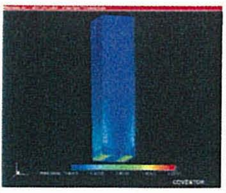
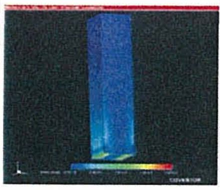
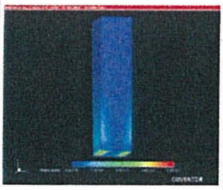
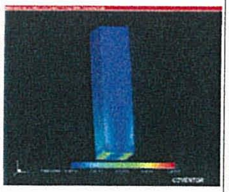
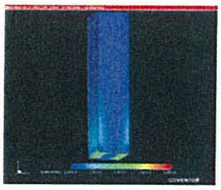
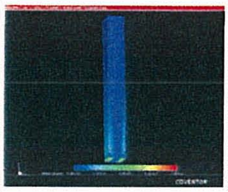
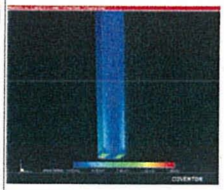
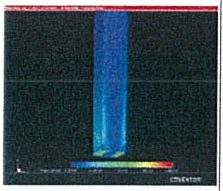
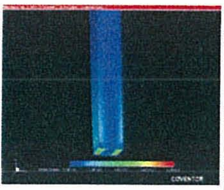
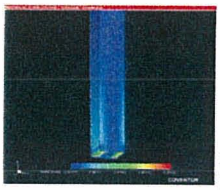
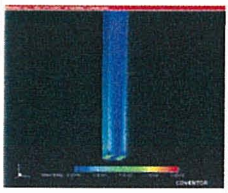
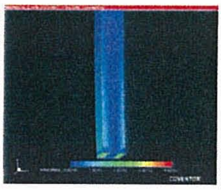
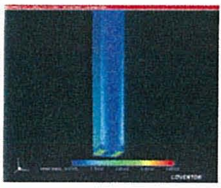
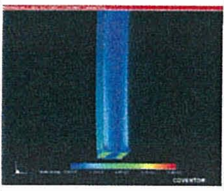
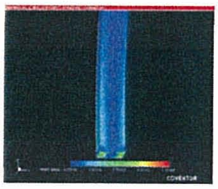
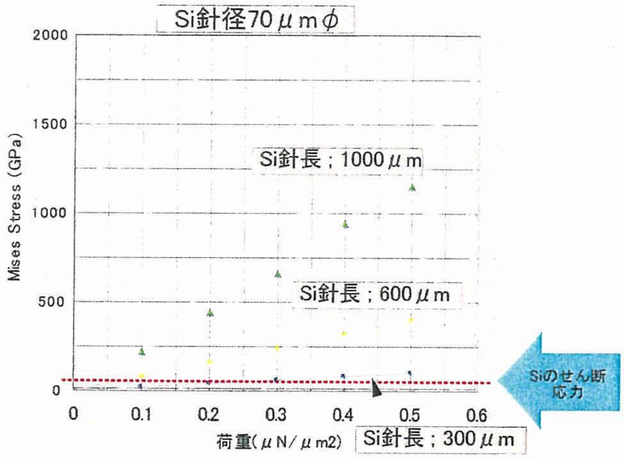
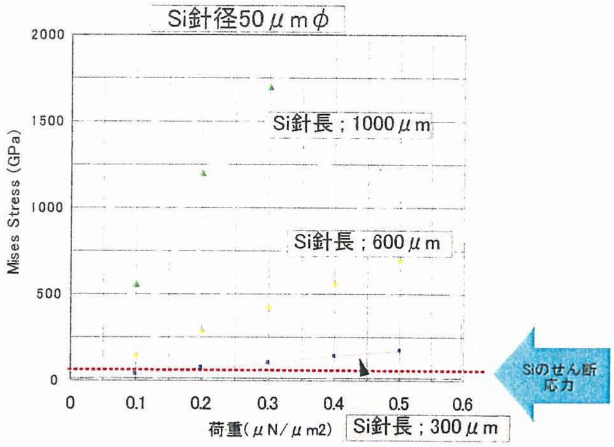
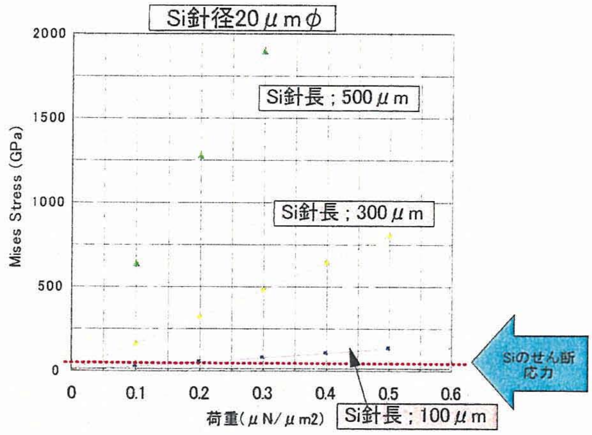
	荷重:0．1µN／µm2	荷重:0．2µN／µm2	荷重:0．3µN／µm2	荷重:0．4µN／µm2	荷重:0．5µN／µm2
針長： 300µm					
針長： 600µm					
針長： 1000µm					

また、せん断応力の針長さと、印加荷重依存性のシミュレーション結果を示す。







針径に対する許容針高さの推測

Si針径　70µφ　の場合　：　高さ　300µm　程度

Si針径　50µφ　の場合　：　高さ　300µm　未満

Si針径　20µφ　の場合　：　高さ　200µm　以下

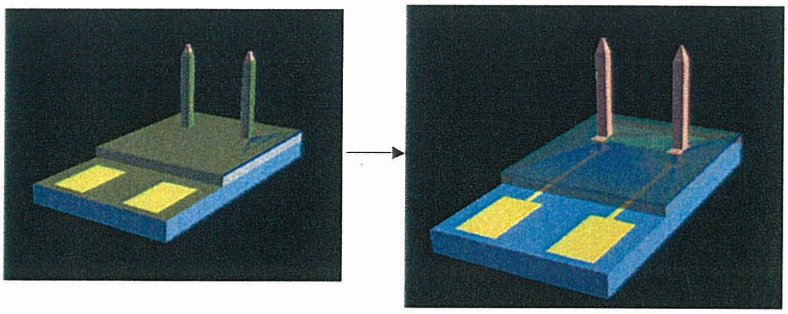
以上のシミュレーション結果から、次のように考察された。

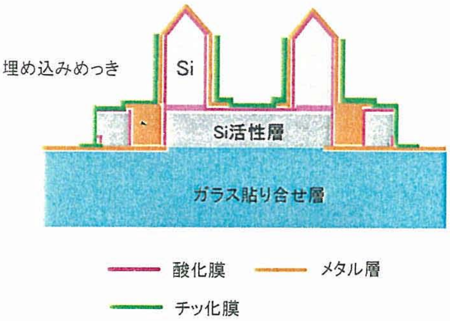
１）針部分に発生する応力は針底部（Ｂｏｘ－ＳｉＯ２膜と接着している面）に最大応力が発生する。

２）応力は針長さの増加に比例しているため、神経記録装置の機械強度を保つには針の長さを適切に設定して折れにくい構造にする必要がある。

３）しかしながら、単結晶Ｓｉのせん断応力（～２０ＧＰａ）を考慮すると、今回のシミュレーションから得られる装置寸法仕様は、針長さ３００μｍ程度、最大印加荷重０．２μＮ／μm2以下と思われる。ヒト自律神経モニターに用いる神経は直径0.5－1.5 cmであり、針長は、少なくとも1mm程度の長さが必要である。従って、Ｓｉ針は、実用化に不向きであると考えられた。

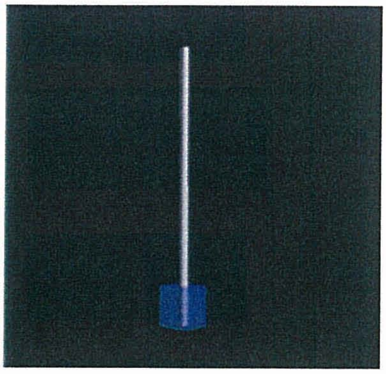
参考までに、導通電極付Ｓｉ針を有する神経電極構造体について、貫通電極なし構造体試作結果から、導通電極を有するＳｉ材による神経電極記録装置の実際のプロセスに即した構造概略を下図に示す。これは、Ｓｉピン全体にメタル層がついており、メタル層（先端のみ露出／ＳｉＮ膜カバー）はフィードスルーメッキ電極を介してガラス上の配線を経由して電極パッドへつながっている。ピン太さは５０～７０μｍ径、長さは３００～５００μｍ程度である。





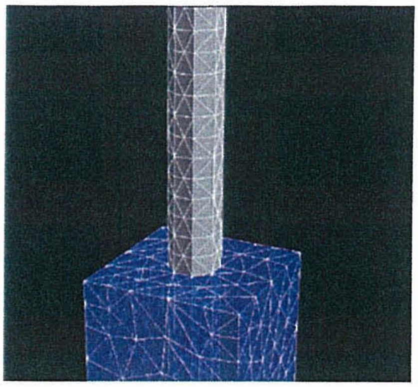
＜タングステン製鍼の機械強度シミュレーション＞

タングステン（W）製自律神経活動記録装置の断面形状シミュレーションし、針にかかる応力を計算した。シミュレーションには、下記のような３次元ソリッドモデルと、メッシュを用いた。



W針長さ1000μmの場合

Glass基板を半透明にし、W差込み部が見えるようにしています

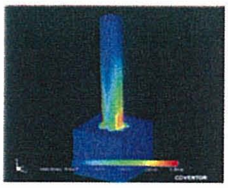
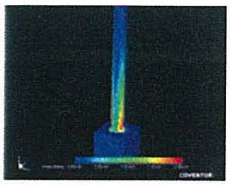
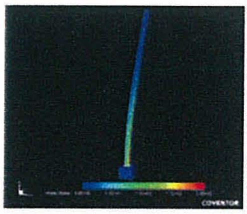
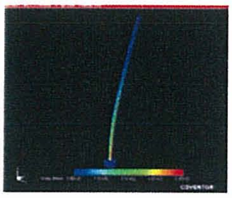


メッシュ型	テトラヘドロン
メッシュサイズ	20〜40μm

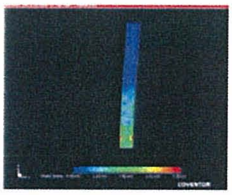
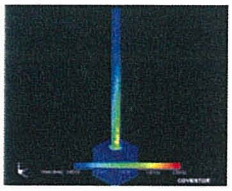
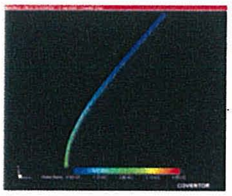
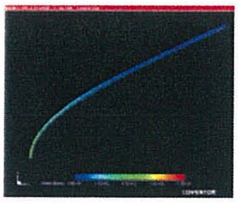
※せん断応力面指定のため、実際には針形状は8角形にしている。

針部分に発生した応力をカラー表示した、シミュレーション結果を下に示す。

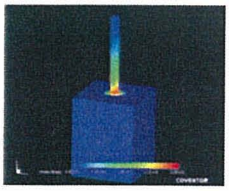
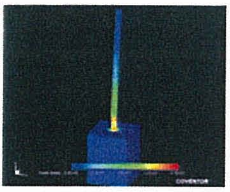
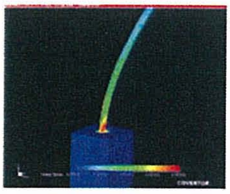
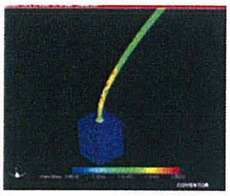
針太さ　80μmφ

	荷重:0.1μN／μm2
針長: 500μm	
針長: 1000μm	
針長: 3000μm	
針長: 5000μm	

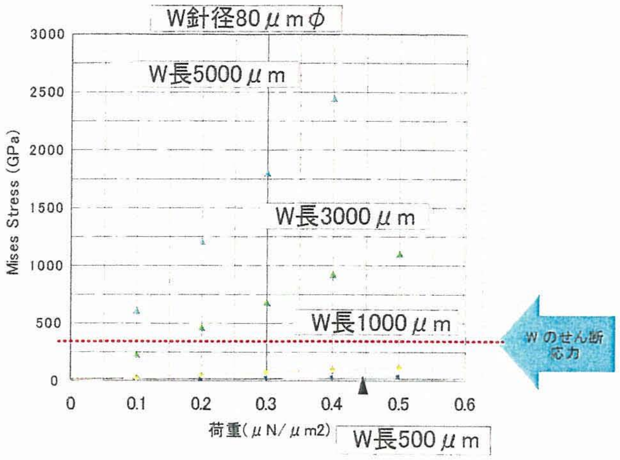
針太さ　50μmφ

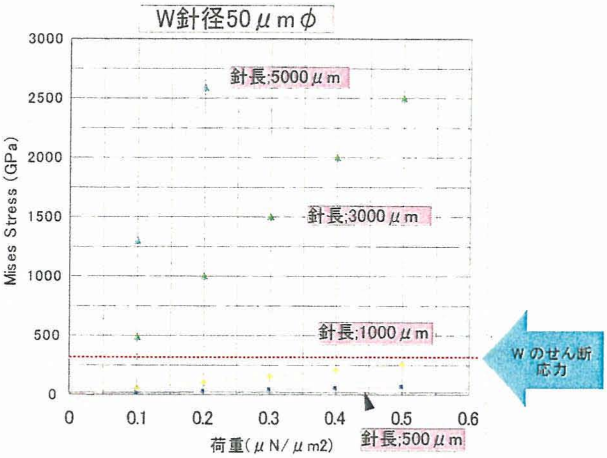
	荷重:0.1μN／μm2
針長: 500μm	
針長: 1000μm	
針長: 3000μm	
針長: 5000μm	

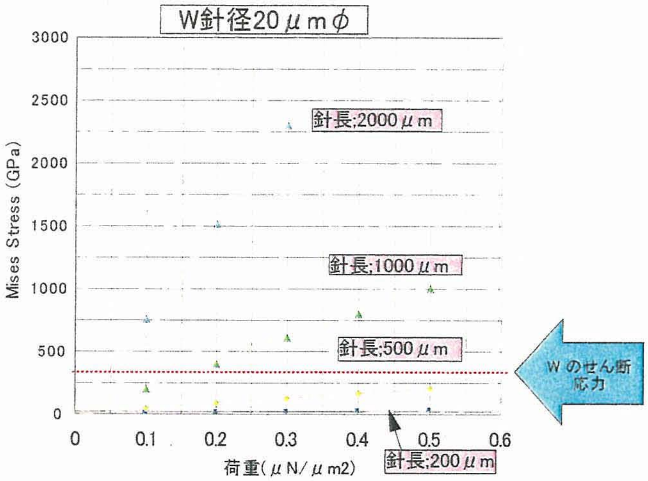
針太さ　20μmφ

	荷重:0.1μN／μm2
針長: 200μm	
針長: 500μm	
針長: 1000μm	
針長: 2000μm	

また、せん断応力の針長さと、印加荷重依存性のシミュレーション結果を示す。







針径に対する許容針高さの推測

W針径　80μφ　の場合　：　高さ　3mm　程度まで

W針径　50μφ　の場合　：　高さ　2mm　程度まで

W針径　20μφ　の場合　：　高さ　1mm　程度まで

以上のシミュレーションから、次のように考察された。

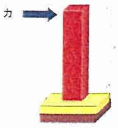
1）針部分に発生する応力は針の基板出口部分を支点にしているが、出口付近から根元上まで応力が分散し、Siよりも弾性があることが分かる。

2）応力は針の長さが増加するに従い増加するため、神経記録装置の機械強度を保つためには極力針の長さを短くするべきであるが、Si針よりは弾性力も大きく、耐せん断応力値も高いので、針の長さを長く設定できる。

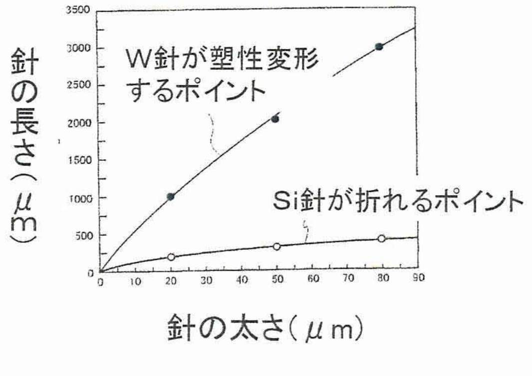
3）Wのせん断応力（～300ＧＰａ）を考慮すると、今回のシミュレーションから得られる装置寸法仕様は、針長さ1～3mm（針径20～80μm）/ 最大印加荷重　０．２μＮ／μｍ２であると考えられる。

上記の、ＳｉとWを材質とした針の、シミュレーション結果から、Siのストレート鍼とWストレート鍼では、同じ太さで約1桁の耐応力性の差があることが分かった。Siは加工しやすくより小型化したデバイス製作には有効であると考えられるが、Si鍼を使用する場合はストレート構造では折れやすく、神経束中で折れた場合

には神経障害へ発展する危険性も想定される。従って、神経活動を安全かつ的確に記録し、且つ刺激する実用型デバイスとして、W鍼を針材とするのが望ましいと考えられた。



有限要素法応力シミュレーションによる機械的強度の検討

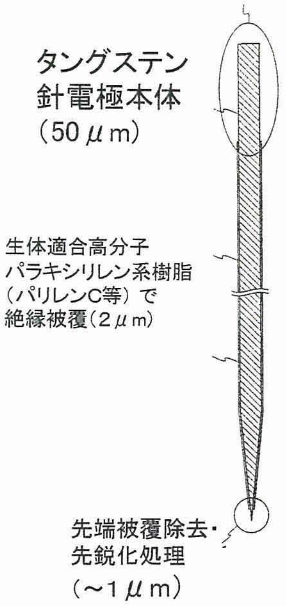


- Si針は、機械的強度が弱く、実用に不向き
- W針は、機械的強度・耐剪断応力性に優れ（針全体に応力分散）自由な針長の微細電極アレイを製作可能

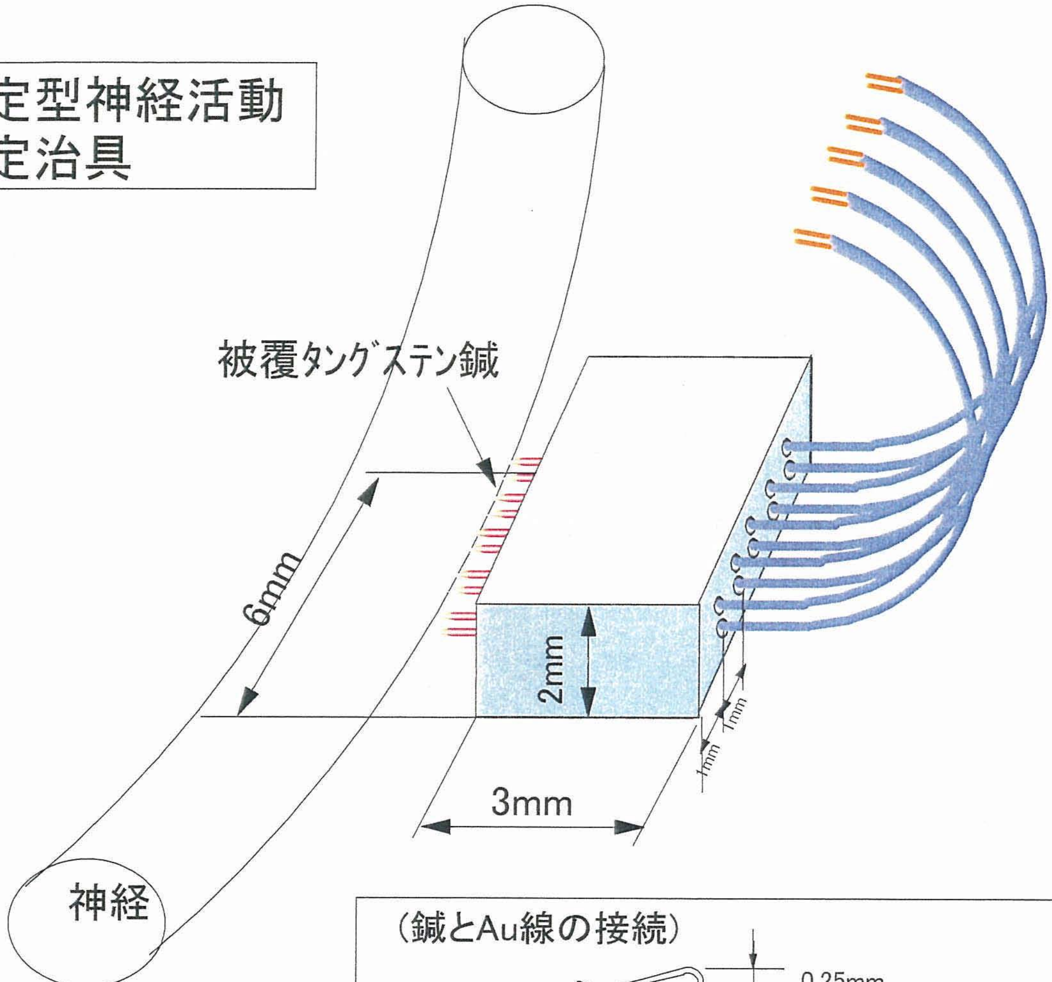
２．W針電極アレイの１次試作

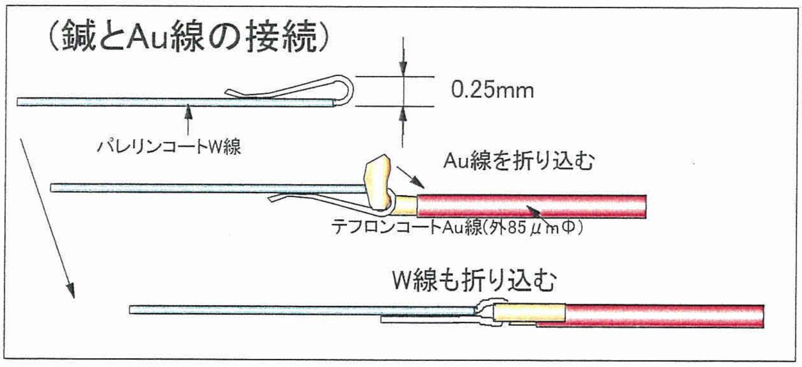
針材をタングステンに決定し、安全生体材料（パリレン）を薄膜コート（2μm）し、電解エッチングで先端ナノ先鋭化処理し、ウサギ腓骨・脛骨神経への刺入テストによって、神経膜貫通性や強度の点から針シャフト径を50μmと決定した。

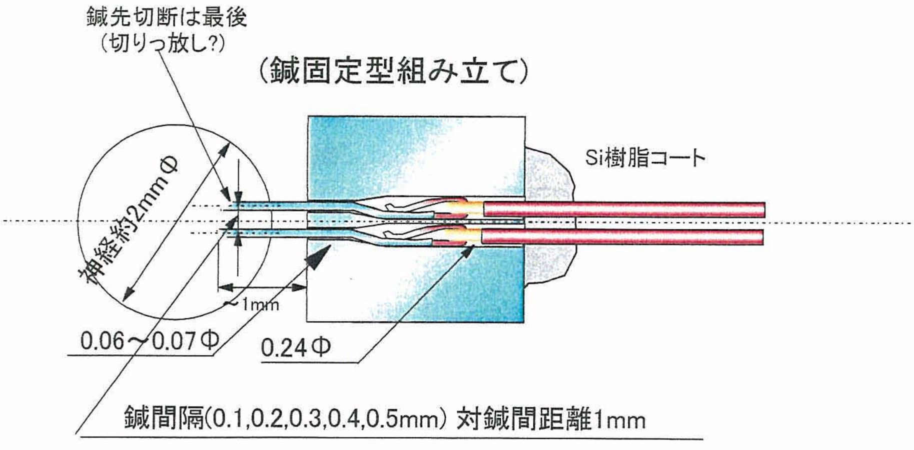
先ず実際に動物神経でテストするためのW鍼用の台座構造案を考えた。下図に示すように、一対をなすW鍼の間隔を100～500μmとし、図のような穴間隔の異なる複数(10本)の鍼を1つの樹脂台座にAu線を絡めた上体で植え込み、Si接着剤などで固定する構造を考えた。

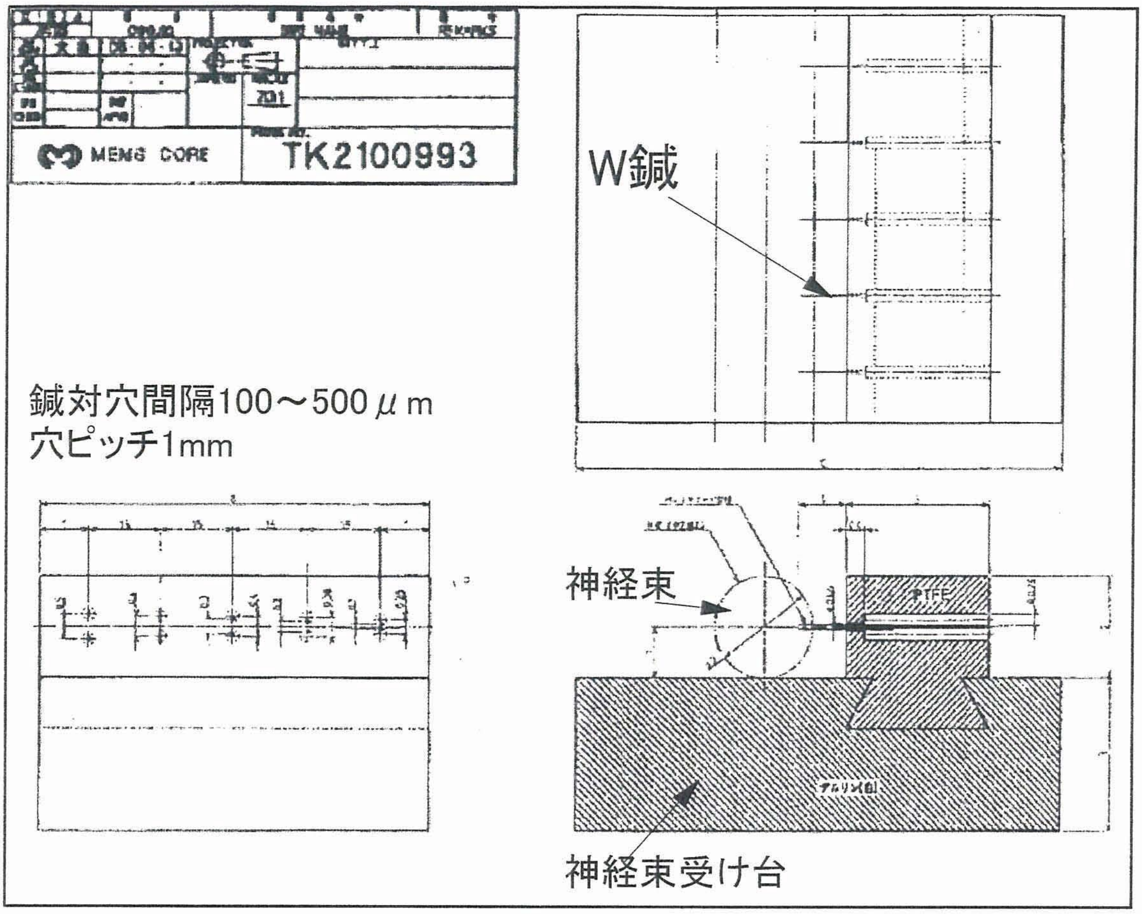


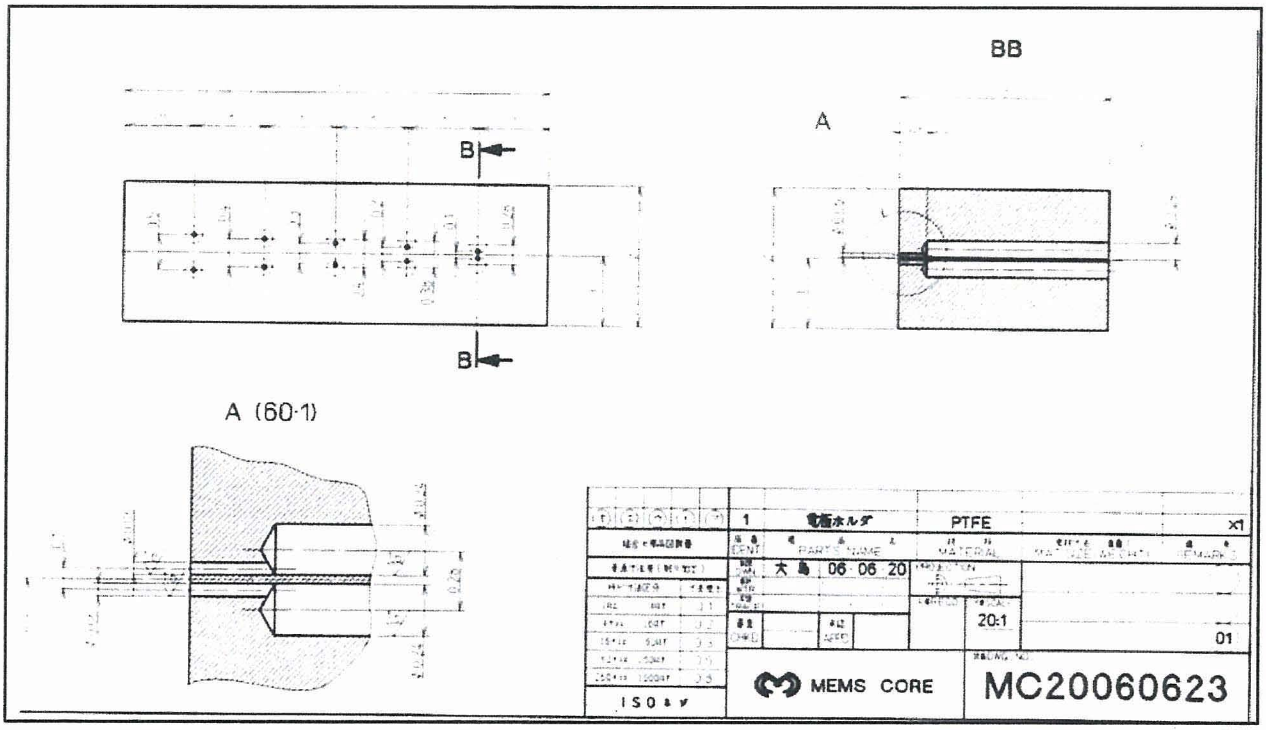
固定型神経活動測定治具











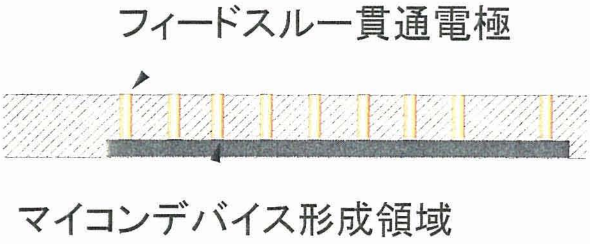
上記電極アレイを、ウサギ腓骨・脛骨神経への刺入するテストによって、神経膜貫通性や強度の点から針シャフト径を50μmと決定した。また、神経束内に交感神経線維が大半を占めると考えられる

腎臓交感神経に電極アレイを装着した所、腎臓交感神経活動を記録できた。そこで、装置の２次へと開発を進めた。

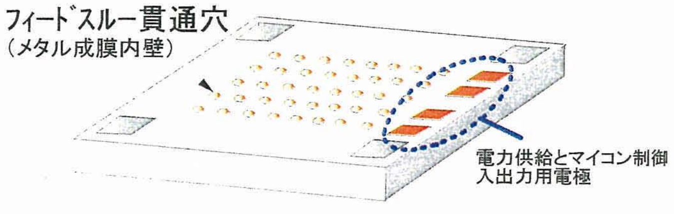
３．W針電極アレイの２次試作

２次試作装置の基本構成と製作プロセス概略を示す。

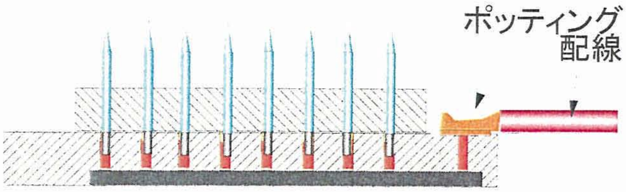
プロセス１
Ｓｉフィードスルー電極付マイコンデバイスチップ

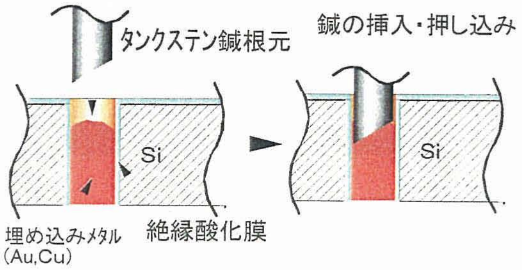


プロセス２
Ｓｉフィードスルー－Ｃｕ埋め込み



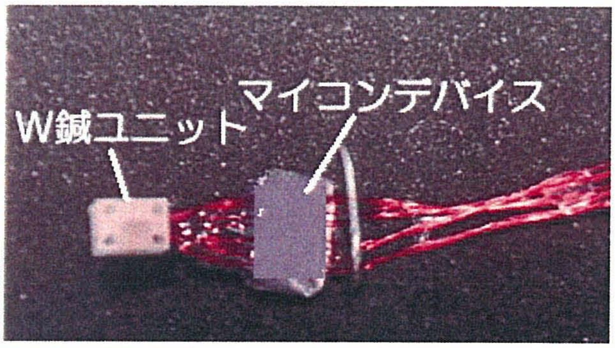
プロセス３
鍼付ベースとマイコンチップの合体
（鍼電極とマイコンの接続）

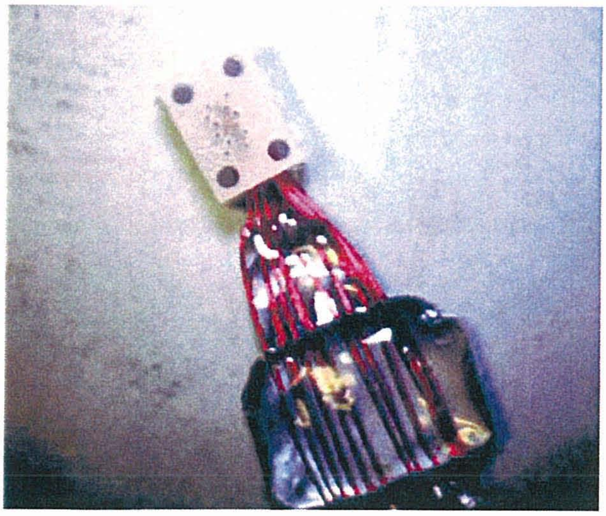




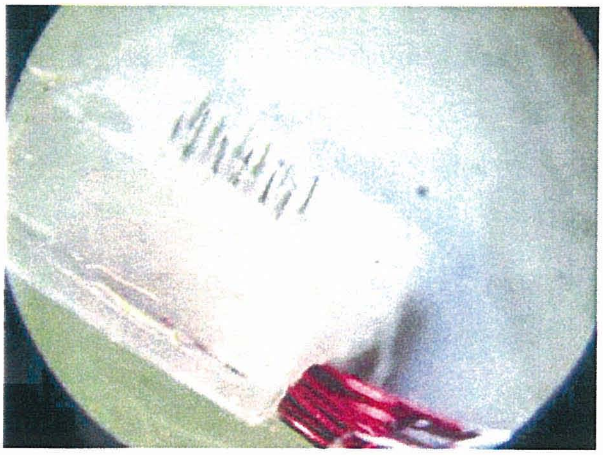
実際に、W針を電極間隔100μmに2列12ピン集積化した装置を下に示す。これは、世界最小レベルの電極アレイの神経装置である。ヒト自律神経モニターに用いる神経は直径0.5－1.5 cmであり、この仕様で50―150本の針電極を１本の神経に挿入できるため、この電極アレイは、実用に十分な空間分解能（神経線維選択能）であると考えられる。

＜装置全体＞





＜電極アレイ＞



＜針電極＞

＜倫理面への配慮＞

本研究の動物実験は、日本生理学学会の動物実験の指針に沿い、実験動物の数と侵襲を最小にするよう、また、動物愛護上においても、十分配慮して行われた。また、国立循環器病センター研究所実験動物委員会に承認のもとに、行われた。

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Ｆ．知的財産権の出願・登録状況

１．特許取得

【発明の名称】神経信号用プローバ、神経信号出力装置、神経信号記録装置、神経刺激装置及び神経信号入出力装置

【名称】神経信号用プローバ、神経信号出力装置、神経信号記録装置、神経刺激装置及び神経信号入出力装置

【発明者】神谷厚範、杉町　勝、桜井史敏、慶光院利映

【出願日】平成19年2月1日

【出願番号】特願2007-023501

２．実用新案登録

なし。

３．その他

なし。

研究成果の刊行に関する一覧表

書籍

なし

雑誌

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Hypothermia reduces ischemia- and stimulation-induced myocardial interstitial norepinephrine and acetylcholine releases

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Atsunori Kamiya,[1] Kazunori Uemura,[1] Hidezo Mori,[2] and Masaru Sugimachi[1]**
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Kawada T, Kitagawa H, Yamazaki T, Akiyama T, Kamiya A, Uemura K, Mori H, Sugimachi M. Hypothermia reduces ischemia- and stimulation-induced myocardial interstitial norepinephrine and acetylcholine releases. *J Appl Physiol* 102: 622–627, 2007. First published November 2, 2006; doi:10.1152/japplphysiol.00622.2006.—Although hypothermia is one of the most powerful modulators that can reduce ischemic injury, the effects of hypothermia on the function of the cardiac autonomic nerves in vivo are not well understood. We examined the effects of hypothermia on the myocardial interstitial norepinephrine (NE) and ACh releases in response to acute myocardial ischemia and to efferent sympathetic or vagal nerve stimulation in anesthetized cats. We induced acute myocardial ischemia by coronary artery occlusion. Compared with normothermia ($n = 8$), hypothermia at 33°C ($n = 6$) suppressed the ischemia-induced NE release [63 nM (SD 39) vs. 18 nM (SD 25), $P < 0.01$] and ACh release [11.6 nM (SD 7.6) vs. 2.4 nM (SD 1.3), $P < 0.01$] in the ischemic region. Under hypothermia, the coronary occlusion increased the ACh level from 0.67 nM (SD 0.44) to 6.0 nM (SD 6.0) ($P < 0.05$) and decreased the NE level from 0.63 nM (SD 0.19) to 0.40 nM (SD 0.25) ($P < 0.05$) in the nonischemic region. Hypothermia attenuated the nerve stimulation-induced NE release from 1.05 nM (SD 0.85) to 0.73 nM (SD 0.73) ($P < 0.05$, $n = 6$) and ACh release from 10.2 nM (SD 5.1) to 7.1 nM (SD 3.4) ($P < 0.05$, $n = 5$). In conclusion, hypothermia attenuated the ischemia-induced NE and ACh releases in the ischemic region. Moreover, hypothermia also attenuated the nerve stimulation-induced NE and ACh releases. The Bezold-Jarisch reflex evoked by the left anterior descending coronary artery occlusion, however, did not appear to be affected under hypothermia.

vagal nerve; sympathetic nerve; cardiac microdialysis; cats

HYPOTHERMIA IS ONE OF THE most powerful modulators that can reduce ischemic injury in the central nervous system, heart, and other organs. The general consensus is that hypothermia induces a hypometabolic state in tissues and balances energy supply and demand (25). With respect to the myocardial ischemia, the size of a myocardial infarction correlates with temperature (6), and mild hypothermia can protect the myocardium against acute ischemic injury (9). The effects of hypothermia on the function of the cardiac autonomic nerves in terms of neurotransmitter releases, however, are not fully understood. Because autonomic neurotransmitters such as norepinephrine (NE) and ACh directly impinge on the myocardium, they would be implicated in the cardioprotection by hypothermia.

In previous studies from our laboratory, Kitagawa et al. (16) demonstrated that hypothermia attenuated the nonexocytotic NE release induced pharmacologically by ouabain, tyramine, or cyanide. Kitagawa et al. (15) also demonstrated that hypothermia attenuated the exocytotic NE release in response to vena cava occlusion or to local administration of high K^+. The effects of hypothermia on the ischemia-induced myocardial interstitial NE release, however, were not examined in those studies. In addition, the effects of hypothermia on the ischemia-induced myocardial interstitial ACh release have never been examined. Because both sympathetic and parasympathetic nerves control the heart, simultaneous monitoring of the myocardial interstitial releases of NE and ACh (14, 31) would help integrative understanding of the autonomic nerve terminal function under hypothermia in conjunction with acute myocardial ischemia.

In the present study, the effects of hypothermia on the ischemia-induced and nerve stimulation-induced myocardial interstitial neurotransmitter releases were examined. We implanted a dialysis probe into the left ventricular free wall of anesthetized cats and measured dialysate NE and ACh levels as indexes of neurotransmitter outputs from the cardiac sympathetic and vagal nerve terminals, respectively. Based on our laboratory's previous results (15, 16), we hypothesized that hypothermia would attenuate the neurotransmitter releases in response to acute myocardial ischemia and to electrical nerve stimulation.

MATERIALS AND METHODS

Surgical Preparation and Protocols

Animals were cared for in accordance with the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences*, approved by the Physiological Society of Japan. All protocols were reviewed and approved by the Animal Subjects Committee of National Cardiovascular Center. Adult cats were anesthetized via an intraperitoneal injection of pentobarbital sodium (30–35 mg/kg) and ventilated mechanically through an endotracheal tube with oxygen-enriched room air. The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (1–2 $mg \cdot kg^{-1} \cdot h^{-1}$) through a catheter inserted from the right femoral vein. Mean arterial pressure (MAP) was measured using a pressure transducer connected to a catheter inserted from the right femoral artery. Heart rate (HR) was determined from an electrocardiogram.

Protocol 1: acute myocardial ischemia. We examined the effects of hypothermia on the ischemia-induced myocardial interstitial releases of NE and ACh. The heart was exposed by partially removing the left fifth and/or sixth rib. A dialysis probe was implanted transversely into

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the anterolateral free wall of the left ventricle perfused by the left anterior descending coronary artery (LAD) to monitor myocardial interstitial NE and ACh levels in the ischemic region during occlusion of the LAD (13). Another dialysis probe was implanted transversely into the posterior free wall of the left ventricle perfused by the left circumflex coronary artery to monitor myocardial interstitial NE and ACh levels in a nonischemic region. Heparin sodium (100 U/kg) was administered intravenously to prevent blood coagulation. Animals were divided into a normothermic group ($n = 8$) and a hypothermic group ($n = 6$). In the hypothermic group, surface cooling with ice bags was performed until the esophageal temperature decreased to 33°C (15, 16). A stable hypothermic condition was obtained within ~2 h. In each group, we occluded the LAD for 60 min and examined changes in the myocardial interstitial NE and ACh levels in the ischemic region (i.e., the LAD region) and nonischemic region (i.e., the left circumflex coronary artery region). Fifteen-minute dialysate samples were obtained during the preocclusion baseline condition and during the periods of 0–15, 15–30, 30–45, and 45–60 min of the LAD occlusion.

Protocol 2: sympathetic stimulation. We examined the effects of hypothermia on the sympathetic nerve stimulation-induced myocardial interstitial NE release ($n = 6$). A dialysis probe was implanted transversely into the anterolateral free wall of the left ventricle. The bilateral cardiac sympathetic nerves originating from the stellate ganglia were exposed through a second intercostal space and sectioned. The cardiac end of each sectioned nerve was placed on a bipolar platinum electrode for sympathetic stimulation (5 Hz, 10 V, 1-ms pulse duration). The electrodes and nerves were covered with mineral oil to provide insulation and prevent desiccation. A 4-min dialysate sample was obtained during the sympathetic stimulation under the normothermic condition. Thereafter, hypothermia was introduced using the same cooling procedure as in *protocol 1*, and a second 4-min dialysate sample was obtained during the sympathetic stimulation.

Protocol 3: vagal stimulation. We examined the effects of hypothermia on the vagal nerve stimulation-induced ACh release ($n = 5$). A dialysis probe was implanted transversely into the anterolateral free wall of the left ventricle. The bilateral vagi were exposed through a midline cervical incision and sectioned at the neck. The cardiac end of each sectioned nerve was placed on a bipolar platinum electrode for vagal stimulation (20 Hz, 10 V, 1-ms pulse duration). To prevent severe bradycardia and cardiac arrest, which can be induced by the vagal stimulation, the heart was paced at 200 beats/min using pacing wires attached to the apex of the heart during the stimulation period. A 4-min dialysate sample was obtained during the vagal stimulation under the normothermic condition. Thereafter, hypothermia was introduced using the same cooling procedure as in *protocol 1*, and a second 4-min dialysate sample was obtained during the vagal stimulation.

Because of the relatively intense stimulation of the sympathetic or vagal nerve, the stimulation period in *protocols 2* and *3* was limited to 4 min to minimize gradual waning of the stimulation effects. At the end of the experiment, the animals were killed by increasing the depth of anesthesia with an overdose of pentobarbital sodium. We then confirmed that the dialysis probes had been threaded in the middle layer of the left ventricular myocardium.

Dialysis Technique

The dialysate NE and ACh concentrations were measured as indexes of myocardial interstitial NE and ACh levels, respectively. The materials and properties of the dialysis probe have been described previously (2, 3). Briefly, we designed a transverse dialysis probe. A dialysis fiber (13-mm length, 310-μm outer diameter, 200-μm inner diameter; PAN-1200, 50,000 molecular weight cutoff; Asahi Chemical) was connected at both ends to polyethylene tubes (25-cm length, 500-μm outer diameter, 200-μm inner diameter). The dialysis probe was perfused with Ringer solution containing a cholinesterase inhibitor eserine (10^{-4} M) at a rate of 2 μl/min. We started dialysate sampling from 2 h after the implantation of the dialysis probe(s), when the dialysate NE and ACh concentrations had reached steady states. The actual dialysate sampling was delayed by 5 min from the collection period to account for the dead space volume between the semipermeable membrane and the sample tube. Each sample was collected in a microtube containing 3 μl of HCl to prevent amine oxidation. The dialysate ACh concentration was measured directly by HPLC with electrochemical detection (Eicom). The in vitro recovery rate of ACh was ~70%. With the use of a criterion of signal-to-noise ratio of higher than three, the detection limit for ACh was 3 pg per injection. The dialysate NE concentration was measured by another HPLC-electrochemical detection system after the removal of interfering compounds by an alumina procedure. The in vitro recovery rate of NE was ~55%. With the use of a criterion of signal-to-noise ratio of higher than three, the detection limit for NE was 200 fg per injection.

Statistical Analysis

All data are presented as means and SD values. For *protocol 1*, we performed two-way repeated-measures ANOVA using hypothermia as one factor and the dialysate sampling periods (the effects of ischemia) as the other factor. For *protocols 2* and *3*, we compared stimulation-induced releases of NE and ACh before and during hypothermia using a paired *t*-test. For all of the statistics, the difference was considered significant when $P < 0.05$.

RESULTS

Figure 1*A* illustrates changes in myocardial interstitial NE levels in the ischemic region during LAD occlusion obtained from *protocol 1*. The *inset* shows the magnified ordinate for the

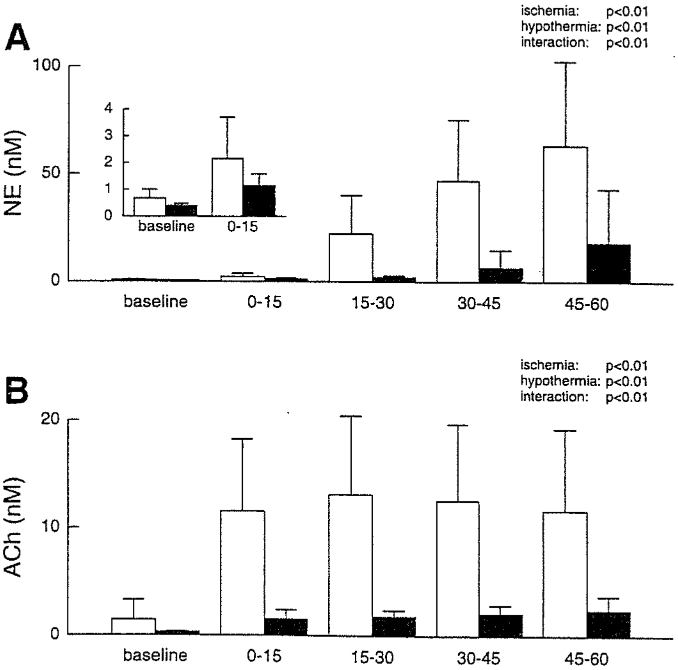

Fig. 1. *A*: ischemia-induced myocardial interstitial norepinephrine (NE) release in the ischemic region. Acute myocardial ischemia caused a progressive increase in the level of myocardial interstitial NE. Hypothermia attenuated the ischemia-induced NE release. *Inset*: magnified ordinate for the baseline and the 0- to 15-min period of ischemia. *B*: ischemia-induced myocardial interstitial ACh release in the ischemic region. Acute myocardial ischemia increased the myocardial interstitial ACh levels. Hypothermia attenuated the ischemia-induced ACh release. Open bars: normothermia; solid bars: hypothermia.

baseline and the 0- to 15-min period of ischemia. In the normothermic group (open bars), the LAD occlusion caused an ~94-fold increase in the NE level during the 45- to 60-min interval. In the hypothermic group (solid bars), the LAD occlusion caused an ~45-fold increase in the NE level during the 45- to 60-min interval. Compared with normothermia, hypothermia suppressed the baseline NE level to ~59% and the NE level during the 45- to 60-min period to ~29%. Statistical analysis indicated that the effects of both hypothermia and ischemia on the NE release were significant, and the interaction between hypothermia and ischemia was also significant.

Figure 1*B* illustrates changes in myocardial interstitial ACh levels in the ischemic region during the LAD occlusion. In both the normothermic (open bars) and hypothermic (solid bars) groups, the LAD occlusion caused an approximately eightfold increase in the ACh level during the 45- to 60-min interval. Compared with normothermia, however, hypothermia suppressed both the baseline ACh level and the ACh level during the 45- to 60-min period of ischemia to ~20%. Statistical analysis indicated that the effects of both hypothermia and ischemia on the ACh release were significant, and the interaction between hypothermia and ischemia was also significant.

Figure 2*A* illustrates changes in myocardial interstitial NE levels in the nonischemic region during the LAD occlusion. Note that scale of the ordinate is only one-hundredth of that in Fig. 1*A*. The LAD occlusion decreased the NE level in the normothermic group (open bars); the NE level during the 45- to 60-min interval was ~59% of the baseline level. The LAD occlusion also decreased the NE level in the hypothermic group (solid bars); the NE level during the 45- to 60-min interval was ~64% of the baseline level. Although the LAD occlusion resulted in a decrease in the NE level under both conditions, the NE level under hypothermia was nearly twice that measured under normothermia. The statistical analysis indicated that the effects of both hypothermia and ischemia on the NE release were significant, whereas the interaction between hypothermia and ischemia was not significant.

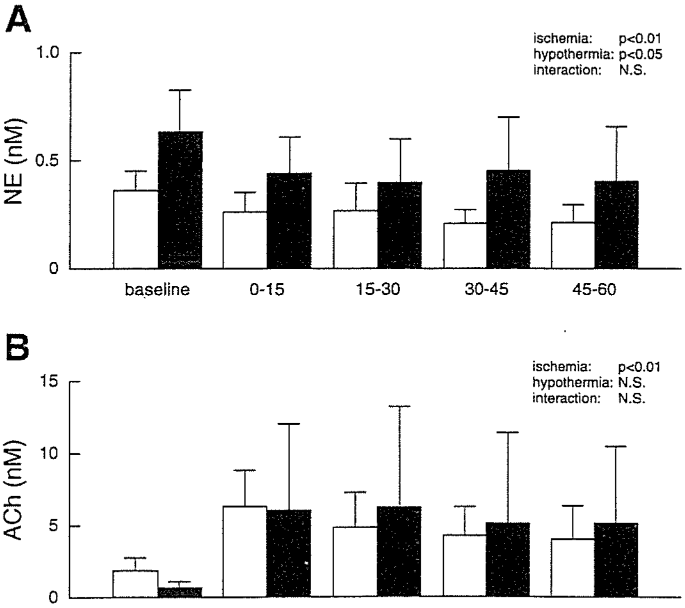

Fig. 2. *A*: changes in the myocardial interstitial NE levels in the nonischemic region. Acute myocardial ischemia decreased the level of myocardial interstitial NE from the baseline level. Hypothermia increased the myocardial interstitial NE levels in the nonischemic region. *B*: changes in the myocardial interstitial ACh levels in the nonischemic region. Acute myocardial ischemia increased the myocardial interstitial ACh level. Hypothermia did not attenuate the increasing response of ACh to the left anterior descending coronary artery occlusion. Open bars: normothermia; solid bars: hypothermia. NS, not significant.

Table 1. *Mean arterial pressure during acute myocardial ischemia obtained in protocol 1*

	Baseline	5 min	15 min	30 min	45 min	60 min
Normothermia	108 (23)	102 (28)	101 (24)	101 (20)	102 (21)	102 (21)
Hypothermia	108 (11)	80 (17)	87 (10)	85 (10)	86 (10)	91 (11)

Values are means (SD) (in mmHg) obtained during preocclusion baseline period and 5-, 15-, 30-, 45-, and 60-min periods of coronary artery occlusion. Ischemia: $P < 0.01$; hypothermia: not significant; interaction: $P < 0.01$.

Figure 2*B* illustrates changes in myocardial interstitial ACh levels in the nonischemic region during the LAD occlusion. The LAD occlusion caused an ~3.4-fold increase in the ACh level during the 0- to 15-min interval in the normothermic group (open bars). The LAD occlusion caused an approximately ninefold increase in the ACh level during the 0- to 15-min interval in the hypothermic group (solid bars). These effects of ischemia on the ACh release were statistically significant. Although hypothermia seemed to attenuate the baseline ACh level, the overall effects of hypothermia on the ACh level were insignificant.

Tables 1 and 2 summarize the MAP and HR data, respectively, obtained in *protocol 1*. Acute myocardial ischemia significantly reduced MAP ($P < 0.01$) and HR ($P < 0.01$). Hypothermia did not affect MAP but did decrease HR ($P < 0.01$). The interaction between ischemia and hypothermia was significant for MAP but not for HR by the two-way repeated-measures ANOVA.

For *protocol 2*, hypothermia significantly attenuated the sympathetic stimulation-induced NE release to ~70% of the level observed during normothermia (Fig. 3*A*). Under normothermia, the sympathetic stimulation increased MAP from 114 mmHg (SD 27) to 134 mmHg (SD 33) ($P < 0.01$) and HR from 147 beats/min (SD 9) to 207 beats/min (SD 5) ($P < 0.01$). Under hypothermia, the sympathetic stimulation increased MAP from 117 mmHg (SD 11) to 136 mmHg (SD 22) ($P < 0.05$) and HR from 125 beats/min (SD 16) to 164 beats/min (SD 10) ($P < 0.01$).

For *protocol 3*, hypothermia significantly attenuated the vagal stimulation-induced ACh release to ~70% of the level observed during normothermia (Fig. 3*B*). Hypothermia did not change MAP [117 mmHg (SD 18) vs. 118 mmHg (SD 27)] but

Table 2. *Heart rate during acute myocardial ischemia obtained in protocol 1*

	Baseline	5 min	15 min	30 min	45 min	60 min
Normothermia	183 (26)	160 (18)	163 (16)	163 (18)	166 (20)	165 (21)
Hypothermia	146 (25)	116 (19)	113 (19)	126 (39)	112 (20)	97 (31)

Values are means (SD) (in beats/min) obtained during preocclusion baseline period and 5-, 15-, 30-, 45-, and 60-min periods of coronary artery occlusion. Ischemia: $P < 0.01$; hypothermia: $P < 0.01$; interaction: not significant.

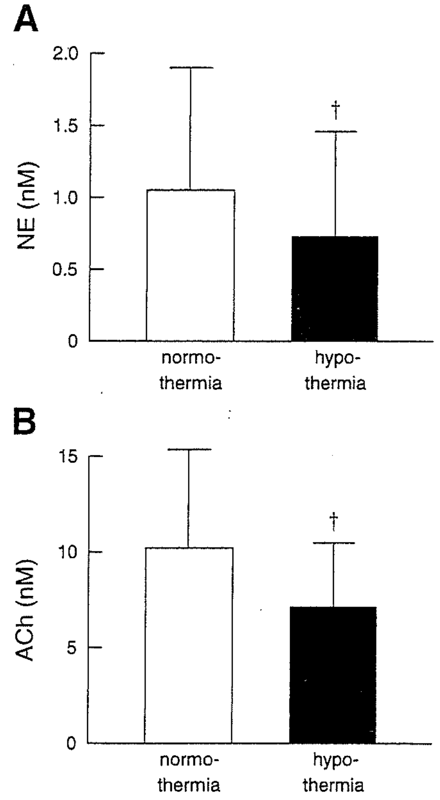

Fig. 3. *A*: efferent sympathetic nerve stimulation-induced release of myocardial interstitial NE before and during hypothermia. †Hypothermia significantly attenuated the stimulation-induced NE release. *B*: efferent vagal nerve stimulation-induced release of myocardial interstitial ACh before and during hypothermia. †Hypothermia significantly attenuated the stimulation-induced ACh release.

did decrease HR from 202 beats/min (SD 24) to 179 beats/min (SD 15) ($P < 0.05$) during the prestimulation, unpaced condition. MAP during the stimulation was 105 mmHg (SD 19) under normothermia and 93 mmHg (SD 33) under hypothermia.

DISCUSSION

A cardiac microdialysis is a powerful tool to estimate neurotransmitter levels in the myocardial interstitium in vivo (2, 3, 14, 19, 20, 31). The present study demonstrated that hypothermia significantly attenuated the myocardial interstitial releases of NE and ACh in the ischemic region during the LAD occlusion. In contrast, the increasing response in the ACh level from its baseline level and the decreasing response in the NE level from its baseline level observed in the nonischemic region were maintained under hypothermia. To our knowledge, this is the first report showing the effects of hypothermia on the myocardial interstitial releases of NE and ACh during acute myocardial ischemia in vivo. In addition, the present study showed that hypothermia significantly attenuated nerve stimulation-induced myocardial interstitial NE and ACh releases in vivo.

Effects of Hypothermia on Ischemia-induced NE and ACh Releases in the Ischemic Region

Acute myocardial ischemia causes energy depletion, which leads to myocardial interstitial NE release in the ischemic region (Fig. 1*A*). The NE release can be classified as exocytotic or nonexocytotic (18, 24). Exocytotic release indicates NE release from synaptic vesicles, which normally occurs in response to nerve discharge and subsequent Ca^{2+} influx through voltage-dependent Ca^{2+} channels. On the other hand, nonexocytotic release indicates NE release from the axoplasm, such as that mediated by a reverse transport through the NE transporter. A neuronal uptake blocker, desipramine, can suppress the ischemia-induced NE release (19, 24). Whereas exocytotic release contributes to the ischemia-induced NE release in the initial phase of ischemia (within ~20 min), carrier-mediated nonexocytotic release becomes predominant as the ischemic period is prolonged (1). Hypothermia significantly attenuated the ischemia-induced NE release (Fig. 1*A*). The NE level during the 45- to 60-min period of ischemia under hypothermia was ~20% of that obtained under normothermia. The NE uptake transporter is driven by the Na^+ gradient across the cell membrane (23). The loss of the Na^+ gradient due to ischemia causes NE to be transported out of the cell by reversing the action of the NE transporter. Hypothermia inhibits the action of the NE transporter and also suppresses the intracellular Na^+ accumulation (8), thereby reducing nonexocytotic NE release during ischemia. The present results are in line with an in vitro study that showed hypothermia suppressed nonexocytotic NE release induced by deprivation of oxygen and glucose (30). The present results are also consistent with a previous study from our laboratory that showed hypothermia attenuated the nonexocytotic NE release induced by ouabain, tyramine, or cyanide (16).

Acute myocardial ischemia increases myocardial interstitial ACh level in the ischemic region, as reported previously (Fig. 1*B*) (13). The level of ischemia-induced ACh release during 0- to 15-, 15- to 30-, 30- to 45-, or 45- to 60-min period of ischemia is comparable to that evoked by 4-min electrical stimulation of the bilateral vagi (Fig. 3*B*). Compared with the normothermic condition, hypothermia significantly attenuated the ischemia-induced myocardial interstitial release of ACh in the ischemic region. Our laboratory's previous study indicated that intracellular Ca^{2+} mobilization is essential for the ischemia-induced release of ACh (13). Hypothermia may have prevented the Ca^{2+} overload, thereby reducing the ischemia-induced ACh release. Alternatively, hypothermia may reduce the extent of the ischemic injury, which in turn suppressed the ischemia-induced ACh release. Because ACh has protective effects on the cardiomyocytes against ischemia (11), the suppression of ischemia-induced ACh release during hypothermia itself may be unfavorable for cardioprotection.

There is considerable controversy regarding the cardioprotective effects of β-adrenergic blockade during severe ischemia, with studies demonstrating a reduction of infarct size (10, 17) or no effects (7, 27). The β-adrenergic blockade seems effective to protect the heart only when the heart is reperfused within a certain period after the coronary occlusion. The β-adrenergic blockade would reduce the myocardial oxygen consumption through the reduction of HR and ventricular contractility and delay the progression of ischemic injury. Hence the infarct size might be reduced when the heart is reperfused before the ischemic damage becomes irreversible. The ischemia-induced NE release reached nearly 100 times the baseline NE level under normothermia (Fig. 1*A*), which by far exceeded the NE level attained by electrical stimulation of the bilateral stellate ganglia (Fig. 3*A*). Because high NE levels have cardiotoxic effects (22), ischemia-induced NE release might aggravate the ischemic injury. However, catecholamine depletion by a reserpine treatment fails to reduce the infarct size (26, 29), throwing a doubt on the involvement of catecholamine toxicity in the progression of myocardial damage during ischemia. It is, therefore, most likely that the hypothermia-induced reductions in NE and ACh are the result of reduced myocardial damage or a direct effect on nerve endings.

Van den Doel et al. (28) showed that hypothermia does not abolish necrosis, but rather delays necrosis during sustained ischemia, so that hypothermia protected against infarction produced by a 30-min occlusion but not against infarction produced by a 60-min occlusion in the rat heart. At the same time, they mentioned that hypothermia was able to reduce the infarct size after a 60-min coronary occlusion in the dog, possibly because of the significant collateral flow in the canine hearts. Because the feline hearts are similar to the canine hearts in that they have considerable collateral flow compared with the rat hearts (21), hypothermia should have protected the feline heart against the 60-min coronary occlusion in the present study.

Effects of Hypothermia on the NE and ACh Releases in the Nonischemic Region and on the Electrical Stimulation-induced NE and ACh Releases

The NE and ACh levels in the nonischemic region may reflect the sympathetic and parasympathetic drives to this region. As an example, myocardial interstitial ACh levels increase during activations of the arterial baroreflex and the Bezold-Jarisch reflex (14). In the present study, acute myocardial ischemia decreased the NE level from its baseline level, whereas it increased the ACh level from its baseline level (Fig. 2). Ischemia also decreased MAP and HR (Tables 1 and 2), suggesting that the Bezold-Jarisch reflex was induced by the LAD occlusion under both normothermia and hypothermia. Taking into account the fact that electrical stimulation-induced ACh release was attenuated to ~70% (Fig. 3), similar ACh levels during ischemia imply the enhancement of the parasympathetic outflow via the Bezold-Jarisch reflex under hypothermia. These results are in line with the study by Zheng et al. (32), where pulmonary chemoreflex-induced bradycardia was maintained under hypothermia. Hypothermia increased the NE level in the nonischemic region, suggesting that sympathetic drive to this region also increased. Hypothermic stress is known to cause sympathetic activation, accompanying increases in MAP, HR, plasma NE, and epinephrine levels (4). In the present study, because the effect of hypothermia on MAP was insignificant (Table 1) and HR decreased under hypothermia (Table 2), the sympathetic activation observed in the nonischemic region might have been regional and not systemic.

Hypothermia attenuated the releases of NE and ACh in response to respective nerve stimulation to ~70% of that observed under normothermia (Fig. 3). The suppression of the exocytotic NE release by hypothermia is consistent with a previous study from our laboratory, where hypothermia attenuated the myocardial interstitial NE release in response to vena cava occlusion or to a local high K^+ administration (15). The suppression of NE release by hypothermia is consistent with an in vitro study by Kao and Westhead (12) in which catecholamine secretion from adrenal chromaffin cells induced by elevated K^+ levels increased as the temperature increased from 4 to 37°C. On the other hand, because hypothermia inhibits the neuronal NE uptake, the NE concentration at the synaptic cleft is expected to be increased if the level of NE release remains unchanged. Actually, Vizi (30) demonstrated that hypothermia increased NE release in response to field stimulation in vitro. In the present study, however, the suppression of NE release might have canceled the potential accumulation of NE due to NE uptake inhibition. The present study also demonstrated that the ACh release was suppressed by hypothermia. In the rat striatum, hypothermia decreases the extracellular ACh concentration and increases the choline concentration (5). Hypothermia may inhibit a choline uptake transporter in the same manner as it inhibits a NE uptake transporter. The inhibition of the choline transporter by hypothermia may have hampered the replenishment of the available pool of ACh and thereby contributed to the suppression of the stimulation-induced ACh release.

Limitations

In *protocol 1*, because we did not measure the infarct size in the present study, the degree of myocardial protection by hypothermia was undetermined. Whether the reduction of ischemia-induced neurotransmitter release correlates with the reduction of infact size requires further investigations. In *protocols 2* and *3*, baseline NE and ACh levels were not measured. The reduction of stimulation-induced NE and ACh release by hypothermia might be partly due to the reduction of baseline NE and ACh levels. However, because transection of the stellate ganglia (31) or vagi (3) reduces the baseline NE and ACh levels, changes in the baseline NE and ACh levels by hypothermia in *protocols 2* and *3* could not be as large as those observed under innervated conditions in *protocol 1* (Figs. 1 and 2).

In conclusion, hypothermia attenuated the ischemia-induced releases of NE and ACh in the ischemic region to ~30 and 20% of those observed under normothermia, respectively. Hypothermia also attenuated the nerve stimulation-induced releases of NE and ACh to ~70% of those observed during normothermia. In contrast, hypothermia did not affect the decreasing response in the NE level and the increasing response in the ACh level in the nonischemic region, suggesting that the Bezold-Jarisch reflex evoked by the LAD occlusion was maintained.

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