Neuronal Messengers in Vascular Function
A. Nobin, C. Owman and B. Arneklo-Nobin (Eds.)

9

Cryopreservation of isolated blood vessels for pharmacological studies: experiments on canine and human veins

Else Müller-Schweinitzer and Carlo Tapparelli

Preclinical Research, Sandoz Ltd., CH-4002 Basel, Switzerland

INTRODUCTION

Organ bath studies with isolated blood vessels generally require freshly obtained vascular tissue, and usually the supply provides more material than can be used within the available time. Furthermore, the availability of human vascular tissue, though it can be easily obtained from surgery, is often unpredictable and irregular. Thus the advantages of a simple, reliable storage method for ensuring the supply of adequate vascular preparations for pharmacological studies are readily apparent. We therefore investigated the pharmacological behaviour of canine and human saphenous veins after various freezing and storage methods.

MATERIALS AND METHODS

Segments of freshly obtained canine and human veins (about 15–20 mm length) were placed either in Krebs–Henseleit solution (mmol/l: 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 5% CO_2 in oxygen, or immersed in 2 ml liquid nitrogen ampoules filled with foetal calf serum (FCS) containing various cryoprotective agents. Usually the ampoules were then placed in a styropor box (11×11×22 cm) and slowly frozen at a mean cooling rate of about 0.6 °C in a freezer maintained at −70 °C. After 4–24 h the ampoules were transferred into liquid nitrogen (−190 °C) where they were stored for several weeks. Before being used the tissues were exposed for 30–60 min to −70 °C and then thawed by placing the ampoules

for 2.25 min in a 37 °C water bath. Thereafter the vessel segments were rinsed in a dish containing Krebs–Henseleit solution at 37 °C and cut into helical strips for isometric recording as described previously (Müller–Schweinitzer, 1984; Müller–Schweinitzer and Tapparelli, 1986).

Investigations on the release of tritiated noradrenaline in response to electrical stimulation were performed on canine saphenous vein strips (25 × 2 mm), incubated for 1 h in Krebs–Henseleit solution containing 50 nmol/l 1-(ring-2,5,6-[^{3}H]noradrenaline (spec. act. = 41.2–48.5 Ci/mmol) and 0.3 μmol/l ascorbic acid. The strips were suspended between two platinum electrodes (2 mm apart, 24 mm long) and superfused with Krebs–Henseleit solution (37 °C, 3 ml/min), containing 10 μmol/l cocaine, 10 μmol/l hydrocortisone, 30 μmol/l pargyline and 0.3 μmol/l indomethacin to inhibit neuronal and extraneuronal uptake, monoamine oxidase and endogenous prostaglandin synthesis, respectively. The initial tension was set at 1.5 g and changes in tone were recorded isometrically. The superfusate was collected at 2-min intervals without and during sustained electrical stimulation (2 Hz, 0.1 ms, 150 mA), provided by a Grass S44 stimulator. Samples (6 ml) of the superfusate were added to 12 ml Luma Gel scintillation fluid (Fakola, Basel, Switzerland) and the radioactivity was measured in a liquid scintillation counter (W+W Beta Counter model MR 300 TPO). Each sample was counted for 5 min.

DRUGS

The following agents were used: (–)-noradrenaline hydrogen tartrate (Hoechst, Frankfurt/Main, F.R.G.), 1-(ring-2,5,6-[^{3}H]noradrenaline (New England Nuclear), 5-hydroxytryptamine creatinine sulfate (5-HT), hydrocortisone acetate (Fluka, Buchs, Switzerland), cocaine hydrochloride (Lehner, Muttenz, Switzerland), indomethacin (Merck and Co., Inc., Darmstadt, F.R.G.), pargyline hydrochloride (Sigma, Munich, F.R.G.), acetylsalicylic acid (Sandoz Ltd., Basel, Switzerland), dimethyl sulfoxide (DMSO), polyethylene glycole 400 (PEG 400, Merck-Schuchardt, Hohenbrunn, F.R.G.). All compounds were dissolved just before use. Drug concentrations are given as molar concentrations. Statistical analysis of data was performed using Student's *t*-test.

RESULTS AND DISCUSSION

Preliminary experiments. When living mammalian cells are frozen in physiological media the formation of ice crystals in intracellular and/or extracellular spaces, generally causes severe cell destructions and only few or even no cells survive. Cryoprotective agents such as DMSO and many other compounds

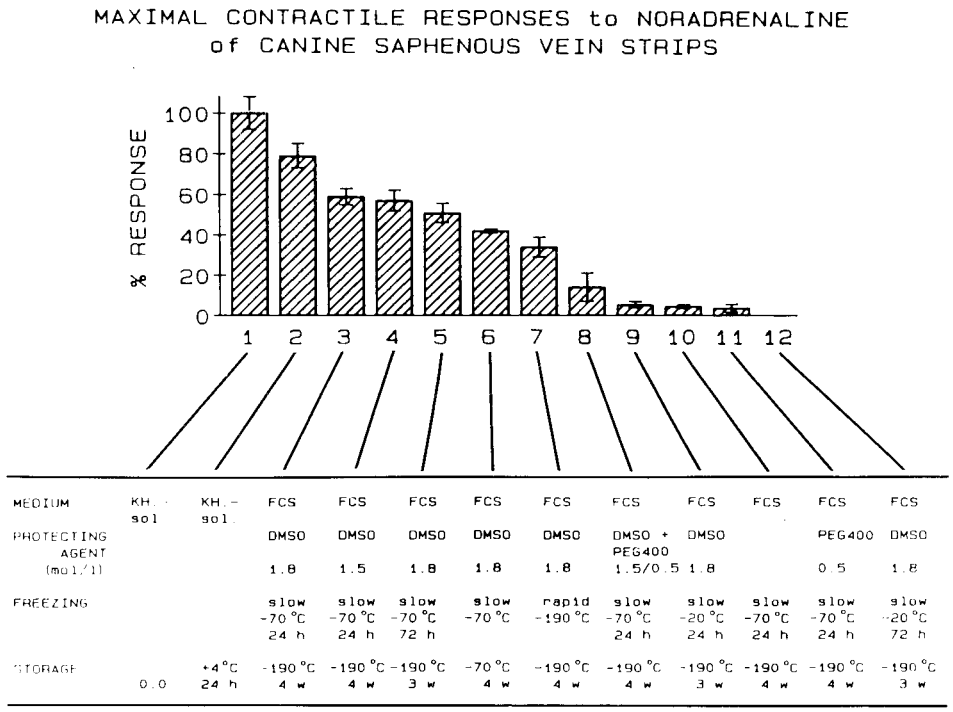

Fig. 1. Contractile force of canine saphenous vein strips after various storage methods, expressed as percentages of the maximum response to noradrenaline of unfrozen vein strips. The bars represent means ± S.E.M.

known to protect mammalian cells from damage during freezing and thawing, may reduce the amount of ice formed at any subzero temperature thereby increasing the recovery rate of frozen stored cells. However, different cell types differ in their requirements with regard to cooling rate, warming rate as well as with regard to the optimal cryoprotectant and its concentration. Consequently the presence of many different cell types within a tissue or organ implies that no one procedure can satisfy them all (Pegg, 1985).

Therefore, preliminary experiments were performed to find a useful method of storing isolated blood vessels for pharmacological studies. The influence of various cryoprotective agents, storage temperatures and freezing and thawing methods on the contractile force of canine saphenous vein strips was determined as assessed by the maximal responses to noradrenaline (Fig. 1). Compared to the maximum contractile response to noradrenaline of unfrozen veins, storage in Krebs–Henseleit solution for 24 h at 4 °C diminished already the contractile force of the vein strips to about 80%. Analysis of data obtained with frozen/thawed veins revealed that the best recovery i.e., a contractile force of about

60%, was obtained when veins had been immersed in FCS containing 1.5 or 1.8 mol/l DMSO as the cryoprotective agent, frozen slowly within 4–24 h to −70 °C and stored at −190 °C. The contractile force of veins was diminished to 50% when the ampoules were kept for 72 h at −70 °C before being transferred into liquid nitrogen, and storage of the ampoules for 4 weeks at −70 °C resulted in a further reduction of the contractile force to about 40%. Similarly, rapid freezing of the tissues by placing the ampoules directly into liquid nitrogen, diminished the contractile force of the veins to 35%, supporting the suggestion that rapid freezing induces the formation of intracellular ice which may lead to cell damage (Pegg, 1985). Only negligible or even no recovery was obtained when veins were kept for 24 or 72 h at −20 °C before being transferred into liquid nitrogen. The same applies for veins which had been immersed in FCS containing 0.5 mol/l PEG 400 or in FCS without any protecting agent. The marked loss of recovery of veins immersed in FCS containing 1.5 mol/l DMSO plus 0.5 mol/l PEG 400 indicates that PEG 400 is toxic.

Validation studies. Based on these data, further validation studies were performed on veins which had been frozen slowly to −70 °C and stored at −190 °C while being immersed in FCS containing 1.8 mol/l DMSO. Comparative pharmacological studies on unfrozen and frozen/thawed *canine saphenous veins* revealed that under these conditions after thawing the pD_2 values of both noradrenaline and 5-HT were slightly diminished while that of tryptamine was unchanged. Further experimental data suggest that main biochemical properties such as monoamine oxidase activity, endogenous prostaglandin synthesis and neuronal uptake mechanisms are well preserved (Müller-Schweinitzer and Tapparelli, 1986). More direct evidence for an unimpaired neuronal uptake mechanism was obtained by measurements of the basal and stimulation-induced tritium overflow from canine saphenous vein strips after pre-incubation with [^{3}H]noradrenaline (Fig. 2). Though the basal outflow from frozen/thawed vein strips was slightly higher than that observed with unfrozen veins, the absolute tritium overflow during sustained electrical stimulation was similar in both groups.

The same method of cryopreservation was applied to store samples of *human saphenous veins.* Functional studies on human saphenous veins thawed after storage at −190 °C revealed that the contractile force development of frozen stored human veins was unchanged as compared to that produced by unfrozen preparations. Furthermore, there is a good correlation between the pD_2 values of various 5-HT receptor agonists (Fig. 3) on unfrozen and frozen/thawed veins and the same applies for the blocking activities of all antagonists tested up to now against 5-HT and noradrenaline on unfrozen and frozen/thawed human saphenous veins (Müller-Schweinitzer et al., 1986).

In conclusion, slow freezing to −70 °C and storage in liquid nitrogen of canine

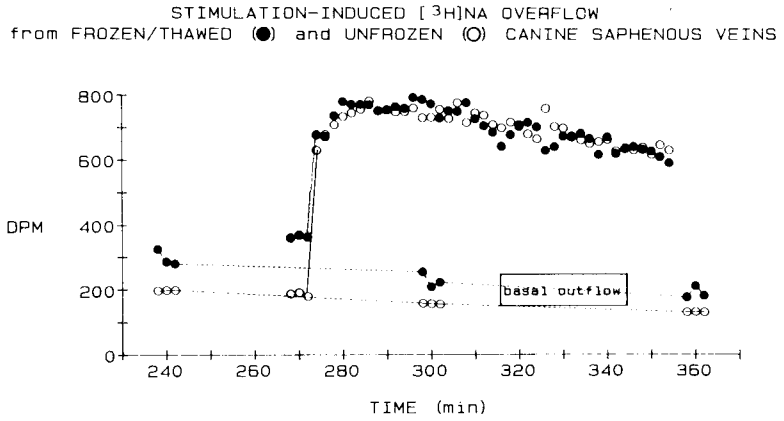

Fig. 2. Spontaneous and stimulation-induced tritium overflow from canine saphenous vein strips after incubation with 1 μmol/l [^{3}H]noradrenaline during superfusion with Krebs–Henseleit solution containing 10 μmol/l cocaine, 0.3 μmol/l indomethacin, 10 μmol/l hydrocortisone and 30 μmol/l pargyline. Frozen/thawed (n=9) and unfrozen (n=6) canine saphenous vein strips were stimulated with square wave pulses of 2 Hz, 0.1 ms and 150 mA.

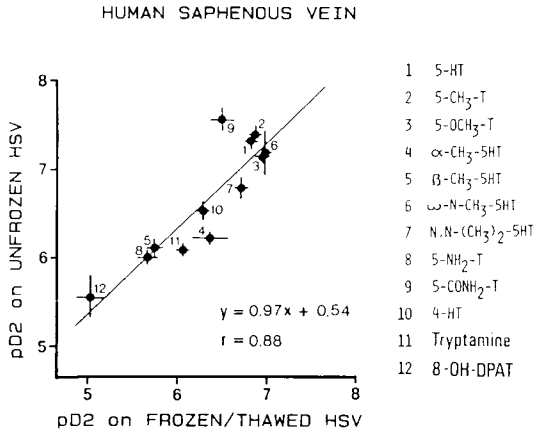

Fig. 3. Correlation between pD_2 values of 8-OH-DPAT and various 5-hydroxytryptamine (5-HT) receptor agonists on helical strips from human saphenous veins (HSV) thawed after storage for 4–5 weeks at −190 °C and those determined on unfrozen preparations. The bars represent means ± S.E.M. The parameters were compared by linear regression analysis and the correlation coefficient is given in the figure. Data from Müller-Schweinitzer et al. (1986).

and human venous tissue immersed in FCS containing 1.8 mol/l DMSO yields an excellent cryopreservation of the main biochemical and functional properties and may be considered an effective means of preserving and storing vascular tissues for pharmacological investigation.

SUMMARY

Canine saphenous veins were immersed in foetal calf serum containing various cryoprotective agents, slowly frozen and stored for several weeks at subzero temperatures. Pharmacological investigations of frozen/thawed tissues revealed considerable attenuation of the contractile force of frozen stored veins as compared to unfrozen veins. The best recovery after thawing of frozen stored canine veins was obtained on tissues which had been frozen slowly to −70 °C and stored in liquid nitrogen while being immersed in FCS containing 1.8 mol/l dimethyl sulfoxide. Though the maximum response to noradrenaline of helical strips prepared from these veins was diminished to about 60%, the evidence suggests that there may be a very good preservation of the main biochemical properties, such as monoamine oxydase activity, endogenous prostaglandin synthesis and neuronal uptake mechanism in veins stored under these conditions. The same method of cryopreservation was applied to store samples of human veins. Comparison of the pD_2 values for various agonists and of the blocking activities of various antagonists of both 5-HT receptors and α-adrenoceptors yielded an excellent correlation between the parameters determined on frozen/thawed and unfrozen human veins. It is concluded that freezing isolated blood vessels may be considered an effective means of preserving and storing vascular tissues for pharmacological investigations.

REFERENCES

Müller-Schweinitzer, E. (1984) Alpha-adrenoceptors, 5-hydroxytryptamine receptors and the action of dihydroergotamine in human venous preparations. *Naunyn Schmiedeberg's Arch. Pharmacol.*, 327: 299–303.

Müller-Schweinitzer, E. and Tapparelli, C. (1986) Pharmacological studies on frozen stored canine saphenous veins and basilar arteries. *Naunyn Schmiedeberg's Arch. Pharmacol.*, 332: 74–78.

Müller-Schweinitzer, E., Tapparelli, C. and Victorzon, M. (1986) Functional studies on human veins after storage at −190 °C. *Br. J. Pharmacol.*, 88: 685–687.

Pegg, D. (1985) Principles of tissue preservation. In P.J. Morris and N.L. Tilney (Eds.) *Progress in Transplantation*, Churchill Livingstone, Edinburgh, pp. 69–105.