doses used were adopted from previous studies (Thandroyen, 1982; Opie & Thandroyen, 1983; Winslow *et al.*, 1983), which were 1, 3, and 10 nmol for verapamil and 10, 30 and 50 nmol for nifedipine. The antiarrhythmic effects of verapamil at 10 nmol and nifedipine at 50 nmol reached a plateau. When the concentration was calculated, taking into account the flow rate, the final concentrations ranged from 10^{-7}–10^{-6} M for verapamil and 10^{-6}–5×10^{-6} M for nifedipine. They were administered 10 min before U50,488H 400 nmol. The ECG was continuously monitored with a positive electrode hooked into the apex of the heart and a negative electrode at the aorta.

Nifedipine was dissolved in 1 ml of dimethyl sulphoxide (DMSO) at a concentration of 0.1%. So the final concentration of DMSO in the perfusate was 0.002–0.0025%. Perfusion of DMSO at this concentration had no effect on the cardiac rhythm.

Measurements of $[Ca^{2+}]_i$

Ventricular myocytes were isolated from the hearts of male Sprague-Dawley rats (190–210 g) using a collagenase perfusion method described previously (Dong *et al.*, 1993). Immediately after decapitation, the hearts were rapidly removed from rats and perfused in a retrograde manner at a constant flow rate (10 ml min^{-1}) with oxygenated Joklik modified Eagle's medium supplemented with 1.25 mM $CaCl_2$ and 10 mM HEPES, pH 7.2, at 37°C for 5 min followed by 5 min in the same medium without Ca^{2+}. Type I collagenase was added to the medium to a concentration of 125 u ml^{-1} with 0.1% (w/v) BSA. After 35–45 min of perfusion with medium containing collagenase, the atria were discarded and the ventricular tissue was dissociated by shaking in the same oxygenated collagenase solution for 5 min at 37°C. The ventricular tissue was cut into small pieces with a pair of scissors followed by stirring with a glass rod for 5 min, which separated the ventricular myocytes from each other. The residue was filtered through 250 μm mesh screens, sedimented by centrifugation at 100 *g* for 1 min and resuspended in fresh Joklik solution with 2% BSA. More than 70% of the cells were rod-shaped and not trypan-blue permeable. Ca^{2+} concentration of the Joklik solution was increased gradually to 1.25 mM in 30 min.

Ventricular myocytes were incubated with fura-2/AM (4 μM) in Joklik solution supplemented with 1.25 mM $CaCl_2$ for 25 min. The unincorporated dye was removed by washing the cells twice in fresh incubation solution. The loaded cells were kept at room temperature (24°C–26°C) for 60 min before measurements of $[Ca^{2+}]_i$ to allow the fura-2/AM in the cytosol to de-esterify. Then, the extracellular Ca^{2+} of loaded myocytes was removed by washing with Joklik solution.

The ventricular myocytes loaded with fura-2/AM were transferred to the stage of an inverted microscope (Nikon) in a superfusion chamber at room temperature. The inverted microscope was coupled with a dual-wavelength excitation spectrofluorometer (Photo Technical International, NJ, U.S.A.). Myocytes were perfused with Ca^{2+}-free buffer (Krebs bicarbonate buffer) containing (mM): NaCl 118, KCl 5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11, with 2 mM EGTA, 1% dialyzed BSA and a gas phase of 95% O_2/5% CO_2. Fluorescent signals obtained at 340 nm (F340) and at 380 nm

(F380) excitation wavelength were stored in computer for data processing and analysis. The F340/F380 ratio was used to represent $[Ca^{2+}]_i$ changes in the myocytes.

The choice of a concentration of U50,488H at 50 μM was based on previous studies in two laboratories (Tai *et al*., 1992; Ventura *et al*., 1992; 1994; Sheng & Wong, 1996). For CCBs the dose used in isolated myocytes was 10^{-5} M according to previous studies also in single isolated myocytes (Lee & Tsien, 1983 Sipido & Wier, 1991; Bassani *et al*., 1992).

Arrhythmia scoring system

Since the arrhythmias in this experimental model were mainly atrial and ventricular ectopic beats, a scoring system modified from previous scoring systems (Curtis & Walker, 1988; Wong *et al*., 1990) with special emphasis on these two types of arrhythmias was employed to enable quantitative comparison. The details of the scoring system are as follows: 0 = no arrhythmias; 1 = occasional premature atrial contraction (OPAC); 2 = frequent PAC (FPAC); 3 = occasional premature ventricular contraction (OPVC) and 4 = frequent PVC (FPVC). Occasional was less than 6 beats min^{-1}, while frequent was six and above. In the present study we found that most hearts that exhibit PAC and/or PVC fell into two categories: 1–4 beats min^{-1} or over 10 beats min^{-1}. In the present study we used 6, a number between 1–4 and 10, to distinguish occasional and frequent.

Drugs and chemicals

The following substances: U69593, U50,488H, nifedipine, nicardipine, diltiazem, verapamil, fura-2/AM, type I collagenase, ryanodine were purchased from Sigma Chemicals Co. (U.S.A.). Nor-BNI was from Tocris Cookson Ltd (U.K.). [^{3}H]-U69593 (47.4 Ci $mmol^{-1}$) and [^{3}H]-nitrendipine (79.4 Ci $mmol^{-1}$) were purchased from New England Nuclear (U.S.A.). Fura-2/AM, nifedipine and nicardipine were dissolved in DMSO and other chemicals were dissolved in distilled water.

Statistical analysis

Data are expressed as mean ± s.e.mean. For the analysis of effect of drug, the non-parametric Kruskall-Wallis test was employed. For evaluating the difference in the incidence of arrhythmias, Chi-square test was used.

Results

Effects of CCBs on [^{3}H]-U69593 binding in the rat cardiac sarcolemma

Four CCBs, nifedipine, nicardipine, diltiazem and verapamil, were used to displace the binding of [^{3}H]-U69593 in the rat cardiac sarcolemma. The specific binding of [^{3}H]-U69593 was inhibited significantly by these four CCBs in a dose-dependent manner (Figure 1). The IC_{50} (μM) values of nifedipine, nicardipine, diltiazem and verapamil were 152 ± 20, 7.2 ± 2.0, 3.8 ± 0.9 and 2.3 ± 0.3 ($n = 6$ in all groups), while the K_i (μM) values of the respective CCBs were 82 ± 11, 4.6 ± 1.3, 2.5 ± 0.6 and 1.5 ± 0.3 ($n = 6$). The K_i value of nifedipine was 55 times that of verapamil; the difference was statistically significant.

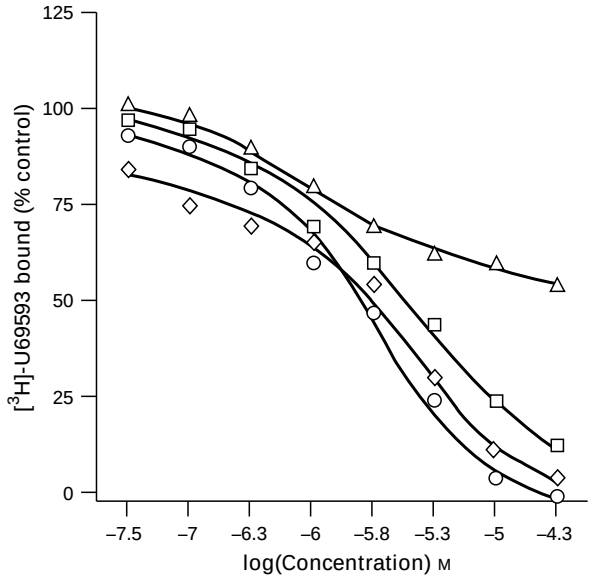

Figure 1 Inhibition of [^{3}H]-U69593 binding to the rat cardiac sarcolemma by verapamil (○), diltiazem (◇), nicardipine (□) and nifedipine (△). The concentration of [^{3}H]-U69593 used was 4 nM. Values shown are mean ± s.e.mean of 6 experiments.

Table 1 Effect of verapamil and nifedipine on U50,488H-induced arrhythmias in the rat isolated perfused heart

	n	*Arrhythmias*	*OPAC*	*FPAC*	*OPVC*	*FPVC*	*Arrhythmia score*[1]	*ED_{50} (nmol)*
Control	8	0	0	0	0	0		
U50,488H								
80 nmol	8	4*	1	1	2	0	1.13 ± 0.48	
400 nmol	11	10**	6*	5*	3	1	2.18 ± 0.33	
800 nmol	11	10**	4	4	5*	3	2.82 ± 0.35	
U50,488H 400 nmol after pretreatment with:								
Norbinaltorphimine								
100 nmol	6	0##	0	0	0	0		
Verapamil								
1 nmol	6	5	3	1	1	1	1.83 ± 0.6	
3 nmol	9	5	4	0#	1	0	0.78 ± 0.32##	2.75
10 nmol	9	4#	4	0#	0	0	0.44 ± 0.18##	
Nifedipine								
10 nmol	6	5	2	3	2	0	2.00 ± 0.45	
30 nmol	9	7	3	3	1	1	1.56 ± 0.44	63.7
50 nmol	8	6	3	3	0	0	1.13 ± 0.29#	

[1]Results shown are means ± s.e.mean. Statistically different from the control group without administration of drug at $*P < 0.05$ and $**P < 0.01$. Statistically different from the corresponding group with U50,488H only at $\#P < 0.05$ and $\#\#P < 0.01$, respectively.

It is worth noting that three specific κ-receptor agonists, U50,488H, U69593 and dynorphine$_{1-13}$ had no effect on the binding of the [^{3}H]-nitrendipine, a specific CCB (data not shown).

Effects of U50,488H on cardiac rhythm in the rat isolated perfused heart

U50,488H at a dose range of 80–800 nmol induced atrial and ventricular arrhythmias (Table 1). At 400 and 800 nmol it induced FPVC. The arrhythmogenic effect of U50,488H was dose-dependant (Table 1). Nor-BNI 100 nmol, which itself had no effect on the cardiac rhythm, completely abolished the arrhythmias induced by U50,488H 400 nmol (Table 1).

Effects of verapamil and nifedipine on U50,488H-induced arrhythmias

Only verapamil and nifedipine were used in the study as they were the most and least potent in displacing the binding of [^{3}H]-U69593, respectively. After administration of verapamil at doses of 10 and 50 nmol, both FPAC and PVC induced by U50,488H were abolished. On the other hand only at the dose of 50 nmol did nifedipine abolish PVC (Table 1). The ED_{50} for verapamil was 2.75 nmol, 1/23 of that (63.7 nmol) for nifedipine (Table 1).

Effects of nifedipine and verapamil on U50,488H-induced elevation in [Ca^{2+}]$_i$ in rat single ventricular myocytes in the absence of external Ca^{2+}

In order to test further that CCBs may modify the effect of κ-receptor stimulation, we studied whether verapamil and nifedipine also attenuated the elevation of $[Ca^{2+}]_i$ in response to κ-receptor stimulation in the single isolated ventricular myocytes in the absence of external Ca^{2+}. In agreement with observations in the previous studies (Tai *et al*., 1992; Ventura *et al*., 1992; 1994), perfusion of a quiescent cell with 50 μM U50,488H caused a small, but sustained increase in the resting fura-2 fluorescence ratio with a mean Ca^{2+} transient amplitude of 0.053±0.007 (n=12) (Figure 2a). This effect was abolished by pretreatment with 5 μM nor-BNI (Figure 2c), or after perfusion for 10 min with 5 μM ryanodine (Figure 2b), which is known to deplete Ca^{2+} from its intracellular store (Endo, 1977; Konishi *et al*., 1984). Pretreatment with verapamil 1 and 10 μM abolished the effect of U50,488H in 37.5% (3 out of 8) and 100% (12 out of 12) of the cells studied, respectively (Figure 2d), whereas nifedipine 10 and 100 μM did not produce a significant attenuation of U50,488H-induced elevation in $[Ca^{2+}]_i$. In agreement with the observations of previous studies (Sheu *et al*., 1986; Bassani *et al*., 1992), neither verapamil nor nifedipine exerted any significant effect on the caffeine-induced Ca^{2+} transient (Figure 3).

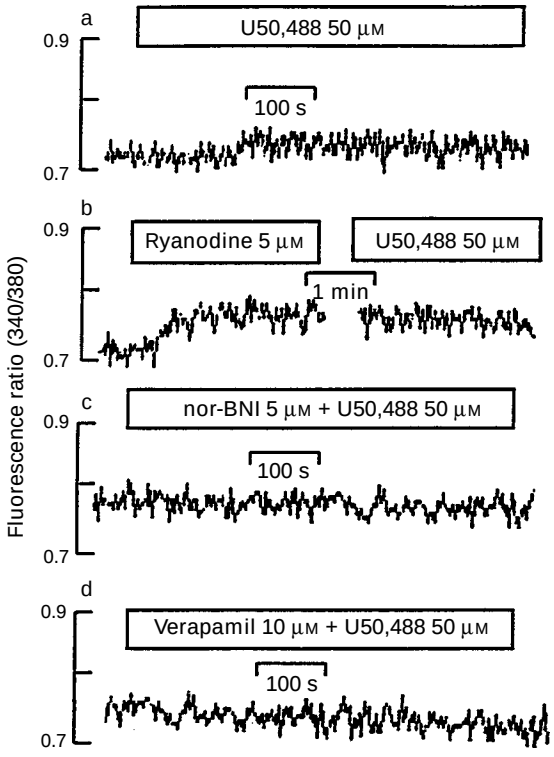

Figure 2 Effect of 5 μM ryanodine (b), 5 μM norbinaltorphimine (c), and 10 μM verapamil (d) on U50,488H-induced $[Ca^{2+}]_i$ (a) in rat fura-2 loaded single ventricular myocytes in the absence of extracellular Ca^{2+}. Each trace is representative of 12 experiments.

Discussion

In the present study we examined the effect of CCBs on the binding and physiological effects of κ-receptor agonists in the heart. Hence, we were able to correlate the binding with the physiological effects resulting from the direct action of a substance with the receptor. This is a clear advantage over previous studies, which looked at the effects of CCBs on either κ-receptor binding or physiological response to κ-receptor stimulation. The important observations from the present study are: (a) CCBs inhibited the binding of [^{3}H]-U69593, a selective κ-agonist, in agreement with the previous observation of Niwa *et al*. (1992); (b) two CCBs, verapamil and nifedipine, inhibited the arrhythmogenic actions of the κ-receptor agonist, U50,488H, in the rat isolated perfused heart with relative potencies directly related to their inhibitory potencies of the κ-receptor binding, but inversely related to their known inhibitory potencies on Ca^{2+} influx across the sarcolemma. The results suggest that the inhibitory effects of the CCBs on the actions of U50,488H may result from inhibition of κ-agonist binding. It is important to note that the inhibitory potency of verapamil was 55 times higher than that of nifedipine, while the antiarrhythmic potency of the former was only 23 times higher based on their ED_{50} values. The observations suggest that at an equipotent concentration, nifedipine may be more effective in blocking the arrhythmogenic effect of κ-receptor stimulation. Whether this implicates an interaction between the CCBs and κ-opioid receptor needs further study.

Since κ-receptor stimulation elevates $[Ca^{2+}]_i$ (Tai *et al*., 1992; Ventura *et al*., 1992; 1994; Wong *et al*., 1995) and an elevation of $[Ca^{2+}]_i$ has been shown to induce cardiac arrhythmias in isolated ventricular myocytes (Thandroyan *et al*., 1991), we also studied the effect of κ-receptor stimulation on $[Ca^{2+}]_i$ after treatment with CCBs. In agreement with the previous observation (Ventura *et al*., 1992), U50,488H induced a small, but sustained increase in $[Ca^{2+}]_i$. The elevation of

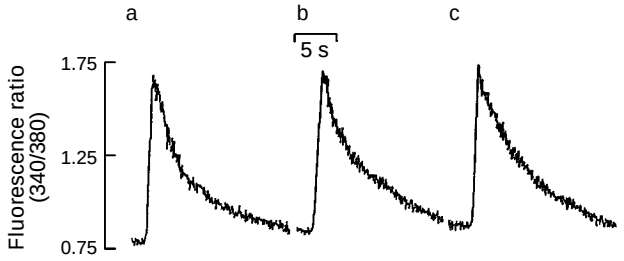

Figure 3 The lack of effect of (b) 10 μM verapamil and (c) 10 μM nifedipine on caffeine-induced cytosolic Ca^{2+} transient in rat fura-2 loaded single ventricular myocytes in the absence of extracellular Ca^{2+}. Caffeine, 10 mM, induced a Ca^{2+} transient with an amplitude of 0.904±0.076 (n=8) (a). The amplitudes of the caffeine-induced Ca^{2+} transients following pretreatment with verapamil (b) and nifedipine (c), were 0.854±0.085 (n=8) and 0.872±0.176 (n=4), respectively.

$[Ca^{2+}]_i$ was shown to result from mobilization of Ca^{2+} from its intracellular pool (Tai *et al*., 1992; Ventura *et al*., 1992). This interpretation was supported by two observations in the present study. Firstly, U50,488H increased $[Ca^{2+}]_i$ in the absence of external Ca^{2+}. Secondly, U50,488H failed to do so following prior treatment with ryanodine. Interestingly, verapamil significantly attenuated the effects of κ-receptor stimulation on the elevation of $[Ca^{2+}]_i$ in the absence of external Ca^{2+}. The effect did not result from a direct action on the intracellular Ca^{2+} store as verapamil exerted no effect on the Ca^{2+} transient induced by caffeine, known to mobilize Ca^{2+} from the sarcoplasmic reticulum. This is in agreement with previous observations that verapamil (10^{-5} M) is ineffective in inhibiting calcium-induced contractions in skinned cardiac fibres or in fibres without slow channels (Fleckenstein, 1977; Nayler & Grinwald, 1981; Miller *et al*., 1985), and does not block the accumulation of ^{45}Ca by isolated sarcoplasmic reticulum in a concentration much higher than that required for such an effect at the cell membrane (Nayler & Szeto, 1972). These results support the suggestion that verapamil inhibits κ-receptor binding, thus attenuating the effects of κ-receptor stimulation on the mobilization of $[Ca^{2+}]_i$. It should be noted that nifedipine did not inhibit the effect of U50,488H on $[Ca^{2+}]_i$. Further studies are needed to explain this latter effect.

It has been shown that verapamil at a concentration range 72–1320 ng ml^{-1} is able to reverse parosyxmal supraventricular tachycardia in man (Singh *et al*., 1983). In the present study the concentrations of verapamil that inhibited arrhythmias induced by κ-receptor stimulation were in the range 36–360 ng ml^{-1} (calculated according to the amount injected in five minutes at a perfusion rate of 10 ml min^{-1}). It is possible that the antiarrhythmic effect of verapamil in man may be due, at least in part, to the inhibition of κ-receptor stimulation.

One major concern of the present study is the high concentrations of U50,488H used. The effects of U50,488H at the concentrations used on the cardiac rhythm in the rat isolated perfused heart and on $[Ca^{2+}]_i$ in the isolated ventricular myocytes were completely abolished by a selective κ-receptor antagonist, nor-BNI, which itself did not produce any effect on the cardiac rhythm. This is in agreement with previous observations, which showed that the effects of U50,488H were antagonized by the κ-receptor antagonists, MR2266 (Wong *et al*., 1990; Tai *et al*., 1992; Xia *et al*., 1994; Sheng & Wong, 1996), MR1452 (Ventura *et al*., 1992; 1994) and nor-BNI (Sheng *et al*., 1997). In addition, the effects of U50,488H on $[Ca^{2+}]_i$ in the ventricular myocyte exhibited tolerance upon chronic treatment with the agonist (Sheng & Wong, 1996), a characteristic feature of a receptor-mediated action. Hence, the effects of U50,488H at the concentration range used in the present study are probably mediated by κ-receptors. The antagonist-reversible actions of U50,488H at 10^{-5} M have also been seen in neural tissues (Clark *et al*., 1986; Baraban *et al*., 1995; Lawrence *et al*., 1995). It is also not uncommon to find that very high concentrations of non-opioid subtances produce a receptor-mediated action. For example, carbachol 10^{-4} M has been shown to increase the force of contraction of the chick atrium (Tajima *et al*., 1987) and Ca^{2+} current in the guinea-pig ventricular myocytes (Gallo *et al*., 1993), which were blocked by atropine and an M_1 muscarinic receptor antagonist, pirenzepine, respectively. Previous binding studies in our laboratory (Jin *et al*., 1995; Zhang *et al*., 1996) showed that in the heart there are numerous κ-receptor binding sites and they consist of the κ_1 and κ_2 subtypes. The κ_1 subtype has low and high affinity sites. It may be possible that U50,488H, a selective κ_1-receptor-agonist, acts at the low affinity site; K_i values of 0.11 and 0.275 μM have been obtained for this site in sarcolemmal preparations (Zhang *et al*., 1996) and crude membrane homogenates of the heart (Jin *et al*., 1995), respectively.

In previous studies it was found that U50,488H inhibited the binding of [^{3}H]-nimodipine, a CCB, in the brain (Gandhi & Ross, 1987; 1988). In the present study we did not obtain any inhibitory effect of U50,488H on the binding of another CCB, [^{3}H]-nitrendipine in the heart. Further studies are needed to address this discrepancy.

In conclusion, the present study has shown that CCBs (a) inhibit κ-receptor binding in the heart and (b) attenuate the effects of κ-receptor stimulation on cardiac rhythm and $[Ca^{2+}]_i$. Both the displacing and antiarrhythmic potencies of two CCBs, verapamil and nifedipine, were inversely related to their known potencies in inhibiting Ca^{2+} influx. The observations suggest that CCBs may inhibit the actions of κ-receptor stimulation at the receptor level. Further studies are needed to elucidate the nature of inhibition of κ-opioid receptor stimulation by CCBs.

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