perpendicular section just through the edge of the papilla, the presence of varicose meshworks of SP-like immunoreactive fibers among ciliated cells was clearly shown (fig. 5). The meshwork with varicose profiles of various sizes was localized only in the ciliated cell group and covered the whole of it. Our immunoelectron-microscopic study confirmed the presence of nerve endings of SP-like immunoreactive fibers which made contact with the ciliated cells and contained numerous vesicles of various sizes (60–125 nm in diameter) (figs 6, 7). These findings strongly suggest that SP-containing fibers influence the ciliated cell proper. Little is known about the presence of ciliated cells in the taste organs of other animals. They may be supplementally involved in gustatory processes by propelling fluid or mucous films over the surface of the epithelium, because the structure of the cilia is typical of those commonly described as being motile[6] (fig. 6).

Interestingly, SP-containing fibers are distributed in other organs including motile cilia[10, 11]. The present study revealed that SP-like immunoreactive fibers in the frog tongue were distributed in each fungiform papilla, especially among the ciliated cells surrounding the gustatory epithelium.

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1 Lundberg, J.M., Hökfelt, T., Änggard, A., Pernow, B., and Emson, P., Acta physiol. scand. *107* (1979) 389.
2 Nagy, J.I., Goedert, M., Hunt, S.P., and Bond, A., Neuroscience *7* (1982) 3137.
3 Yamasaki, H., Kubota, Y., Takagi, H., and Thoyama, M., J. comp. Neurol. *227* (1984) 380.
4 Henry, J.L., in: Substance P in the Nervous System, p. 206. Ciba Foundation Symposium 91, Pitman, London 1982.
5 Somogyi, P., and Takagi, H., Neuroscience *7* (1982) 1779.
6 Graziadei, P.P.C., and DeHan, R.S., Acta Anat. *80* (1971) 563.
7 Hökfelt, T., Johansson, O., Ljungdahl, A., Lundberg, J.M., and Schultzberg, M., Nature *284* (1980) 515.
8 Beidler, L., and Smallman, R.L., J. Cell Biol. *27* (1965) 263.
9 Robbins, N., Exp. Neurol. *17* (1967) 364.
10 Lundblad, L., Lundberg, J.M., Brodin, E., and Änggard, A., Acta otolaryngol. *96* (1983) 485.
11 Lundberg, J.M., Hökfelt, T., Martling, C.R., Saria, A., and Cuello, C., Cell Tiss. Res. *235* (1984) 251.

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Calcium binding in chemically skinned fibers of rat myocardium during force development

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Summary. The amout of bound calcium and force were measured in chemically skinned rat myocardial fibers during isometric contraction at different concentrations of free Ca^{2+}. The data obtained suggest that calcium binding by cardiac myofibrils is cooperative and probably depends on mechanical tension.
Key words. Ca^{2+} binding; force; myofibrils; myocardial contraction.

It is well known that the contraction of cross-striated muscle is initiated by calcium ions binding to troponin. However, it is still not clear what is the exact quantitative relation between the degree of occupation of Ca^{2+} binding sites and muscle mechanical response, in particular the value of isometric tension. The results of a number of studies[2-5] suggest that the parameters of calcium binding by myofibrils are not constant, but depend on actomyosin interaction. If this is the case, then the characteristics of Ca^{2+} binding to troponin in the muscle, generating force, might be different from those for isolated troponin or myofibril suspensions. In some studies[6-9] calcium binding by skinned fibers of skeletal muscle was measured at different pCa values; however, the developed force was not determined. The only study of both Ca^{2+} binding and force at different pCa values was carried out by Fuchs and Fox using glycerinated skeletal muscle fibers[10]. There have been no similar studies on cardiac muscle. Therefore, the aim of the present study was to measure Ca^{2+} binding by chemically skinned fibers of rat myocardium during force development at different concentrations of free Ca^{2+}.

Materials and methods. The bundles of fibers, 0.3–0.4 mm in diameter, were isolated from left ventricular endocardial surface and incubated for 2 days at 0 °C in a calcium-free solution, with EGTA and 1% Triton X-100 to ensure that destruction and removal of the sarcolemma and the intracellular membrane structures would be as complete as possible. All the solutions used in the study contained 3 mM free Mg^{2+}, 5 mM MgATP, 15 mM phosphocreatine, 20 mM imidazole, 80 mM potassium propionate, 5 mM glucose, 100 μM Ca, 0.3 mM dithiothreitol, pH 7.0, and amounts of EGTA to adjust the pCa to the desired value between 7 and 4.5. The composition of the solutions was calculated by using the set of equations[11] with dissociation constant values given by Fabiato[12]. The solutions were made using deionized water. The contamination of the solution with admixed calcium was assessed by plasma emission spectrometry. The fiber bundles were fixed with tungsten clamps. One clamp was connected to the force transducer (FT.03, Grass Instrument Co.). The relaxed fibers were stretched to the point at which tension appeared, and additionally by 20% of this length. The preparations were incubated in 1 ml of test-solution with ^{45}Ca and ^{3}H-glucose (a marker for solvent space) for 5 min, then the labeled substances were extracted for 10 min in 1 ml of 'cold' solution with pCa 7. Then the procedure was repeated with another test-solution. Incubation and extraction were performed with intensive stirring of the solutions.

In preliminary experiments the time-course of elution of the bound ^{45}Ca from the fiber bundles into the extraction solution with pCa 7 was determined (fig. 1). It can be seen that after 10 min the label exchange between the preparation and the solution is completed. Therefore, it is clear that 10 min are sufficient for extraction of practically all the labeled molecules.

All the bundles were passed through 13 test-solutions with pCa from 7 to 4.5. The experiments were performed at room temperature (22–23 °C). The radioactivity of the incubation solutions and the extracts was determined using a 'Rackbeta' liquid scin-

tillation counter (LKB). Protein content in the fiber bundles after boiling for 1.5 h in an alkaline solution was measured according to Lowry et al.[13].

Results and discussion. After successive possages through the test-solutions the bundles started to develop mechanical tension at pCa 6.2–6.0, and the maximal force was observed at pCa 5.0–4.8 (fig. 2). Calcium binding by the bundles with pCa decrease rose stepwise (fig. 2). At [Ca^{2+}] less than 1 μM, a rather noticeable amount of Ca (about 1 nmol/mg protein) bound to the preparations, not causing any mechanical response. As Ca^{2+} concentration increased > 1 μM, i.e., when the active tension appears, the steepness of the calcium binding curve rose sharply. The inset to figure 2 shows the developed force and bound Ca in the pCa range of 6.4–5.0 plotted in Hill coordinates. One can see that Hill coefficients coincide, that is, the force develops in parallel to Ca^{2+} binding in this range of [Ca^{2+}]. At pCa values less than 5, Ca^{2+} binding increased very significantly and did not reach saturation. A similar considerable increase in bound Ca^{2+} at [Ca^{2+}] more than 10 μM was observed in isolated cardiac myofibrils[14,15]. This permits the assumption that cardiac myofibrils possess a number of Ca^{2+} sites of relatively low affinity, possibly located on the thick filament and not participating in the regulation of mechanical activity.

A definite change of slope of the Ca^{2+}-binding curve, in that range of [Ca^{2+}] values where active force starts to develop, was not revealed in the experiments with isolated cardiac myofibrils[14,15]. On this basis one can suggest that the Ca^{2+}-binding characteristics are different in systems generating and not generating force. Thus, the point of view that calcium binding by myofibrils depends on muscle mechanics is confirmed.

Another difference between calcium binding by isolated myofibrils and skinned fibers developing active tension should be noted. It was found in the present study that the major amount of Ca^{2+} binds to the fibers at a pCa less than 6, and at pCa 7 only about 10% is bound as compared with pCa 5. Glycerinated rabbit skeletal muscle fibers also bind only 20–30% Ca^{2+} at pCa 7[10]. At the same time, according to recent studies[15,16], isolated cardiac myofibrils at pCa 7 bind about half of the amount of Ca^{2+} bound at pCa 5. It is interesting to note that there is similarity between the characteristics of Ca^{2+} binding by isolated myofibrils and glycerinated skeletal muscle fibers which are in rigor and not developing active force[9].

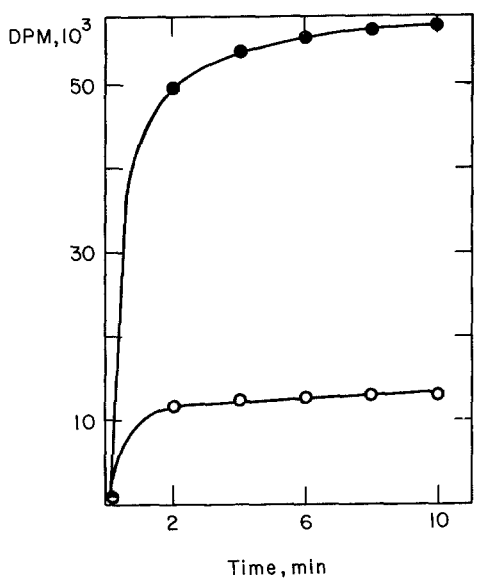

Figure 1. Activity of ^{45}Ca in extraction solution (pCa 7) depending on the time of the extraction of the isotope from a bundle of skinned fibers after preliminary activation by labeled solutions with pCa 5.6 (○) and 4.5 (●). The activity of dissociated calcium was calculated after subtraction of the amount of ^{45}Ca in the solvent space of the fibers determined by a space marker (^{3}H-glucose).

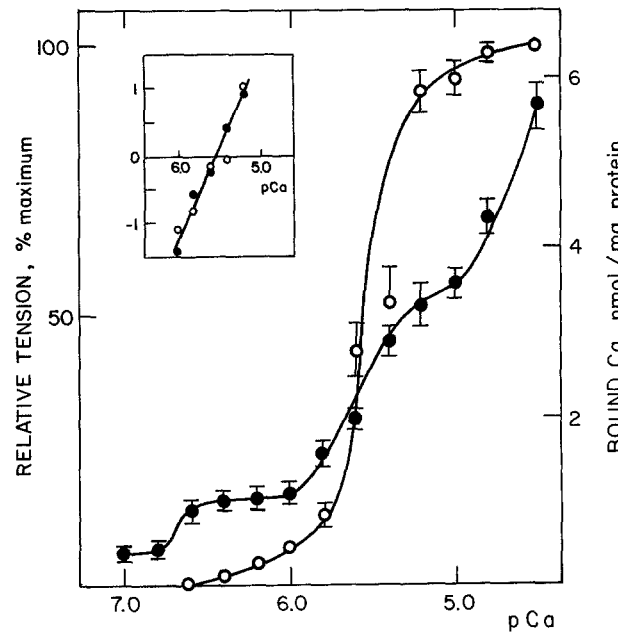

Figure 2. Relative tension (○) and Ca^{2+} bound (●) by bundles of skinned fibers at different pCa values (n = 7). Inset: values of relative tension (○) and bound Ca^{2+} (●) plotted in Hill coordinates. For minimal and maximal values for Ca^{2+} binding the values at pCa 6.4 and 5.0, respectively, were taken.

The data obtained do not permit a clear answer to be given to the question, to which particular binding sites on the myofibrils calcium is bound in the range of pCa values at which force develops. The situation is also complicated by the fact that the absolute amount of bound Ca^{2+} determined in the present study exceeds the amount found in isolated canine cardiac myofibrils[14,15] and in glycerinated rabbit skeletal muscle fibers[6–10]. These discrepancies may be caused by species differences as well as by differences between the experiments in the conditions used for the determination of bound calcium.

Summarizing the results it can be stated that the data presented testify to the parallelism between Ca^{2+} binding by cardiac myofibrils and force development. The relatively high Hill coefficient for Ca^{2+} binding in the pCa range 6.4–5.0 indicates a cooperativity of the binding, probably influenced by cross-bridge formation during the contraction process. Thus, it is likely that there is an effect of the mechanical state of the muscle on the myofibrillar Ca^{2+}-binding parameters. Further studies are necessary to elucidate the details of the mechanism responsible for the feedback between the state of the contractile apparatus and calcium activation.

Cooperative binding of Ca^{2+} to myofibrils suggests significant changes in the amount of bound Ca^{2+} in a relatively narrow range of concentration of free Ca^{2+}. The sharp increase in the amount of myofibril-associated calcium, which accompanies force development by cardiac cells in vivo, probably leads to a considerable decrease in the free Ca^{2+} concentration. Myofibrils thus represent a rather powerful buffer for intracellular [Ca^{2+}] in the micromolar range. Such a buffer might make a significant contribution to the shifts in intracellular Ca^{2+} metabolism in cases when myofibrillar calcium-binding characteristics change (for instance, with shifts of pH or degree of troponin I phosphorylation).

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2 Bremel, R.D., and Weber, A., Nature, New Biol. *238* (1972) 97.

3 Brandt, P.W., Cox, R.N., and Kawai, M., Proc. natn. Acad. Sci. USA *77* (1980) 4717.
4 Brandt, P.W., Cox, R.N., Kawai, M., and Robinson, T., J. gen. Physiol. *79* (1982) 997.
5 Ridgway, E.B., and Gordon, A.M., J. gen. Physiol. *83* (1984) 75.
6 Fuchs, F., Biochim. biophys. Acta *491* (1977) 523.
7 Fuchs, F., Biochim. biophys. Acta *585* (1979) 477.
8 Fuchs, F., and Black, B., Biochim. biophys. Acta *622* (1980) 52.
9 Blanchard, E.M., Pan, B.-S., and Solaro, R.J., J. biol. Chem. *259* (1984) 3181.
10 Fuchs, F., and Fox, C., Biochim. biophys. Acta *679* (1982) 110.
11 Fabiato, A., and Fabiato, F., J. Physiol., Paris *75* (1979) 463.
12 Fabiato, A., J. gen. Physiol. *78* (1981) 457.
13 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. biol. Chem. *193* (1951) 265.
14 Solaro, R.J., Wise, R.M., Shiner, J.S., and Briggs, F.N., Circulation Res. *34* (1974) 525.
15 Blanchard, E.M., and Solaro, R.J., Circulation Res. *55* (1984) 382.
16 Solaro, R.J., and Rüegg, J.C., Circulation Res. *51* (1982) 290.

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Stabilisation of collagen by betel nut polyphenols as a mechanism in oral submucous fibrosis

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Summary. Treatment of reconstituted collagen fibrils and pieces of rat dermis with the crude extract, purified tannins or (+)-catechin from betel nut *(Areca catechu)* increases their resistance to both human and bacterial collagenases in a concentration-dependent manner. These tanning agents may stabilise collagen in vivo following damage to the oral epithelium, and promote the sub-epithelial fibrosis which occurs in betel nut chewers.
Key words. Collagen; oral submucous fibrosis; tannins; flavanoids; collagenase.

Oral submucous fibrosis (OSF) is a chronic disabling disease characterised by epithelial atrophy and accumulation of collagenous scar tissue within the oral mucosa. It occurs predominantly in India, Pakistan and South East Asia and although its aetiology is obscure, OSF is closely associated with the habit of chewing betel nuts *(Areca catechu)* [1,2]. Betel nuts contain a variety of pharmacologically active compounds including arecoline, which is the most common member of a group of alkaloids responsible for the psychotropic effects of the nut. We have previously demonstrated that crude extracts from the nuts stimulate collagen synthesis by buccal mucosa fibroblasts in vitro[3] and that the alkaloids appear to be responsible for this stimulation[4]. Quantitatively, the most important group of pharmacologically active compounds are the polyphenols, which constitute more than 50% of the water-extractable compounds. These polyphenols are predominantly the flavanoid (+)-catechin, and monomeric and polymeric leucocyanidins (tannins)[5]. They are of potential importance in OSF as they can interact with proteins, conferring mechanical and chemical stability to the complexes. (+)-catechin has been shown to increase the thermal shrinkage temperature of rat skin and tendon in vitro[6], to increase the resistance of soluble collagen to degradation by mammalian collagenase[7] and to exert a protective effect against the action of lathyrogenic drugs in vivo[8].

Here we report that treatment of reconstituted collagen fibrils and rat dermal polymeric collagen with crude extracts, purified tannins or (+)-catechin from betel nuts increases their resistance to both human and bacterial collagenases.

Materials and methods. a) Preparation of betel nut extracts. 100g of raw betel nuts were powdered and defatted in petroleum ether. The residue was extracted twice in 400 ml of 70% ethanol for 2 h. Part of this extract was lyophilised and termed 'crude extract'. From the remainder, tannins were purified by the method of Wall et al.[9], briefly, an aqueous solution of the crude extract was filtered twice through celite to remove any phlobaphens, and the tannins precipitated by addition of a saturated solution of caffeine. The caffeine was removed by washing with chloroform. (+)-catechin was obtained from BDH (Poole, Dorset) and used without further purification.

b) Preparation of collagen substrates. Radioactively labeled reconstituted collagen fibrils were prepared from a solution of purified, pepsin-extracted rat skin collagen which had been ^{3}H-acetylated by the method of Gislow and McBride[10]. The collagen was dissolved in 50 mM Tris-HCl (pH 7.5), 0.165 M NaCl, 5 mM $CaCl_2$ to give a 1 mg/ml solution, sp. act. 120,000 dpm/mg. Fibrils were reconstituted from this solution by gelling at 37°C for 18 h, dispersed by vigorous shaking, and washed three times in fresh buffer. Fine dispersion was achieved by forcing the suspension through an 18-gauge hypodermic needle.

1-ml aliquots of suspended fibrils were mixed with equal volumes of crude extract, tannin and (+)-catechin solutions (1-1000 µg/ml), or buffer alone, and incubated for 18 h at 37°C. The fibrils were washed three times with buffer and exposed to either bacterial or human collagenase (see below).

Fragments of rat dermis were prepared by scraping the epidermis, fat and muscle from frozen rat skins, and mincing with scalpels to a fragment size of approximately 1 mm^3. This tissue was washed twice for 2 h at 4°C in buffer (as above). Aliquots of approximately 100 mg wet wt were incubated in 5-ml volumes of tannin solutions (1, 10 and 100 µg/ml) or in buffer alone for 18 h at 37°C. The fragments were rinsed three times in buffer before exposure to bacterial collagenase.

c) Degradation with collagenase. Triplicate 50-µl aliquots of reconstituted ^{3}H-labeled collagen fibrils were incubated at 37°C with either a) bacterial collagenase (Sigma Type II, purified by the method of Peterkofsky and Diegelmann[11]) at a final concentration of 1 mg/ml until complete dissolution of the control (untanned) fibrils had occurred, or b) human collagenase from tissue cultures of dental cyst capsule. The latent collagenase in the culture medium (Eagle's Minimal Essential Medium supplemented with 10% foetal bovine serum) was activated by incubation with 0.2% trypsin for 20 min at 25°C, and the trypsin subsequently inactivated with a 3-fold excess of soybean trypsin inhibitor (Sigma), 100 µl of this activated collagenase preparation was added to each of the collagen fibril aliquots and incubated for 5 h at 37°C. Controls contained buffer or fresh culture medium with trypsin and trypsin inhibitor in place of the collagenase preparation. After incubation the samples were centrifuged (10,000 × g for 5 min), the supernatants mixed with 3 ml Unisolve 1 scintillation cocktail (Koch Light, Colne, Middlesex), and radioactivity measured on a LKB 'Rackbeta' with external standardisation. Collagen lysis was calculated from the radioactivity released into the supernatant and expressed as the mean percentage of the total radioactivity in the tubes.