Effects of Prolonged Cold Storage on Purinergic and Adrenergic Components of Sympathetic Co-transmission in Isolated Canine Splenic Arteries

Xiao-Ping Yang and Shigetoshi Chiba*

Department of Pharmacology, Shinshu University School of Medicine, Matsumoto 390–8621, Japan

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ABSTRACT—The present study demonstrated the progressive inhibition by prolonged cold storage (4, 7 and 14 days at 4°C) on prejunctional and postjunctional functions of purinergic and adrenergic components of double-peaked vasoconstrictor responses to periarterial electrical nerve stimulation in the isolated canine splenic artery. After the cold storage for 4 days, the first phase constriction was markedly decreased, whereas the second response was not significantly modified. Furthermore, after the 7 days of cold storage, the first phase was substantially depressed at low frequencies, but at high frequencies, a low level of contractile responses was still observed. On the other hand, the second phase in the cold stored artery for 7 days largely remained at any used frequencies. Moreover, the 14 days of cold storage almost completely inhibited the vasoconstrictor responses to nerve stimulation. Tyramine-induced constrictions were progressively decreased in the stored-days dependent manner, although the ATP and the noradrenaline-induced one was not modified for 4 and 7 days of the cold storage. In conclusion, 1) the 4°C cold stored artery for 4 days may show preferential injury of its tyramine-dependent noradrenaline releasing mechanisms, whereas nerve excited ones might well remain; and 2) the prejunctional contractile response of purinergic transmission might be damaged more preferentially than that of adrenergic transmission within 7 days storage.

Keywords: Cold storage, Tyramine, Sympathetic nerve stimulation, Purinergic transmission, Splenic artery

Several lines of morphological evidence have shown that prolonged cold storage produces a more preferential degeneration of adrenergic nerve fibers than smooth muscle fibers in sympathetically innervated tissues (1, 2). The functional data have also demonstrated that prolonged cold storage preferentially abolishes nerve stimulation-induced contractile responses but the responses to exogenous agonists were well maintained (2–7). These observations indicated that the prolonged cold storage may exert a dissociation of the prejunctional from the postjunctional responses in sympathetically innervated tissues. However, several investigators observed that the prejunctional responses to an indirect sympathomimetic agonist tyramine were well retained after 5–7 days of cold storage (4°C) in the canine intermediate auricular artery (8), 3–9 days of cold storage in the canine femoral artery (9) and 3–5 days of cold storage in the monkey skeletal muscle artery (10). It is demonstrated that the

vasoconstrictor response of the canine splenic artery to nerve stimulation is mediated by the release of sympathetic co-transmitters noradrenaline (NA) and adenosine 5'-triphosphate (ATP) (11–13). In the canine splenic artery, periarterial electrical nerve stimulation readily induced double-peaked vasoconstrictions where the first phase response might contain mainly a purinergic component and the second phase, mostly an adrenergic component (14, 15). More recently, Yang and Chiba reported that the 7 days of 4°C cold storage of the isolated canine splenic artery depressed the first peaked responses to a much greater extent than the second ones (16). In the present study, we demonstrated that in 4 days of 4°C cold storage, only the first peaked responses are clearly and significantly depressed, but the second peaked ones are not significantly affected. Thus, it is possible that investigations on the inhibition by cold storage may provide new pharmacological insight into the mechanisms of sympathetic co-transmission. The purpose of this study was to extend the length of cold storage and to re-examine

* To whom correspondence should be addressed.

whether progressive inhibition by prolonging the length of cold storage produces a dissociation of inhibitory effect on prejunctional and postjunctional responses of purinergic and adrenergic components of sympathetic co-transmission in the isolated, perfused canine splenic artery.

MATERIALS AND METHODS

Arterial preparations

Mongrel dogs of either sex, weighing 9 to 18 kg, were anesthetized with sodium pentobarbitone (30 mg/kg, i.v.). After treatment with sodium heparin (200 units/kg, i.v.), the dogs were killed by rapid exsanguination from the right femoral artery. The arterial main branches of the splenic artery were isolated, and side branches of the artery were tied with silk threads. Then, the artery (0.8–1.2 mm in an outer diameter) was cut into segments (15–20 mm in length), and each segment was cannulated and set up for perfusion as described previously (17, 18). During cold storage in the refrigerator, the isolated artery was kept in the Krebs-Henseleit solution without supplying exogenous oxygen at a constant temperature of 4°C for 4, 7 or 14 days. The cold Krebs-Henseleit solution was exchanged at 4 days. After the cold storing period, segments were cut from arteries and prepared for the experiments in the same manner as a fresh artery. Briefly, a stainless steel cannula was inserted into the arterial segment from the distal to the proximal end. A proximal portion of the segment was fixed to the distal portion of a needle-type cannula with silk threads. The cannula was 3–4 cm long and 0.6–1 mm in outer diameter and had small side holes 5 mm from the distal sealed end. The cannulated arterial segment was placed in a cup-shaped glass bath and was perfused by a roller pump (Tokyo Rikakikai, Tokyo) with Krebs-Henseleit solution gassed with 95% O₂ and 5% CO₂. The solution contained 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃ and 10 mM glucose. The flow rate was kept at approximately 2 ml/min. The perfusion pressure was continuously measured with an electric manometer (MPU-0.5A; Nihon Kohden, Tokyo) and recorded with a rectigraph (WT-685G, Nihon Kohden). After a stabilization period of 60 min, the preparation was removed from the bath solution and fixed in a horizontal position. The preparation was perfused at a constant flow rate during the experiment. The basal perfusion pressure was within 35–80 mmHg.

For electrical stimulation of the periarterial sympathetic nerve terminals, two platinum electrodes were placed on the extraluminal side of the arterial wall. Electrical stimulation was delivered by an electric stimulator (SEN-7203, Nihon Kohden) using 30-s trains of pulses at 10-V

amplitude, 1-ms pulse duration, in a frequency range of 1–10 Hz. The organ bath was sealed with the plastic film to maintain the preparation at 37°C. Ten-minute intervals between electrical stimulation periods were needed to obtain a reproducible response. The intervals between frequency-response curves were 60 min. The drug solution was administered into the rubber tubing close to the cannula in a volume of 0.01–0.03 ml by using microinjectors.

Drugs

Drugs used were tyramine hydrochloride (Wako, Osaka); disodium adenosine 5'-triphosphate and prazosin hydrochloride (Sigma, St. Louis, MO, USA); *dl*-noradrenaline hydrochloride (Sankyo, Tokyo); α,β -methylene adenosine 5'-triphosphate lithium salt (α,β -MeATP; Research Biochemicals International, Natick, MA, USA). All drugs were dissolved in physiological saline before the start of the experiment. The stock solutions were kept at –20°C until used.

Fig. 1. Double peaked vasoconstrictor responses of an isolated, perfused canine splenic arterial preparation and the effects of prolonged cold storage (4°C) for 4, 7 and 14 days. The vessel was electrically stimulated by 30-s trains of pulses at 10-V amplitude and 1-ms pulse duration, with a frequency range of 1, 4 or 10 Hz. ES: Electrical nerve stimulation.

Statistical analyses

Vasoconstrictor responses to electrical stimulation or an agonist are expressed as the maximal changes in perfusion pressure (mmHg) from their basal levels. The data are presented as the mean $\pm$ S.E.M. An analysis of variance with Bonferroni's test was used for the statistical analysis of multiple comparisons of data. P values < 0.05 were considered statistically significant.

Fig. 2. Effects of the prolonged cold storage (4, 7 and 14 days at 4°C) on the first phase (A) and the second phase (B) of the biphasic vasoconstrictor responses to electrical nerve stimulation (10-V amplitude, 1-ms pulse duration and 30-s trains of pulses at stated frequencies) in the canine splenic arteries. ○: fresh arteries, ●: 4-day cold-stored arteries, △: 7-day cold-stored arteries, ▲: 14-day cold-stored arteries. Data are presented as the mean $\pm$ S.E.M., $n = 8-10$; * $P < 0.05$, ** $P < 0.01$, as compared with the fresh arteries.

RESULTS

Vascular responses to periarterial electrical nerve stimulation in cold stored arteries

The periarterial electrical stimulation (30-s trains of pulses) induced a double peaked vasoconstriction (2 phases of the vasoconstriction) in fresh splenic artery in a frequency-related manner and usually separated by an intervening dip in increasing perfusion pressure (Figs. 1A and 3A). The double-peaked vasoconstrictor responses at the used stimulation conditions in fresh splenic arteries were abolished by 30 nM tetrodotoxin (data not shown). Figure 1 shows the original tracings of contractile force from typical experiments showing the effects of the prolonged cold storage for 4, 7 and 14 days on the double-peaked vasoconstrictor responses to electrical nerve stimulation. As shown in Fig. 2A, the cold storing for 4, 7 and 14 days significantly inhibited the first phase responses in a stored-days-dependent manner. After 7 days of cold storage, the first response induced by 1 Hz was inhibited by 87%, whereas the one induced by 10 Hz was inhibited by 56% (Fig. 2A). The inhibitory effects of 4 and 7 days of cold storage on the first phases were irreversible even after perfusion with Krebs-Henseleit solution for 2 h (data not shown). Furthermore, the prolonged cold storage for 14 days blocked the first phase responses at the used frequencies (Fig. 2A). On the other

Fig. 3. Effects of prazosin and α, β -MeATP on the double-peaked vasoconstrictions in a 7-day cold-stored canine splenic artery. The vessel was electrically stimulated by 30-s trains of pulses at 10-V amplitude and 1-ms pulse duration, with a frequency range of 1, 4 or 10 Hz. ES: Electrical nerve stimulation.

hand, the cold storage for 4 days did not modify the second phase responses, but the second ones at the used frequencies were significantly inhibited in the 7-day cold stored arteries ($P < 0.05$, Fig. 2B). After 7 days of cold storage, the 1 Hz-induced second response was inhibited by 38% and 10 Hz-induced one, by 19% (Fig. 2B). Moreover, the cold storage for 14 days almost completely inhibited the second responses at any used frequencies (Fig. 2B).

Fig. 4. Effects of prazosin and α,β -MeATP on the first phase (A) and the second phase (B) vasoconstrictor responses to electrical nerve stimulation (10-V amplitude, 1-ms pulse duration and 30-s trains of pulses at stated frequencies) in the 7-day cold-stored canine splenic arteries. ○: fresh arteries, ●: 7-day cold-stored arteries, △: after 0.1 μ M prazosin in the 7-day cold-stored arteries, ▲: after 0.1 μ M prazosin and 1 μ M α,β -MeATP in the 7-day cold-stored arteries. Data are presented as the mean $\pm$ S.E.M., $n = 6-8$; * $P < 0.05$, ** $P < 0.01$, as compared with the 7-day cold-stored arteries and # $P < 0.05$, ## $P < 0.01$, as compared with the 0.1 μ M prazosin in the 7-day cold-stored arteries.

Effects of an α_1 -adrenoceptor antagonist, prazosin, and a $P2X$ -purinoceptor desensitizer, α,β -MeATP, on the remaining responses after cold storage for 7 days

The treatment with 0.1 μ M prazosin abolished the vasoconstrictor responses to exogenous NA (0.03–3 nmol) in the fresh arteries, but did not affect the contractile responses to ATP (0.01–1 μ mol). On the other hand, the perfusion with α,β -MeATP (1 μ M) blocked the contractile responses to ATP, but not to NA (data not shown). Figure 3 shows the original tracings of contractile force from typical experiments showing the effects of prazosin and α,β -MeATP on the remained responses to electrical nerve stimulation after cold storage for 7 days. As shown in Fig. 4A, the treatment with 0.1 μ M prazosin did not significantly affect the first phase responses at low frequencies (1–3 Hz) in the 7-day cold stored arteries, but significantly inhibited the first ones at high frequencies (8–10 Hz) ($P < 0.05$, Fig. 4A). A subsequent administration of α,β -MeATP (1 μ M) abolished the remaining responses after treatment with 0.1 μ M prazosin in the cold stored arteries for 7 days (Fig. 4A). On the other hand, in the 7-day cold-stored arteries, prazosin (0.1 μ M) almost completely inhibited the second phase responses at low frequencies (1–3 Hz) and substantially suppressed the second ones at high frequencies (6–10 Hz) (Fig. 4B). The remaining second responses at high frequencies after treatment with prazosin in the 7-day cold-stored arteries were blocked by a subsequent application of α,β -MeATP (1 μ M).

Fig. 5. Effects of the prolonged cold storage (4, 7 and 14 days at 4°C) on the vasoconstrictor response to tyramine in the isolated canine splenic arteries. ○: fresh arteries, ●: 4-day cold-stored arteries, △: 7-day cold-stored arteries, ▲: 14-day cold-stored arteries. Data are presented as the mean $\pm$ S.E.M., $n = 10-16$; * $P < 0.05$, ** $P < 0.01$, as compared with the fresh arteries.

Vascular responses to tyramine

Figure 5 shows the vasoconstrictor responses to tyramine of fresh and 4-, 7- or 14-day cold-stored isolated canine splenic arteries. When tyramine (0.01 – $1 \mu\text{mol}$) was intraluminally administered in fresh and 4- or 7-day cold-stored arteries, vasoconstrictions were produced in a dose-related manner. In fresh preparations, the contractile threshold concentration of tyramine was approximately $0.01 \mu\text{mol}$, and $0.3 \mu\text{mol}$ of tyramine caused a strong vasoconstriction such as one of over 250 mmHg . On the other hand, in the 4-day cold-stored preparations, the threshold concentration was approximately $0.03 \mu\text{mol}$, and $1 \mu\text{mol}$ of tyramine also induced a marked contractile response of over 250 mmHg . This shows that the concentration-response curve for tyramine in the 4-day cold-stored artery is shifted to the right in almost

a parallel manner. However, in the 7-day cold-stored artery, the maximal contractile responses to $1 \mu\text{mol}$ tyramine were obviously decreased, although $3 \mu\text{mol}$ tyramine consistently induced a marked vasoconstrictor response of about 250 mmHg . The tyramine-induced contractile response was abolished in the 14-day cold-stored preparation.

Vascular responses to NA and ATP

When a relatively large dose of ATP (0.01 – $1 \mu\text{mol}$) was given intraluminally, marked vasoconstrictions were introduced in a dose-related manner. After the cold storage for 4 or 7 days, the vasoconstrictions induced by low doses of ATP (0.01 – $0.1 \mu\text{mol}$) slightly increased, whereas the vasoconstrictor response of the 7-day cold-stored artery to a high dose of ATP ($1 \mu\text{mol}$) was slightly decreased. However, there were no significant differences between the fresh and 4- and 7-day cold-stored arteries. On the other hand, The vasoconstrictor responses to ATP were almost completely inhibited in the 14-day of cold-stored artery (Fig. 6A)

A bolus injection of NA (0.03 – 3 nmol) also induced a marked vasoconstriction in a dose-related manner. The vasoconstrictor responses to low doses of NA (0.1 – 0.3) were slightly increased in the 4- or 7-day cold-stored artery, but the contractile response to a high dose of NA (3 nmol) was slightly reduced after 7 days of cold storage. There were also no significant differences for the fresh and 4- or 7-day cold-stored arteries. Furthermore, after the 14 days of cold storage, the constrictor responses to NA were almost completely inhibited as shown in Fig. 6B.

DISCUSSION

Observations obtained in sympathetic nerve innervating blood vessel preparations have shown that a prolonged cold storage preferentially causes an irreversible degeneration of adrenergic nerve fibers, whereas post-junctional contractile responses to agonists might be relatively well maintained (2, 7). In 1993, Kristek et al. (2) reported that contraction of aortic rings in rabbits in response to transmural nerve stimulation was entirely abolished after 6 days of cold storage (4°C), whereas contractile responses to exogenous NA were unaffected. The similar results were also observed with the cold storage of the rat mesenteric small arteries for 5–8 days (4°C) (7). Recently, we reported that cold storing for 7 days did not abolish double-peaked vasoconstrictions in isolated canine splenic artery, with over 50% constrictor responses to periarterial nerve stimulation remaining, and contractile responses to exogenous ATP or NA were not influenced at all (16). The present experiments further

Fig. 6. Effects of the prolonged cold storage (4, 7 and 14 days at 4°C) on the vasoconstrictor responses to ATP (A) and NA (B) in the isolated canine splenic arteries. $\circ$: fresh arteries, $\bullet$: 4-day cold-stored arteries, $\triangle$: 7-day cold-stored arteries, $\blacktriangle$: 14-day cold-stored arteries. Data are presented as the mean $\pm$ S.E.M., $n=12$ –18; ** $P < 0.01$, as compared with the fresh arteries.

confirmed that the adrenergic transmission of sympathetic nerve terminals might be largely maintained even in the 4- or 7-day cold-stored preparations, although the purinergic component of response to nerve stimulation was clearly damaged. On the other hand, the cold storage for 14 days simultaneously abolished any vasoconstrictor responses to nerve stimulation and exogenous ATP or NA. These results indicated that presynaptic purinergic transmission may be gradually damaged followed by adrenergic dysfunction within 14 days of storage.

We recently demonstrated that P2X-purinoceptor and α_1 -adrenoceptor mechanisms are involved in the double-peaked vasoconstrictor responses to periarterial electrical nerve stimulation in the isolated canine splenic artery (14). At low frequencies (1–3 Hz), it was confirmed that the first phase constriction involves only a purinergic component because it was not blocked by prazosin, an α_1 -adrenoceptor blocking agent, but almost completely inhibited by treatment with α, β -MeATP (a P2X-purinoceptor desensitizing agent). At high frequencies (6–10 Hz), the first phase vasoconstriction involves largely purinergic and to a small extent some adrenergic components because the responses were partly inhibited by prazosin and the large remaining ones were abolished by α, β -MeATP. The second phase vasoconstriction at the used frequencies was dominantly inhibited by prazosin, and the remaining one was abolished by additional α, β -MeATP, indicating that the second phase response involves mainly NA and partially ATP (14). In the present study, the 4 days of cold storage preferentially inhibited the first phase response that might be mainly mediated via a release of ATP, whereas the second phase that might be mainly mediated via a release of NA was almost unmodified. Moreover, after the cold storage for 7 days, the first phase responses were almost completely inhibited at low frequencies, but at high frequencies, the first phase responses still remained in part. The first-peaked responses at a high frequency after the cold storage for 7 days may partially involve an adrenergic component that was inhibited by prazosin as shown in Fig. 4A. After cold storing for 7 days, the remaining second phase responses were markedly depressed by α_1 -adrenoceptor blockade, suggesting that the adrenergic component might be rather resistant to the cold storage. Since the vasoconstrictor responses to exogenous NA and ATP were not influenced by the 4 or 7 days of cold storage, postsynaptic responses of adrenergic and purinergic components might be not modified in these degrees of cold storage. Therefore, it is reasonable to consider that the cold storage for 4 days may preferentially inhibit the purinergic component, and 7 days of cold storage substantially injures the purinergic component of co-transmission presynaptically.

Kirkpatrick and Burnstock (19) reported that the in-

direct sympathomimetic tyramine releases endogenous ATP as a co-transmitter of NA from the vas deferens of the rat and guinea pig. However, Driessen et al. (20) observed that ATP release elicited by tyramine arises only from extraneuronal sites by the postjunctional action of NA at α_1 -adrenoceptors in the guinea pig vas deferens. This non-neural ATP does not seem to be involved in the contraction response to endogenous NA (20). In the canine splenic artery, the vasoconstrictor response to tyramine was not modified by α, β -MeATP, but blocked by prazosin, suggesting that responsiveness to tyramine would only represent a contractile function of adrenergic transmission (16). In this study, in the 4 days of cold storage, the second phase of constrictions (adrenergic component) to electrical nerve stimulation was not significantly modified at any used frequency as shown in Fig. 2B. However, the tyramine-induced dose-response curve was significantly shifted to the right in parallel as shown in Fig. 5. These results indicated that the 4 days of cold storage depress tyramine-dependent NA releasing mechanisms but do not depress electrical stimulated ones. It is proposed that tyramine releases NA by a mechanism that differs from that of electrical nerve stimulation; namely, the possible contribution of the extravesicular, axoplasmic fraction of NA to the releasing effect of tyramine (21). Thus, it is considered that 4 days of cold storage may exert a preferential inhibition of tyramine-dependent NA-releasing mechanisms over the electrical stimulated ones. We must precisely analyze the difference between these NA releasing mechanisms in the future. Alternatively, since tyramine releases NA from storage sites following uptake into the adrenergic nerve terminal (22), these findings might be explained if 4 days of cold storage produce a preferential inhibition on the uptake mechanism of tyramine.

It is hypothesized that ATP and NA are released as co-transmitters from the same axons (23, 24). The present results that the prolonged cold storage may exert a preferential inhibition on the purinergic component over the adrenergic component of sympathetic co-transmission, suggesting that ATP and NA are possibly released from two separate populations of exocytotic vesicles within the periarterial sympathetic nerve in the canine splenic artery, as proposed by Todorov et al. (25) and Westfall et al. (26). An alternative possibility is that the preferential inhibition of prolonged cold storage on purinergic transmission over adrenergic transmission reflects primarily the differences in susceptibilities of the biosynthesis of the two transmitters and breakdown processes to cold storage. Moreover, a proper cold storage may separate the two different kinds of NA-releasing mechanisms between nerve excitation and tyramine.

In conclusion, the prolonged cold storage of the canine

splenic artery may produce a more preferential inhibition on prejunctional contractile functions of sympathetic co-transmission over the postjunctional one. The 4°C cold storage for 4 days may preferentially damage the tyramine-dependent NA-releasing mechanisms, whereas nerve-excited ones might be resistant. After the cold storage for 7 days, the prejunctional contractile response of purinergic transmission might be depressed more preferentially than that of adrenergic transmission. In the 14-day cold-stored canine splenic artery, postjunctional contractile functions of co-transmission were damaged almost completely.

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