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Arterial Smooth Muscle Function after Prolonged Exposure to a Medium Containing Dimethyl Sulfoxide (Me_2SO) and Storage at −196°C

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Changes in smooth muscle responsiveness were investigated *in vitro* after storage of canine femoral arteries at room temperature (21°C) and in liquid nitrogen (−196°C) in Krebs–Henseleit solution containing 50% fetal calf serum, 2.0 *M* dimethyl sulfoxide, and 0.1 *M* sucrose as cryoprotectants. Both contractile responses to noradrenaline and relaxant effects of bimakalim were unchanged after exposure of arterial smooth muscle preparations for 1 h to the cryomedium without freezing. However, after exposure of the tissues for increasing time periods to the cryomedium with subsequent cryopreservation the post-thaw functional recovery was progressively diminished. Optimal post-thaw functional recovery was obtained with tissues that had been frozen within 10 min of being placed in the cryomedium. The results suggest that exposure to the cryomedium without freezing is well tolerated by the arterial smooth muscle, whereas a progressive reduction of the contractile activity occurs with prolonged exposure of the preparations to room temperature cryomedium before starting the cooling process.

Cryopreservation and storage at −196°C has been shown to offer clear potential for ensuring the supply of rare material such as human tissues, the supply of which is often irregular and unpredictable, for pharmacological studies (6). Recent studies on cryopreservation of isolated blood vessels have revealed that very good post-thaw functional recovery can be obtained with tissues that had been frozen slowly while immersed in Krebs–Henseleit solution containing 50% fetal calf serum, 1.8 *M* dimethyl sulfoxide, and 0.1 *M* sucrose (7, 8). The major tissue function change in most of these studies was a reduction of the post-thaw contractile forces by up to 50%. Furthermore, peripheral arteries appeared to be more susceptible to the freezing/thawing injury than veins (9) or bronchi (11). Besides the cryomedium many factors such as rate of cooling and rewarming and/or rate of addition and removal of the cryoprotectant(s) may modify the post-thaw recovery of cryopreserved tissues. The aim of the following experiments was to investigate the influence of prefreezing exposure of the artery to the cryomedium on post-thaw smooth muscle functions.

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MATERIALS AND METHODS

Tissue Preparation and Storage Method

Femoral arteries were obtained from two male beagle dogs (12 and 13 kg), killed by iv injection of pentobarbitone (50 mg/kg) and exsanguination. The arteries were placed in Krebs–Henseleit (KH) solution (composition m*M:* 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) at room temperature, cleaned of loose connective tissue, and cut into segments (5 mm long). The arterial segments were placed randomly into 2-ml Liquid Nitrogen Storage Ampoules (Gibco AG, Basel, Switzerland) filled with either KH solution or with KH solution containing 50% fetal calf serum (FCS), 2.0 *M* dimethyl sulfoxide (Me_2SO), and 0.1 *M* su-

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crose. The arteries were placed in this medium at different times so that the tissues were exposed for 10, 20, 60, 120, 240, and 360 min at room temperature to the cryomedium before being either investigated *in vitro* as described below or slowly frozen at a mean cooling rate of about 0.6°C/min in a freezer maintained at −70°C. After about 3 h at −70°C the ampoules were transferred into liquid nitrogen (−196°C) where they were stored for 1 week. Before use the tissues were thawed within 2.5 min by placing the ampoules in a 37°C water bath. Thereafter, the tissues were placed in a dish containing about 500 ml KH solution at 37°C and each arterial segment was cut into two rings (about 2–3 mm in length).

Experimental Procedures

The rings were mounted between two stainless steel wire hooks and suspended in 10-ml organ baths containing KH solution at 37°C, gassed continuously with 5% CO_2 in oxygen. The tension of the rings was recorded isometrically under a resting tension of about 1 g with electromechanical transducers (Statham Model UC 3) and a potentiometric recorder. The preparations were allowed to equilibrate for 2 h in the bathing medium. During this time the preparations were challenged once with noradrenaline (10 μ*M*) and the baseline tension of the rings was readjusted to 1 g. Thereafter, concentration–response curves for noradrenaline were determined by cumulative additions, each concentration being added when the maximum effect had been produced by the previous concentration. When relaxant responses to the potassium channel activator bimakalim (2) were investigated, active tone was induced by the addition of a submaximal concentration (30 n*M*) of the thromboxane analogue U46619. At the end of these concentration–response curves papaverine (300 μ*M*) was added to induce complete relaxation of the arterial rings. All effects were expressed in grams.

Drugs

The following pharmacological agents were used: (−)-noradrenaline hydrogentartrate (Fluka, Buchs, Switzerland), U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$), papaverine hydrochloride (Sigma, Munich, FRG), and bimakalim (Merck, Darmstadt, FRG). Both U46619 and bimakalim were dissolved in ethanol and diluted in distilled water to give 1 m*M* solutions containing 60 and 10% ethanol, respectively. Further dilutions were performed in physiologic salt solutions. Aliquots of U46619 (1 μ*M*) were kept frozen at −20°C; all other compounds were prepared just before use.

Data Analysis

Concentration–response curves were analyzed with a linear computer program in RS/1 (BBN Software Products Corporation, Cambridge, MA) and E_{max} (maximal effects) and $-\log EC_{50}$ values (negative logarithm of the molar concentration of the agonist producing 50% of E_{max}) were derived from this analysis. Data are given as means ± SEM. One-way analysis of variance (ANOVA) was performed, followed by the Bonferroni-corrected *t* test to assign differences to individual between-group comparisons when overall significance ($P < 0.05$) was attained.

RESULTS

In the first series of experiments arterial tissues were exposed for 60 min to the cryomedium (KH solution containing 50% FCS, 2.0 *M* Me_2SO, and 0.1 *M* sucrose) at room temperature and investigated *in vitro* with and without freezing. Neither the contractile responses to noradrenaline and U46619 nor the relaxant effects of bimakalim and papaverine were changed when arterial preparations were investigated after 60 min exposure to the cryomedium without freezing. In contrast, there were marked reductions of both contractile and relaxant re-

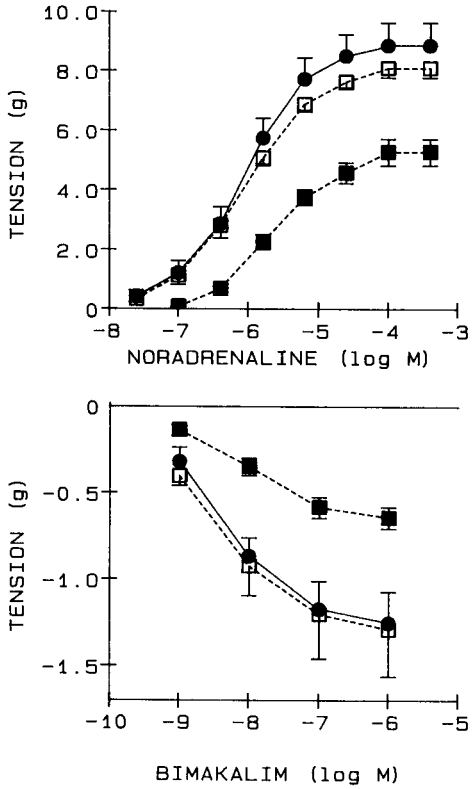

FIG. 1. (Top) Cumulative concentration–response curves for noradrenaline on rings from canine femoral arteries without treatment (●), following 60 min exposure to the cryomedium at room temperature (□), and after cryopreservation following 60 min exposure to the cryomedium (■). (Bottom) Cumulative concentration–response curves for bimakalim on rings from canine femoral arteries without treatment (●), following 60 min exposure to the cryomedium at room temperature (□), and after cryopreservation following 60 min exposure to the cryomedium (■). The developed tensions are expressed in grams. Bars represent means ± SEM; for each curve $n = 4$.

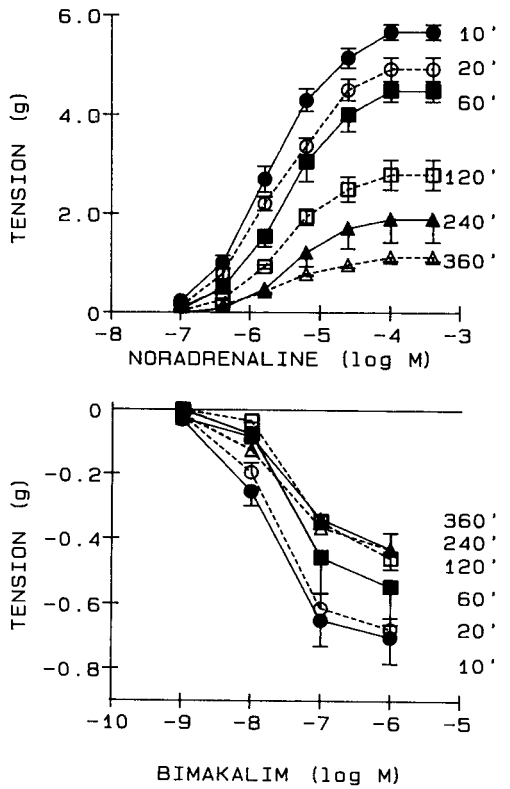

FIG. 2. (Top) Cumulative concentration–response curves for noradrenaline on rings from frozen/thawed canine femoral arteries after prefreezing incubation times of 10–360 min in the cryomedium at room temperature. (Bottom) Cumulative concentration–response curves for bimakalim on rings from frozen/thawed canine femoral arteries stimulated with 30 n*M* U 46619 after prefreezing incubation times of 10–360 min in the cryomedium at room temperature. The developed tensions are expressed in grams. Bars represent means ± SEM; for each curve $n = 4$.

cryomedium. This artery was selected since it appears that arteries are considerably more susceptible to cryoinjury than veins (7–9). Furthermore, it has been suggested that the time allowed to reach equilibrium with the cryomedium might be an important factor determining the post-thaw function of cryopreserved vascular tissues (4, 7, 8). The cryomedium employed in the present study contained two cryoprotecting agents, Me_2SO and sucrose. It is assumed that Me_2SO as a permeating cryoprotective agent protects the cell from damage during freezing by entering the cell and replacing some water (12). Sucrose belongs to the nonpermeating cryoprotectants, functions at the outer surface of the cell, and is suggested to protect the cell by retaining more liquid water at low temperatures, thereby limiting the increase in external electrolyte concentration during the freezing process (5). Exposure of the arterial smooth muscle to this hypertonic medium for 1 h without

TABLE 2
Post-Thaw Parameters for Various Contractile and Relaxant Effects on Rings from Canine Femoral Arteries Cryopreserved after Different Times of Exposure to the Cryomedium (Krebs–Henseleit Solution Containing 50% Fetal Calf Serum, 2.0 *M* Me_2SO, and 0.1 *M* Sucrose) at Room Temperature

	Time (min)					
	10	20	60	120	240	360
E_{max} (g)						
Noradrenaline	5.67 ± 0.16	4.92 ± 0.25	4.49* ± 0.23	2.79* ± 0.31	1.89* ± 0.47	1.12* ± 0.08
U46619 (30 n*M*)	0.39 ± 0.09	0.35 ± 0.06	0.37 ± 0.09	0.39 ± 0.03	0.21 ± 0.09	0.10* ± 0.02
Bimakalim	−0.70 ± 0.08	−0.68 ± 0.04	−0.55 ± 0.12	−0.46 ± 0.04	−0.43 ± 0.02	−0.43 ± 0.05
Papaverine (300 μ*M*)	−0.97 ± 0.13	−1.07 ± 0.07	−0.96 ± 0.18	−1.56* ± 0.08	−1.21 ± 0.12	−0.74 ± 0.09
Passive Resttone	0.30 ± 0.09	0.15 ± 0.01	0.21 ± 0.04	0.21 ± 0.01	0.13 ± 0.02	0.41 ± 0.07
− Log EC_{50} values						
Noradrenaline	5.74 ± 0.06	5.70 ± 0.08	5.48 ± 0.11	5.53 ± 0.04	5.42* ± 0.03	5.58 ± 0.06
Bimakalim	7.74 ± 0.03	7.66 ± 0.05	7.45* ± 0.05	7.38* ± 0.03	7.50 ± 0.07	7.62 ± 0.05

Note. − Log EC_{50}, negative logarithm of the molar concentration producing 50% of maximum response. Data are given as means ± SEM; for each value $n = 4$.
* Significant differences against values determined in tissues with 10 min exposure to the cryomedium.

freezing was well tolerated. Full recovery of functional activities has also been reported for canine jugular veins (13), rabbit aortae (3), and guinea pig taenia coli (1) under similar incubation conditions. However, when these incubation conditions are combined with freezing, considerable reductions of both the contraction and relaxation were observed (Table 1).

Prolonged prefreezing immersion of the arteries in the cryomedium induced time-dependent decreases in post-thaw functional activities. Optimal recovery was obtained with tissues that had been exposed to the cryomedium for only 10 min before starting the cooling process. This rather unexpected finding indicated that prolonged prefreezing exposure of arteries to a Me_2SO-containing cryomedium is not necessary but may be rather toxic.

From the data illustrated in Fig. 2 it appears that relaxant responses mediated through potassium channel opening are less susceptible to incubation of the tissues in the cryomedium than α-adrenoceptor-mediated contractile responses. This apparently better preservation of bimakalim-induced relaxation after prolonged exposure to the cryomedium most probably results from the progressive reduction of arterial smooth muscle contractile responsiveness with increasing medium exposure periods. Responses to bimakalim were recorded during active tone induced by U46619 which were reduced after more than 120 min of cryomedium exposure (Table 2).

Despite significant decreases in post-thaw contractile function, prolonged exposure to the cryomedium did not change the vascular sensitivity to noradrenaline. This was supported by the finding that the − log EC_{50} values for noradrenaline, with the exception of that determined after 4 h cryomedium exposure, were similar. The same applies for bimakalim, the − log EC_{50} values of which were reduced only after 1 and 2 h cryomedium exposure. These data, suggesting that the membrane located recognition sites for both noradrenaline and bimakalim are largely resistant to the freezing/thawing injury, are in line with previous studies showing a significant correlation between drug potencies for various agonists (8) and antagonists (10) in fresh and cryopreserved vascular tissues.

In conclusion, exposure of arterial smooth muscle to the cryomedium without freezing was well tolerated. However, prolonged immersion in the cryomedium before starting the cooling process reduced post-thaw functional activities. For optimal

post-thaw recovery arteries should be frozen within 10 min of room temperature exposure to the cryomedium.

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