twitch produced a 66.8 ± 5.1% (n = 4) depression of the contraction induced by α,β-mATP (fig. 2B).

The tissue was relatively insensitive to exogenous ATP, even high doses eliciting small responses. Exogenous ATP ($> 10^{-4}$ M) gave dose-related contractions which rose to a peak and subsequently declined during continuous perfusion. Subsequent single shocks in the presence of ATP elicited biphasic twitches with both phases significantly ($p < 0.01$) depressed relative to the controls. 5×10^{-4} M ATP depressed the nonadrenergic and adrenergic phases of the twitch by 77.1 ± 4.9% and 80.2 ± 3.8% (n = 6) respectively. This depression was not prevented by 8-phenyltheophylline (10^{-5} M), a presynaptic P_1-purinoceptor antagonist[11]. This suggests that the P_2-purinoceptors are rapidly desensitized on exposure to ATP[12].

Nifedipine or verapamil did not abolish the ATP-induced contraction. In the presence of nifedipine (10^{-5} M) or verapamil (10^{-5} M) the peak size of the contraction to ATP (5×10^{-4} M) was reduced by 51.7 ± 5.2% (n = 5) and 32.7 ± 1.8% (n = 4) respectively. This result contrasts with the effect of these calcium channel blockers on the non-adrenergic phase of single shock responses.

Discussion. In different tissues including the rat vas deferens, nifedipine and verapamil abolish responses which result from the activation of voltage-sensitive Ca^{2+}-channels[13–15]. The results presented in this paper demonstate clearly that transmitters released neurally, compared to exogenous application, have different sensitivities to Ca^{2+}-channel blockers. Although the same post-junctional α_1-adrenoceptors mediate the responses to neurally released NA and to bath applied NA, only the latter is abolished by nifedipine. This suggests that whilst the NA-induced contraction depends on Ca^{2+} influx through nifedipine sensitive Ca^{2+}-channels, the adrenergic slow phase of the twitch probably develops in response to the release of sequestered Ca^{2+}[14]. Replacement of Ca^{2+} with Sr^{2+}, which supports transmitter release, results in the marked depression and subsequent inhibition of only the adrenergic slow phase[16], presumably because the sequestered pool is not being replenished. Furthermore the longer latency of the adrenergic slow phase[17] indicates the involvement of secondary internal processes. This process, although requiring Ca^{2+}, may use a different tension generating mechanism such as myosin phosphorylation[18].

The non-adrenergic rapid phase of the twitch, recently attributed to the action of neurally released ATP on post-synaptic P_2-purinoceptors[8], is abolished by nifedipine but not by verapamil. In contrast the activation of these purinoceptors with ATP or its stable analogue α,β-mATP elicits a contractile response that is not abolished or at best is only partially sensitive to either Ca^{2+}-channel blocker. Again, the responses to neurally released and to exogenously applied transmitters depend on Ca^{2+} influx through different voltage-sensitive Ca^{2+}-channels.

A variety of Ca^{2+}-channels have been identified in different tissues including smooth muscles[2]. The Ca^{2+}-channels involved in spike generation activate and inactivate rapidly, others activate relatively slowly in response to sustained depolarization. Nifedipine selectively abolishes the generation of action potentials and the non-adrenergic phase of the twitch in the guinea pig and rat vasa deferentia[5], probably indicating the involvement of the rapidly activating Ca^{2+}-channels. Activation of post-synaptic P_2-purinoceptors or α_1-adrenoceptors by exogenous ATP or NA respectively produces a depolarization of the smooth muscle cells of the guinea pig vas deferens[19,20]. If this is also true of the rat then the contractions to exogenous ATP, α,β-mATP or NA, which are sensitive to verapamil as well as nifedipine, must result from Ca^{2+} influx through the slowly activating Ca^{2+}-channels. This is consistent with the finding that the influx of labeled Ca^{2+} and the contractions of the rat vas deferens induced by elevated KCl is abolished by both verapamil and nifedipine[21]. Likewise the susceptability of the adrenergic secondary component of the tetanic response contrasts with the relative insensitivity of the adrenergic phase of the twitch to nifedipine[6] and verapamil[22]. Thus where there is a sustained depolorisation the responses appear to depend on slowly activating Ca^{2+} channels which are sensitive to both verapamil and nifedipine. It remains a puzzle why doses of nifedipine which abolished the nonadrenergic phase of the twitch, supposedly purinergic, failed to block the response to exogenous ATP.

In conclusion these results demonstrate that the sources of Ca^{2+} utilized by the smooth muscle, and thus the effectiveness of Ca^{2+}-channel blockers, may depend not only on the transmitter involved but also on the stimulus duration and origin (i.e. neurally released or exogenously applied) of the agonist.

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Diltiazem prevents the damage to cultured aortic smooth muscle cells induced by hyperlipidemic serum

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Summary. Diltiazem, a calcium antagonist, significantly reduced the increased ^{45}Ca uptake and the number of dead cells in cultured aortic smooth muscle cells induced by hyperlipidemic serum.
Key words. Diltiazem; hyperlipidemia; cultured aortic smooth muscle.

Calcium antagonists have been shown to retard atherosclerosis in the cholesterol-fed rabbit[1,2]. However, their mechanism of action remains obscure. These agents reduce the blood pressure and platelet function which are thought to play an important role in the development and progression of atherosclerosis. To avoid these actions, we used cultured aortic smooth muscle cells in this study. We examined the effect of diltiazem, a calcium antagonist, on the damage to cultured aortic smooth muscle cells induced by hyperlipidemic serum (HLS).

Materials and methods. Cultured aortic smooth muscle cells were prepared according to the modified method of Chamley et al.[3]. Thoracic aortas from Japan White rabbits (obtained from the Ohita Lab. Anim. Co., Japan), weighing 2–2.5 kg, were dissected out under sterile conditions. Aortas were placed in Hank's balanced salt solution (HBSS) containing 0.1% collagenase (Worthington Biochem. Co.) at 37 °C for 30 min. The adventitias were completely stripped off using watchmakers' forceps and discarded. After that, the aortas were cut longitudinally and placed in fresh Hank's solution containing 0.1% collagenase at 37 °C for 30 min with occasional gentle agitation. The remaining medias were washed with Dulbecco's modified Eagle's medium (DME) and placed in DME containing 0.1% collagenase and 0.05% elastase (Sigma Chem. Co.) at 37 °C for 120 min with occasional gentle agitation. The cell suspension was centrifuged at 100× g for 10 min. Cells were resuspended in DME and counted. 5×10^5 cells were seeded in plastic culture dishes, 35 mm in diameter. The culture medium was DME with 10% fetal bovine serum (FBS), 0.1 mg per ml of streptomycin and 100 units per ml of penicillin. The cells were incubated at 37 °C in an atmosphere of 5% CO_2, and the culture medium was changed every 2 days.

When the cells were confluent, the culture medium was replaced by one containing 5% FBS for 12 h, and then replaced by one containing 1% FBS for 24 h to stop the proliferation. After that procedure, the culture medium was replaced by either DME, DME with HLS (final cholesterol concentration 1 mg/ml) or DME with HLS and diltiazem (final concentration 1 µg/ml), and incubated for 48 h. HLS was prepared by feeding Japan White rabbits with an atherogenic diet, containing normal rabbit chow with 1% cholesterol (Oriental Yeast Co. Japan) for at least 10 weeks. Blood was collected sterilely and serum was obtained by centrifugation at 250× g for 10 min after blood clot retraction. The serum was inactivated by heating at 56 °C for 30 min and was dialyzed at 4 °C for 48 h against at least 10 volumes of HBSS, pH 7.4. The concentration of cholesterol, triglyceride and HDL-cholesterol in HLS were 1210 mg/dl, 480 mg/dl and 40 mg/dl respectively. Sterile pure powder of diltiazem was supplied by Tanabe Co. Japan.

The cytotoxicity of HLS for the cultured aortic smooth muscle cells was determined by the trypan blue exclusion test in each of the 10 dishes. Cultured aortic smooth muscle cells were dispersed by 0.05% trypsin-EDTA and washed with normal saline by centrifugation. The trpyan blue was added to the cell suspension at a final concentration of 2%. The number of stained cells and unstained cells in five areas on the hemocytometer were counted. The percentage of injured cells was equal to stained cells/stained + unstained cells. We performed a double blind counting of the samples.

The ^{45}Ca activity of cultured aortic smooth muscle cells was determined according to the method of Fenton et al.[4] in each of the 10 dishes. The ^{45}Ca was added to the culture medium (3 µCi/ml) at the time when the culture medium was replaced by one of following media: DME, DME + diltiazem, DME + HLS and DME + HLS + diltiazem. After incubation for 48 h in the presence of ^{45}Ca, the medium was removed and cultured cells were washed 5 times with a total 10 ml of Ca-EGTA solution within 15 s. The washed cultured cells were suspended with 1 ml of 1 N NaOH solution and the radioactivity of an aliquot of the NaOH solution was measured in a liquid scintillation spectrometer (Aloka 903 SP). Another aliquot was used for the measurement of the protein content by the method of Lowry et al.[5]. The ^{45}Ca activities of cultured cells are expressed as a mean±SD cpm/mg protein, and statistical analysis was performed using Student's t-test.

Results. The trypan blue-positive cultured aortic smooth muscle cells, dead cells, were significantly increased by the incubation in a HLS solution for 48 h as compared to those in a control solution (22 ± 2% vs 14 ± 3%: $p < 0.001$). Adding diltiazem to the HLS solution reduced the percentage of dead cells significantly (15 ± 4%) (fig. 1). The ^{45}Ca activity in cultured aortic smooth muscle cells was significantly increased by adding HLS as compared to the control (3061 ± 370 cpm/mg protein: vs 1746 ± 145 cpm/mg protein: $p < 0.001$), and this elevated ^{45}Ca activity was significantly reduced by the addition of diltiazem (2449 ± 402 cpm/mg protein) (fig. 2).

Discussion. It is well-known that atherosclerosis is accompanied by an accumulation of calcium in arterial walls, and a proliferation and necrosis of smooth muscle cells occurs together[6]. Necrosis of smooth muscle cells containing massive amounts of lipid may release membrane-active lipids affecting the membrane function of neighboring cells and accelerate cell proliferation. HLS is known to cause necrosis in cultured aortic smooth muscle cells[7]. If calcium antagonists directly inhibit HLS-induced necrosis of smooth muscle cells containing massive amounts of lipid, some part of the mechanism of protection from atherosclerosis by calcium antagonists in experimental animals may be explained. In this study, diltiazem clearly suppressed the HLS-induced necrosis of cultured aortic smooth muscle cells in the absence of effects of the blood pressure and platelet function. Yokoyama and Henry[8] demonstrated that a high cholesterol environment sensitized isolated arteries to the constrictor effects of calcium, and they suggested that excess cholesterol in the membrane of smooth muscle cells enhanced the permeability for calcium ions. The ^{45}Ca activity of cultured aortic smooth muscle

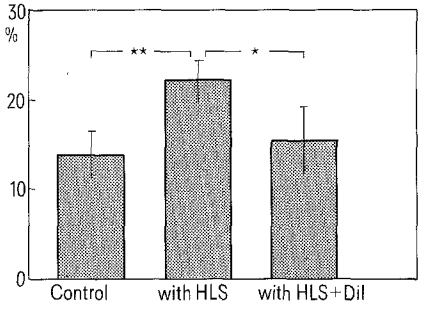

Figure 1. The proportion of dead cells in cultured aortic smooth muscle cells, determined by the trypan blue exclusion test. Diltiazem (Dil) significantly reduced the proportion of dead cells induced by hyperlipidemic serum (HLS). * $p < 0.01$; ** $p < 0.001$.

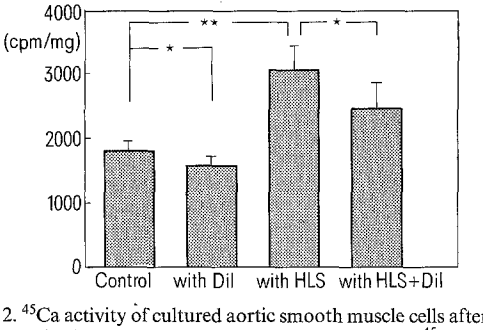

Figure 2. ^{45}Ca activity of cultured aortic smooth muscle cells after incubation for 48 h. Diltiazem (Dil) significantly reduced the ^{45}Ca activity of cultured cells without HLS as well as the elevated ^{45}Ca activity of cultured cells induced by hyperlipidemic serum (HLS). * $p < 0.01$; ** $p < 0.001$.

cells was increased by adding HLS for 48 h in this study, which confirms their suggestion. The increase was not a consequence of an increased number of dead cells containing large amounts of calcium, because most of the dead cells, which were detached from the dish, could be removed by the washing procedure. Calcium overloading is thought to be a final common pathway of cell necrosis[9], and calcium antagonists are well known to reduce the calcium accumulation and cell necrosis in various disorders[10].

We used the concentration of 1 µg/ml of diltiazem in this study, because this dose was reported to bring about an 80% reduction in the sustained tension development of the thoracic aorta induced by ouabain but little change in the resting tension development and action potential duration of the papillary muscle[11,12]. This dose of diltiazem significantly reduced the calcium content of cultured aortic smooth muscle cells without HLS as well as the elevated calcium content of cultured aortic smooth muscle cells with HLS (fig. 2). On the basis of these data, we concluded that diltiazem, a calcium antagonist, suppressed the HLS-induced necrosis of cultured aortic smooth muscle cells by reducing the intracellular calcium content; and this might play an important role in the mechanisms of protection from atherosclerosis by calcium antagonists.

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Temperature dependence of neurotransmitter release in the antarctic fish *Pagothenia borchgrevinki*

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Summary. The quantal contents of endplate potentials from extraocular muscles of an antarctic fish *Pagothenia borchgrevinki* were measured over a range of temperatures. Quantal release was maximal at about 5 °C but showed little dependence on temperature between −2 °C and 10 °C. Above 10 °C quantal content declined until release ceased about 18 °C. In view of the fact that the ambient temperature at which these fish live is constant at −1.9 °C, the results suggest that *Pagothenia borchgrevinki* is only partially adapted to its environment despite 25 million years acclimatization.

Key words. Neuromuscular junction; quantal content; antarctic fish; temperature.

Antarctic fauna became isolated by the establishment of a circum-antarctic ocean circulation in the mid-Oligocene, about 25 million years ago[2]. Since that time, the evolution of antarctic fishes must have been strongly influenced by low temperatures and extensive areas of perennial sea ice. The sea temperature in deep coastal basins such as McMurdo Sound is constant throughout the year at −1.9 °C[3,4]. At this temperature temperate fish become comatose[5] so the very fact that antarctic species such as *Pagothenia borchgrevinki* live in McMurdo Sound must indicate that their nervous systems have become adapted to low temperature function. The temperature dependence of muscle contraction and of MEPP frequency and decay have been studied in *Pagothenia borchgrevinki*[6,7]. In the present experiments we set out to determine the temperature dependence of evoked transmitter release at the neuromuscular junction, to see how well it is tuned to the environment.

All experiments were carried out on the inferior oblique extraocular muscle of the nototheniid fish *Pagothenia borchgrevinki*[8], in a fish hut on the McMurdo Sound sea ice (77°51'S, 166°45'E). Specimens of *P. borchgrevinki* were caught on handlines and immediately decapitated. The inferior oblique muscle and its nerve were dissected free in an ice slurry and pinned in a bath filled with 5 °C physiological saline made up to match major ion concentrations in *P. borchgrevinki* serum[9] (258 mM NaCl, 7.47 mM KCl, 4.05 mM $CaCl_2$, 0.79 mM $MgCl_2$, buffered with 10 mM HEPES, pH 8.4). The nerve was taken up into a close fitting suction electrode and stimulated at 10 Hz with 0.1 ms voltage pulses. Intracellular recordings were made with 10–20 MΩ glass microelectrodes filled with 3 M KCl and using standard techniques, from the band of large white fibers on the midline side of the muscle. In normal saline, nerve stimulation evoked action potentials in the muscle fibers so transmitter release was lowered with Mg^{++} (25–50 mM) until only endplate potentials were recorded. Muscle fibers receiving focal, rather than multiple innervation were selected by stimulating the nerve with pulses of increasing voltage and selecting only muscle fibers where epp amplitude stayed constant rather than increasing in steps with voltage increase as axons were recruited. Usually the large white muscle fibers were focally innervated and the small red fibers multiply innervated by this criterion. Bath temperature was controlled by manual adjustment of a peltier unit under the recording chamber, with fluid temperature being measured by a thermister in the bath. Endplate potentials were stored as FM tape recordings and subsequently analyzed for quantal content by a modification of the method of variance, as described elsewhere[10–12].

Intracellular penetrations were made on a total of 39 muscle fibers in muscles from 17 different fish. Six penetrations were each held for about 5 h, while the temperature was varied up and down between −3 °C and +20 °C and quantal content determinations were made at different temperatures. All of the resulting curves (e.g. fig. 1) had very similar shapes. Quantal content increased slightly from −2 °C to +6 °C but no marked temperature dependence was shown until about +9 °C, when quantal content started to decline. Release ceased at about +18 °C. Resting membrane potential also changed over the experimental temperature range (fig. 2), hyperpolarising as temperatures increased so that epp amplitude remained almost constant until