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Effects of calcium channel blockade in canine saphenous veins after storage at −190°C

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1 Canine saphenous veins were investigated *in vitro* either within 24 h after removal or after storage at −190°C for 4–5 weeks in foetal calf serum containing 1.8 M dimethyl sulphoxide.

2 Contractions and $^{45}Ca^{2+}$ uptake in response to both depolarization and guanfacine were studied in the absence and presence of the calcium channel antagonists diltiazem, verapamil, nifedipine and the two stereoisomers of a 1,4-dihydropyridine derivative, namely the (+)-(S) enantiomer and the (−)-(R) enantiomer of 202-791 (isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2, 6-dimethyl-5-nitro-3-pyridinecarboxylate).

3 Comparison of the data obtained on unfrozen and frozen/thawed veins revealed a good preservation of both contractile responsiveness and $^{45}Ca^{2+}$ uptake mechanisms after storage at −190°C.

4 It is suggested that cryopreservation is a useful technique for storing venous smooth muscle for pharmacological studies.

Introduction

Recently a simple and reliable technique for cryopreservation of isolated blood vessels for pharmacological studies has been described (Müller-Schweinitzer & Tapparelli, 1986; 1987; Müller-Schweinitzer *et al.*, 1986). Using human and canine saphenous veins and canine basilar arteries evidence has been presented that, even after several months of storage at −190°C, important biochemical properties, such as monoamine oxidase activity, endogenous prostaglandin synthesis, catecholamine uptake ($uptake_1$ mechanism), as well as contractile responses to various agonists and the blocking activities of several antagonists at both α-adrenoceptors and 5-hydroxytryptamine (5-HT) receptors are reasonably well maintained.

We now present evidence that, after storage of canine saphenous veins at −190°C, the functional activities and also the calcium uptake processes of both potential-sensitive and receptor-operated calcium channels are well preserved.

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Methods

Storage methods

Canine saphenous veins were obtained from beagle dogs of either sex (7–13 kg), killed by i.v. injection of pentobarbitone (50 mg kg^{-1}) and exsanguination from the femoral arteries. Vein segments of about 15 to 20 mm were distributed into 2 groups. Group 1 consisted of 'unfrozen veins' which were used immediately or after 24 h storage in Krebs-Henseleit solution (mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, EDTA 0.03) gassed with 95% O_2 plus 5% CO_2 at 37°C. The veins of group 2, 'frozen/thawed veins', were placed in 2 ml liquid nitrogen storage ampoules (Gibco AG, Basel, Switzerland) filled with foetal calf serum (FCS) containing 1.8 M dimethyl sulphoxide (DMSO) as a cryoprotective agent. The ampoules were placed in a polystyrol box (11 × 11 × 22 cm) and slowly frozen at a mean cooling rate of about 0.6°C min^{-1} in a freezer maintained at −70°C. After 24 h the ampoules were transferred into liquid nitrogen (−190°C), where they were stored for 30 ± 3 days (mean ± s.e. mean) until use. Before being used the

frozen tissues were exposed for about 30 min to −70°C, thawed by placing the ampoules for 2.25 min in a 37°C water bath and rinsed in a dish containing Krebs-Henseleit solution at 37°C.

Organ bath studies

Organ bath studies were performed as described previously (Müller-Schweinitzer, 1983; Müller-Schweinitzer & Neumann, 1983). Briefly, venous strips (20 × 2 mm) from both unfrozen and frozen/thawed saphenous veins were suspended in 10 ml organ baths containing Krebs-Henseleit solution at 37°C and gassed continuously with 95% O_2 plus 5% CO_2. The tension of the strips was recorded isometrically with electromechanical transducers (Statham model UC3) and a potentiometric recorder. At the beginning of the experiments, the strips were stretched to an initial tension of 1000 mg and then allowed to equilibrate for 2 to 3 h in the bathing medium which was changed every 15 min. During this time the resting tension was adjusted to 200 to 300 mg, which then remained constant throughout the experiment. Concentration-response curves to agonists were determined by cumulative additions, each concentration being added when the maximum effect had been produced by the previous concentration. When constrictor responses to increasing calcium chloride concentrations were investigated, the Krebs-Henseleit solution in the organ baths was replaced by a calcium-free solution containing 60 mM potassium chloride in equimolar exchange for sodium chloride and 50 mM TRIZMA instead of $NaHCO_3$ buffer (pH 7.4), gassed with O_2. Contractile responses to guanfacine were expressed as % of the maximum of a preceding concentration-response curve to noradrenaline. In these experiments neuronal uptake mechanisms and prostaglandin synthesis were blocked by cocaine (30 μM) and indomethacin (0.3 μM) respectively, added to the organ baths 20 min before the agonist. For the calcium channel antagonists, an incubation period of 15 min before the first administration of the agonists was allowed. When phenoxybenzamine was used, an incubation period of 20 min was applied, the drug being washed out from the bathing solution 15 min before the first administration of the agonist. In each experiment six strips of the same vein were investigated at the same time, and at least one was used as a control preparation to correct for any sensitivity change not caused by the antagonists. Each vein strip was exposed only once to an antagonist. When guanfacine was used as the agonist, IC_{50} values were calculated at the level of the maximal effect of the control curve. For purposes of comparison, IC_{50} values against depolarization-induced venoconstrictor responses were calculated at the level of the constrictor response to 1.2 mM $CaCl_2$.

$^{45}Ca^{2+}$ uptake

Tissue $^{45}Ca^{2+}$ uptake was determined according to the method described by Van Breemen *et al.* (1981). Briefly, veins were cleaned of connective tissues, cut into rings (1–3 mm), placed on stainless steel holders and allowed to equilibrate for 60 min in Krebs-Henseleit solution bubbled with 95% O_2 plus 5% CO_2 at 37°C. Thereafter, the rings were placed for 120 min into Krebs-Henseleit solution containing 2 μCi $^{45}Ca^{2+}$ ml^{-1} in addition to the non-radioactive calcium, to label exchangeable cellular calcium stores. This was followed by the addition of the calcium channel antagonist under investigation to the labelling medium. After 15 min the rings were transferred for 60 min to a solution of the same composition but containing either 60 mM KCl in equimolar exchange for NaCl or 10 μM guanfacine, in addition to cocaine (30 μM) and indomethacin (0.3 μM), before being washed for 45 min in ice-cold Ca^{2+}-free Krebs-Henseleit solution containing 2.0 mM EGTA to remove extracellular calcium. The tissues were then blotted, weighed and placed overnight in 3 ml 5 mM Na_2EDTA solution. After addition of 7 ml Lumagel scintillation fluid, the radioactivity was determined by liquid scintillation counting.

Statistical analysis of data was performed by use of Student's unpaired *t* test.

Drugs

The following compounds were used: diltiazem hydrochloride (Sigma, Munich, FRG), nifedipine (Bayer, Leverkusen, FRG), verapamil hydrochloride (Knoll, Ludwigshafen, FRG), phenoxybenzamine hydrochloride (Smith, Kline & French, Philadelphia, USA), (−)-noradrenaline hydrogen tartrate (Hoechst, Frankfurt/Main, FRG), indomethacin (Merck, Darmstadt, FRG), cocaine hydrochloride (Lehner, Muttenz, Switzerland), guanfacine hydrochloride (Sandoz, Basle, Switzerland). The (+)-(S) and the (−)-(R) enantiomers of 202-791 (isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-nitro-3-pyridinecarboxylate) were synthesized as described previously (Hof *et al.*, 1985). When dihydropyridines were investigated, a stock solution of 10 mM of the drug was prepared in 94% ethanol just before use and diluted 1 : 10 in 50% ethanol. Further dilutions were made in distilled water. Drug concentrations are given as molar concentrations throughout.

Table 1 Parameters calculated from functional studies on unfrozen and frozen/thawed canine saphenous veins (CSV)

	Unfrozen CSV	*Frozen/thawed CSV*
$-log\ EC_{50}$ values		
$CaCl_2$	3.16 ± 0.12 (7)	2.94 ± 0.05 (7)
Guanfacine	6.71 ± 0.07 (7)	6.51 ± 0.07 (6)*
$-log\ IC_{50}$ values against $CaCl_2$		
Diltiazem	6.22 ± 0.07 (3)	6.53 ± 0.16 (3)
Verapamil	6.63 ± 0.16 (3)	7.02 ± 0.20 (4)
Nifedipine	8.55 ± 0.21 (6)	8.32 ± 0.13 (7)
(−)-(R)-202-791	7.89 ± 0.23 (6)	8.21 ± 0.15 (6)
$-log\ IC_{50}$ values against guanfacine		
Diltiazem	5.19 ± 0.05 (3)	5.67 ± 0.18 (3)*
Verapamil	5.69 ± 0.16 (6)	6.00 ± 0.18 (6)
Nifedipine	6.78 ± 0.23 (7)	7.71 ± 0.18 (5)**
(−)-(R)-202-791	6.62 ± 0.20 (5)	7.25 ± 0.12 (6)***

Values are means ± s.e.mean (*n*). Difference from unfrozen control significant at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

Results

Contractile responses to calcium chloride

Following exposure of venous strips to calcium-free depolarizing solution, the administration of $CaCl_2$ evoked concentration-dependent increases in tension. The maximal contractile response to $CaCl_2$ of frozen/thawed veins (1.80 ± 0.23 g) was 36% of that produced by unfrozen veins (5.00 ± 0.48 g, means ± s.e.mean, $n = 8$), the difference being significant ($P < 0.0005$). However, as indicated by the calculated EC_{50} values (Table 1), the sensitivity to $CaCl_2$ of frozen/thawed canine saphenous veins was statistically not different from that of unfrozen veins. The same applies for the activities of the calcium channel antagonists when used to inhibit depolarization-induced contractions. Compared to their activities on unfrozen veins most of the investigated calcium channel antagonists appeared more potent on frozen/thawed veins, although the differences between the calculated IC_{50} values did not achieve significance (Figure 1, Table 1). The effects of both enantiomers of 202-791 on the contractile responses to $CaCl_2$ of unfrozen and frozen/thawed veins are illustrated in Figure 3a. On each venous preparation the (−)-(R) enantiomer shifted the curve for $CaCl_2$ to the right, whereas the (+)-(S) enantiomer at 100 nM caused a concentration-dependent leftward shift of the curve. Higher concentrations (above 100 nM) of the (+)-(S) enantiomer failed to elicit further enhancement of the $CaCl_2$ effect, but instead antagonized the contractile responses (not illustrated).

Contractile responses to guanfacine

Compared to noradrenaline, the α_2-adrenoceptor agonist guanfacine proved to be equieffective on both venous preparations eliciting 83.0 ± 0.5% (mean ± s.e.mean, $n = 7$) and 83.0 ± 2.3% (mean ± s.e.mean, $n = 6$) of the maximal contractile response to noradrenaline on unfrozen and frozen/thawed veins, respectively. However, comparison of the absolute tension revealed that the maximal response to guanfacine of frozen/thawed veins (3.21 ± 0.66 g, means ± s.e.mean, $n = 6$) was only 42% ($P < 0.0005$) of that produced by unfrozen preparations (7.66 ± 0.73, mean ± s.e.mean, $n = 7$). Furthermore, as indicated by the pD_2 values, guanfacine was slightly less potent on frozen/thawed veins than on unfrozen veins (Table 1). Phenoxybenzamine (50 nM) reduced the maximal responses to guanfacine of unfrozen vein preparations to the same extent as for frozen/thawed veins (to 31 ± 4%, $n = 4$, and 27 ± 5%, $n = 5$, of the maximal noradrenaline effect, respectively; not illustrated). In both venous preparations the calcium channel antagonists were significantly less potent at inhibiting contractions to guanfacine than to K^+-depolarization (Table 1). Concentration-response curves for guanfacine on both venous preparations in the absence and presence of diltiazem, verapamil and nifedipine are shown in Figure 2. Each of the calcium channel antagonists diminished the maximum response to the α_2-adrenoceptor agonist and was about 4 times more potent on frozen/thawed than on unfrozen veins. The differences between calculated IC_{50} values were significant, except for verapamil (Table 1). In

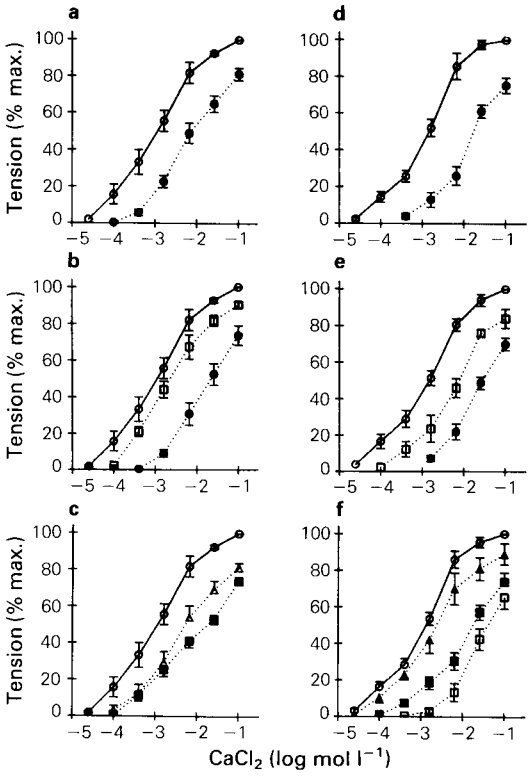

Figure 1 Cumulative concentration-response curves for $CaCl_2$ on helical strips from unfrozen (a–c) and frozen/thawed (d–f) canine saphenous veins without (○) and in the presence of (a, d) diltiazem, (b, e) verapamil and (c, f) nifedipine (●, 1 μM; □, 100 nM; ■, 10 nM; △, 1 nM). Ordinates represent tension as a percentage of the maximum control tension for each series of experiments. Values are means and vertical lines represent s.e.mean, $n = 3$ or 4.

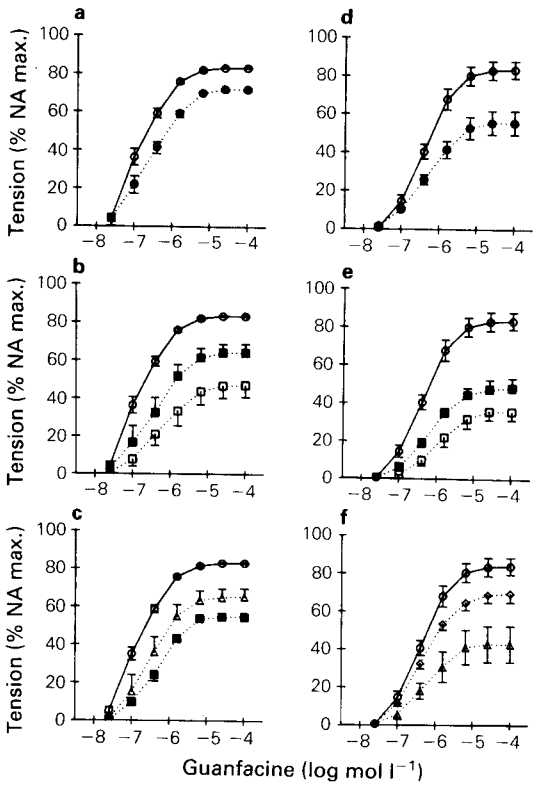

Figure 2 Cumulative concentration-response curves for guanfacine on helical strips from unfrozen (a–c) and frozen/thawed (d–f) canine saphenous veins in the absence (○) and presence of (a, d) diltiazem, (b, e) verapamil and (c, f) nifedipine (●, 1 μM; □, 3 μM; ■, 300 nM; △, 30 nM; ◇, 3 nM). Ordinates represent tension as a percentage of the maximal response to noradrenaline in each series of experiment. Values are means and vertical lines represent s.e.mean, $n = 3$ or 4.

contrast to the observed enhancement of depolarization-induced contractions by the (+)-(S) enantiomer of 202-791, neither on unfrozen nor on frozen/thawed veins did it cause a significant enhancement of the contractile response to guanfacine (Figure 3b).

$^{45}Ca^{2+}$ uptake

Basal $^{45}Ca^{2+}$ uptake as well as the increases in $^{45}Ca^{2+}$ content during stimulation with 60 mM potassium or with 10 μM guanfacine were not statistically different from those in unfrozen veins. Moreover, the guanfacine-induced $^{45}Ca^{2+}$ uptake in the presence of various calcium channel antagonists was similar in both unfrozen and frozen/thawed veins. In contrast, diltiazem, verapamil (100 μM), nifedipine (10 nM) and the (−)-(R) enantiomer of 202-791 were significantly more potent in diminishing the depolarization-induced calcium uptake in frozen/thawed preparations than in unfrozen veins (Table 2). Despite these differences in the two venous preparations, a fairly good correlation was obtained between $^{45}Ca^{2+}$ uptake and the maximal contractile responses to 60 mM KCl or 10 μM guanfacine in the presence and absence of calcium channel antagonists (Figure 4).

At a concentration of 100 nM, the (+)-(S) enantiomer increased significantly the depolarization-induced $^{45}Ca^{2+}$ uptake by both unfrozen and frozen/thawed canine saphenous veins, but failed to increase guanfacine-induced $^{45}Ca^{2+}$ uptake in either venous preparation.

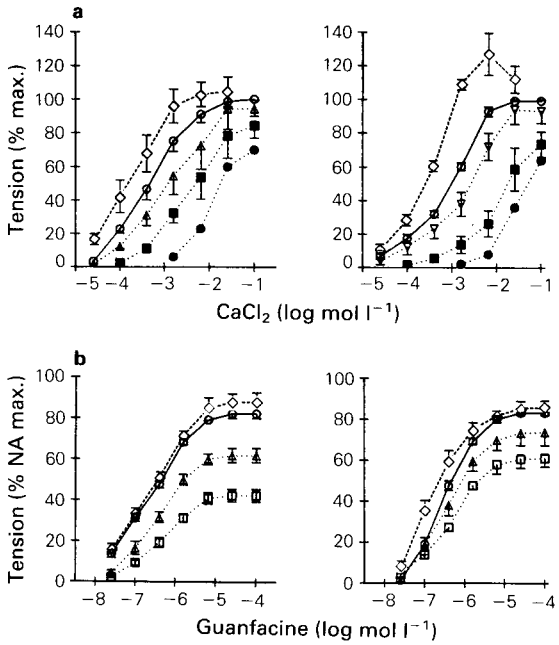

Figure 3 Cumulative concentration-response curves for (a) $CaCl_2$, and (b) guanfacine, in the absence (○) and presence of the (+)-(S) enantiomer (◇, 100 nM) or the (−)-(R) enantiomer of 202-791 (●, 300 nM; □, 100 nM; ■, 30 nM; △, 10 nM; ▽, 3 nM) on helical strips from unfrozen (left) and frozen/thawed (right) canine saphenous veins. The ordinates represent tension as a percentage of maximum control tension elicited by $CaCl_2$ (a) or by noradrenaline (b). Values are means and vertical lines represent s.e.mean from 4 observations.

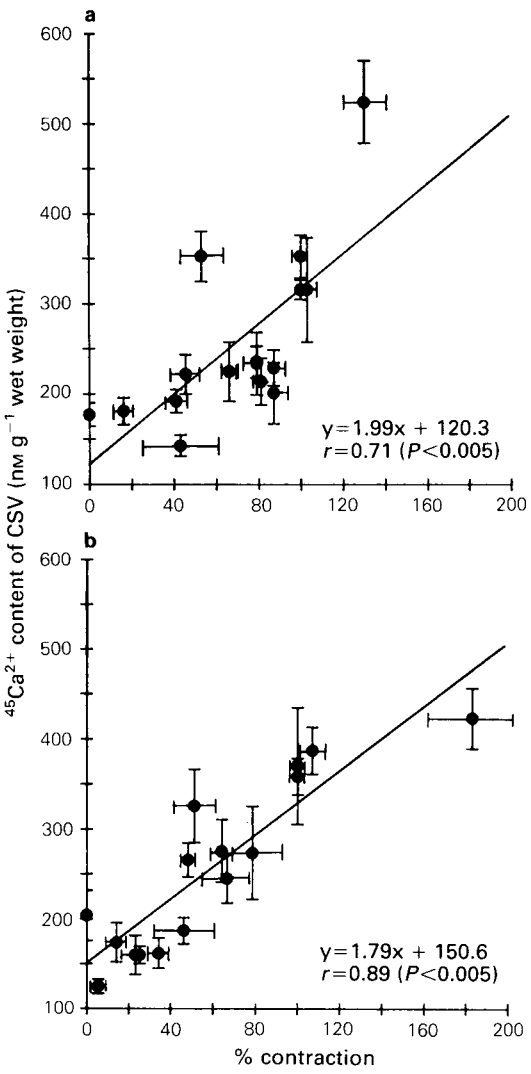

Figure 4 Correlation between the $^{45}Ca^{2+}$ content (nM g^{-1} wet weight) and tension response to stimulation by KCl (60 mM) or guanfacine (10 μM) in the absence and presence of various calcium channel blockers on unfrozen (a) and frozen/thawed (b) canine saphenous veins (CSV). The abscissae represent the maximum tension obtained under experimental conditions expressed as a percentage of the maximum control tension elicited by KCl (60 mM) or guanfacine (10 μM). The data were compared by linear regression analysis and the correlation coefficients are given in the figures.

Discussion

Besides release of intracellular calcium, the activation of vascular smooth muscle involves the influx of extracellular calcium which it has been suggested may be mediated through at least two different populations of calcium channels, namely voltage-dependent and receptor-operated channels, both of which are susceptible to organic calcium channel antagonists (Bolton, 1979; Cauvin *et al.*, 1983; 1984).

During exposure to high concentrations of potassium, contractile responses of smooth muscle to $CaCl_2$ are due to entry of Ca^{2+} ions through voltage-sensitive channels. According to the EC_{50} values for the venoconstrictor activity of $CaCl_2$ obtained in the present study, there was no significant difference in the sensitivity to $CaCl_2$ between unfrozen and frozen/thawed canine veins when exposed to 60 mM potassium. Moreover, the calculated IC_{50} values for inhibition of depolarization-induced contractions suggested that the voltage-dependent calcium channels of both unfrozen and frozen/thawed veins are equally susceptible to blockade by different organic calcium channel antagonists. In contrast, depolarization-induced $^{45}Ca^{2+}$ uptake during partial blockade of

Table 2 $^{45}Ca^{2+}$ content (nM g^{-1} wet weight) of unfrozen and frozen/thawed canine saphenous veins (CSV) following stimulation with 60 mM KCl or 10 μM guanfacine in the absence and presence of various concentrations of calcium channel antagonists

Treatment	*Conc.* (nM)	*Unfrozen CSV*	*Frozen/thawed CSV*
Control		177 ± 13 (12)	204 ± 28 (10)
KCl 60 mM			
KCl alone		316 ± 11 (6)	371 ± 65 (7)
+ Diltiazem	1000	192 ± 13 (6)	160 ± 10 (7)*
+ Verapamil	100	236 ± 18 (6)	187 ± 15 (7)*
+ Verapamil	1000	181 ± 15 (6)	174 ± 22 (7)
+ Nifedipine	1	353 ± 28 (5)	274 ± 52 (6)
+ Nifedipine	10	222 ± 22 (6)	162 ± 17 (7)*
+ Nifedipine	100	156 ± 17 (6)	125 ± 8 (7)
+ (+)-(S) 202-791	100	525 ± 46 (6)	424 ± 34 (7)*
+ (−)-(R) 202-791	30	143 ± 12 (6)	160 ± 22 (7)
Guanfacine 10 μM			
Guanfacine alone		353 ± 24 (6)	359 ± 20 (7)
+ Diltiazem	1000	229 ± 20 (6)	245 ± 27 (7)
+ Verapamil	100	201 ± 34 (6)	266 ± 19 (7)
+ Nifedipine	30	234 ± 35 (6)	326 ± 41 (7)
+ Nifedipine	300	225 ± 33 (6)	212 ± 14 (7)
+ (+)-(S) 202-791	100	316 ± 58 (6)	388 ± 26 (7)
+ (−)-(R) 202-791	30	214 ± 26 (6)	276 ± 35 (7)

Values are means ± s.e.mean (n). Difference between values for frozen/thawed and unfrozen canine saphenous veins significant at * $P < 0.005$.

voltage-dependent channels, by frozen/thawed veins was generally somewhat lower than that by unfrozen preparations. On the other hand, the $^{45}Ca^{2+}$ uptake under control conditions as well as that induced by KCl (60 mM) were not statistically different in the two venous preparations, suggesting that cryopreservation enhanced the susceptibility of voltage-dependent channels to calcium channel antagonists in venous smooth muscle.

In canine saphenous veins α_1- and α_2-adrenoceptor activation may cause both $^{45}Ca^{2+}$ influx and release of intracellular Ca^{2+} (Janssens & Verhaeghe, 1984; Matthews *et al.*, 1984; Jim & Matthews, 1985; Jim *et al.*, 1985). Therefore, in the present study guanfacine, a preferential α_2-agonist (Scholtysik, 1980) with additional affinity for α_1-adrenoceptors in canine saphenous veins (Flavahan *et al.*, 1984), has been used to investigate the effects of calcium channel antagonists on receptor operated mechanisms in both venous preparations. It has been demonstrated that exposure of canine saphenous veins to 50 nM phenoxybenzamine produces selective and irreversible blockade of α_1-adrenoceptors (Constantine *et al.*, 1982). In the present experiments this concentration of phenoxybenzamine reduced the venoconstrictor responses to guanfacine of both unfrozen and frozen/thawed preparations to the same extent, indicating that storage at −190°C did not change the ratio of α_1/α_2-adrenoceptors in canine saphenous veins.

Following stimulation with guanfacine (10 μM), in the absence and presence of different calcium channel antagonists, the content of $^{45}Ca^{2+}$ was similar in both venous tissues. When tested against guanfacine-induced contractions, only verapamil, which is known to compete for both α_1- and α_2-binding sites in addition to its calcium channel blocking activity (Motulsky *et al.*, 1983), was equiactive on both preparations; whereas diltiazem and both 1,4-dihydropyridine derivatives proved to be more potent on frozen/thawed veins than on unfrozen preparations. In connection with the reduced efficacy of guanfacine in frozen/thawed veins, this enhanced susceptibility to calcium channel blockade supports the concept that it is the efficiency of receptor-response coupling that determines the effect of calcium channel blockade on the adrenergic response (Cooke *et al.*, 1985).

Small modifications to a dihydropyridine molecule may produce derivatives with effects diametrically opposite to those of the calcium antagonists (Schramm *et al.*, 1983; Loutzenhiser *et al.*, 1984; Gopalakrishnan *et al.*, 1985). Optical isomers of 1,4-dihydropyridine derivatives, for example, elicit opposite effects on the functional activity of calcium channels in vascular smooth muscle (Franckowiak *et*

al., 1985; Hof *et al.*, 1985). Therefore, the effects of the two, (+)-(S) and (−)-(R), enantiomers of 202-791 were also investigated on our two venous preparations. While the (−)-(R) enantiomer reduced both venoconstriction and $^{45}Ca^{2+}$ uptake in response to stimulation by guanfacine or depolarization, the (+)-(S) enantiomer elicited just the opposite effect when studied in the presence of high potassium. The (+)-(S) enantiomer, however, failed to produce a significant enhancement of either contraction or Ca^{2+} uptake in response to the α_2-agonist guanfacine. The effects of both enantiomers on both voltage-dependent and receptor-operated calcium mechanisms observed in the present study are consistent with the dualistic actions found for derivatives of other 1,4-dihydropyridines in arterial vascular smooth muscle (Schramm *et al.*, 1983; Loutzenhiser *et al.*, 1984; Franckowiak *et al.*, 1985; Hof *et al.*, 1985; Mikkelsen & Nyborg, 1986).

In conclusion, the effects of the investigated calcium channel antagonists on the stimulation-induced $^{45}Ca^{2+}$ uptake by both unfrozen and frozen/thawed canine veins closely reflect the effects of these compounds on tension development, suggesting that cryopreservation of the venous smooth muscle had no deleterious effect on the mechanisms coupling Ca^{2+} entry, through either voltage-dependent or receptor-operated channels, to activation. Thus, the present study provides further pharmacological characterization of cryopreserved canine saphenous veins and presents evidence that in frozen/thawed veins, the coupling of tissue $^{45}Ca^{2+}$ uptake mechanisms and functional activity of the contractile proteins is well preserved.

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