Light and Electron Microscopic Studies Regarding Cell Contractility and Cell Coupling in Light Sensitive Smooth Muscle Cells from the Isolated Frog Iris Sphincter

Klaus V. Wolf*

Fachrichtung 3.2, Physiologie I, Universität des Saarlandes, Homburg/Saar, Bundesrepublik Deutschland

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Light Sensitive Smooth Muscle Cells, Frog Iris, Cell Junctions, Cell Coupling, Fine Structure

(1) In light microscopical studies of living isolated frog irises, it was found that the maximal areas of experimentally light induced contractions in the *m. sphincter pupillae* were located beneath small illuminated regions. There were no visible contractions of muscle cells outside the illuminated areas. It was shown that exposure to light could directly cause contractions of isolated single sphincter muscle cells.

(2) Junctional structures of the iris sphincter cells were studied by means of thin sections and freeze fracture electron microscopy. Intermediate junctions, a few focal tight junctions and occasional small gap junctions were identified. Pit containing intramembranous particles which resemble gap junction connexons were found in large numbers, dispersed over the plasmalemmas of sphincter muscle cells.

From these physiological and morphological observations, it is concluded that sphincter muscle cells of the frog iris may be coupled *via* gap junctions, but that the cell coupling is not sufficiently extensive to form the basis for a functional syncytium.

Introduction

It has been known for more than a hundred years that the musculus sphincter pupillae of lower vertebrates is able to contract directly upon light stimulation [1] (For reviews see [2–4]). Fundamental knowledge about this light-induced contraction of the isolated frog iris, its dependence on different stimulation and other experimental conditions came from physiological experiments which were done ten to twenty years ago [3–5]. These investigations led to the hypothesis that in lower vertebrates the smooth muscle cells themselves were able to perceive the light stimulus. One aim of the present investigation was to test this hypothesis by examining single iris muscle cells of the frog *Rana esculenta* during light stimulation.

The observation of a consensual pupillary reaction in frogs (*c.f.* [3]) and the detection of sympathetic and parasympathetic innervation of the sphincter pupillae in toads [6] revealed that the pupillary response can, in addition, be under nervous control *in vivo*. Moreover, during the evolution of higher vertebrates, the ability of the iris sphincter to contract directly upon light stimulation has largely been rejected (*c.f.* [7, 8]) in favour of the essential need for information carrying structures. For example the importance of nerves as structural prerequisites for the retina mediated pupillary reflex is evident. In higher vertebrates there exists a well developed cell-to-cell communication system between sphincter muscle cells by numerous gap junctions as shown in guinea-pigs [9]. Therefore, the main aim of the present investigation was to evaluate to what extent cell junctions are also present in the iris sphincter of the amphibian *Rana esculenta*. It will be discussed to what extent sphincter muscle cells are coupled in the isolated frog iris.

Materials and Methods

Preparation

Frogs *(Rana esculenta)* were kept at 4 °C. Immediately after decapitation, the eyes were removed and rinsed with Ringer solution, buffered to pH 7.0 with Tris-HCl (20 °C). The remainder of the preparation was carried out in buffered Ringer solution under a dissecting microscope under yellow illumination, to which the iris muscle cells are relatively insensitive. The iris together with the cornea were first removed *in toto*. Then the lens and the cornea were dissected from the iris. Finally the iris was fixed with four

* *Present address:* Dr. Klaus V. Wolf, Nadlerstraße 18, 6800 Mannheim 51.

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needles to a chamber made of transparent Sylgard 184 encapsulating resin (Dow Corning Corp. USA).

The isolated irises were examined with a light microscope either directly using lateral illumination or with transmitted illumination. In the latter case the posterior pigment epithelium and the anterior xanthophores and iridiophores had been removed (Fig. 1a–c).

Single smooth muscle cells were obtained by treating the isolated irises as follows: 1) 0.1% collagenase type I (Sigma) + 0.1% trypsin type III (Sigma) in Ringer solution (30.5 °C, 30 min), 2) 0.05% collagenase in Ringer solution (30.5 °C, 30 min), 3) gentle manual agitation with pipettes in Ringer solution (20 °C, 30 min).

Light microscopy

The *in vitro* experiments were carried out with a light microscope (Zeiss) equipped with an incidental beam condensor. Thus, during the examination of the iris with transmitted light or in lateral illumination (using yellow filters), a second focused light source could be projected onto the specimen. The focused light covered an area of the iris with a minimum diameter of 0.1 mm, when a 10 × objective lens was used. The wavelength of this light was chosen 450–490 nm (blue). Thus, maximum stimulation of the illuminated cells could be observed. (By modifying the filter equipment in the incidental beam the stimulating blue light could alternatively be made visible or filtered away after it was reflected by the specimen surface.)

Electron microscopy

For electron microscopy, freshly prepared irises were fixed in 2% glutaraldehyde (GA) and 1% OsO_4 in cacodylate buffer (pH 7.2, combined osmolarity of buffer and GA, 260 mosm) for 1 min, followed by identical concentrations of GA and buffer but without OsO_4 for 1 h (*c.f.* [10]). After rinsing the irises in cacodylate buffer (pH 7.2, 260 mosm) they were postfixed in 1% OsO_4 in the same buffer, dehydrated in a graded series of ethanol solutions and embedded in Spurr's medium [11]. Some specimens were stained with uranyl acetate in the 70% ethanol step. In a few samples, the extracellular membrane surface was stained with 1% ruthenium red according to Luft [12]. Thin sections were cut on a Reichert OM U3 microtome and poststained with lead citrate [13].

For freeze fracture irises were fixed with the combined OsO_4 glutaraldehyde solution and then infiltrated with increasing concentrations of glycerol up to 30%. Then they were frozen in liquid propane and freeze fractured in a Balzers BAF 300 apparatus according to the method of Moor and Mühlethaler [14]. The samples were etched for 30 sec at −100 °C and at a vacuum pressure of about 10^{-7} mbar followed by unidirectional shadow casting.

Replicas and thin sections were investigated in a Siemens Elmiscop 101. Stereo micrographs were obtained with a Siemens DKL unit (stage tilted through ± 5°).

The membrane facture faces were designated according to the nomenclature of Branton *et al.* [15].

Results

I. Contractile behaviour of sphincter cells in vitro

Illumination of a small area of the pupillary margin with blue light led to an intense contraction of that area (Fig. 1a–c). The neighbouring sphincter cells, which lay outside the illuminated area, were pulled towards the sphincter cells which showed maximal contraction. This is demonstrated in Fig. 1a–c by the distortion of the delineated sphincter cells towards the contraction centre during long time photographic exposures. The contraction centres lay exactly beneath the cone of the blue light, and followed even short lateral (Fig. 1b) and vertical (Fig. 1c) dislocations of the illuminated area.

This contractile response of the isolated frog iris to illumination is based on a direct perception of the light stimulus by single sphincter muscle cells without an essential need for additional nervous or humoral support: Fig. 1d shows a single sphincter cell in normal Ringer solution in yellow light, which underwent a strong contraction after the light was changed to blue (Fig. 1e).

II. Morphological investigations

Thin sections

General appearance

Fig. 2a shows a radial section through a frog iris near the pupillary margin (left) exposing cross sections of the circulary arranged smooth muscle cells of the sphincter pupillae (S). These muscle cells seem to emerge from the posterior layer of the double layered pigment epithelium (P) after it has turned into a

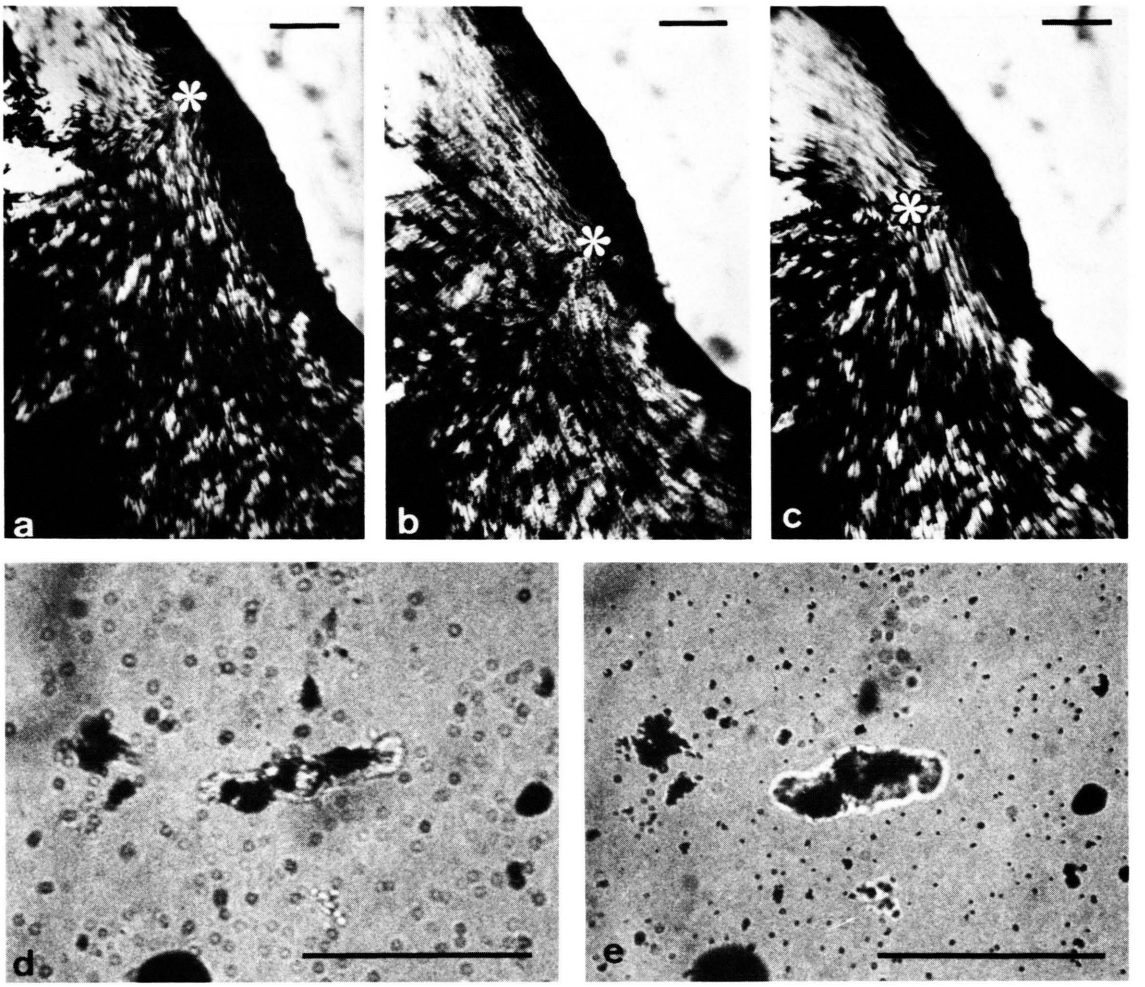

Fig. 1. Contraction experiments under light microscopic control. – (a), (b), (c). A sector of the isolated iris in transmission illumination with yellow light. The double layered pigment epithelium has been prepared away. The pupillary margin is on the right side. Long time photographic exposures during simultaneous stimulation of small areas with blue light bundles: The contraction centres follow lateral (a), (b) and vertical (c) dislocations of the illuminated area (asterisk). One single isolated sphincter cell under yellow (d) and blue (e) illumination. – (a), (b), (c) 90×. – (d), (e) 600×. – Bars (a), (b), (c) 100 μm, (d), (e) 50 μm.

curve to form the pupillary edge (Fig. 2a, arrowhead). Behind that loop, the content of black pigment granules decreases. This indicates that there exists a transition region between pigment cells and sphincter cells (Fig. 2a, asterisk). Nevertheless, even in deeper regions each of the sphincter muscle cells contains a certain number of pigment granules.

Our observations of the fine structure of the pigment epithelial cells revealed that the amount of peripherally arranged contractile filaments increases in this transition region to reach the normal content of such filaments in smooth muscle cells.

In Fig. 2b a higher magnification of the transition region is shown: In this cross section distinct cells can be identified as sphincter cells, when they are at least partially separated from each other by an extracellular basal lamina (arrows). In cross sections of deeper regions of the sphincter pupillae the basal lamina is more extensive and separates individual smooth muscle cells or groups of them (Fig. 2c, BL).

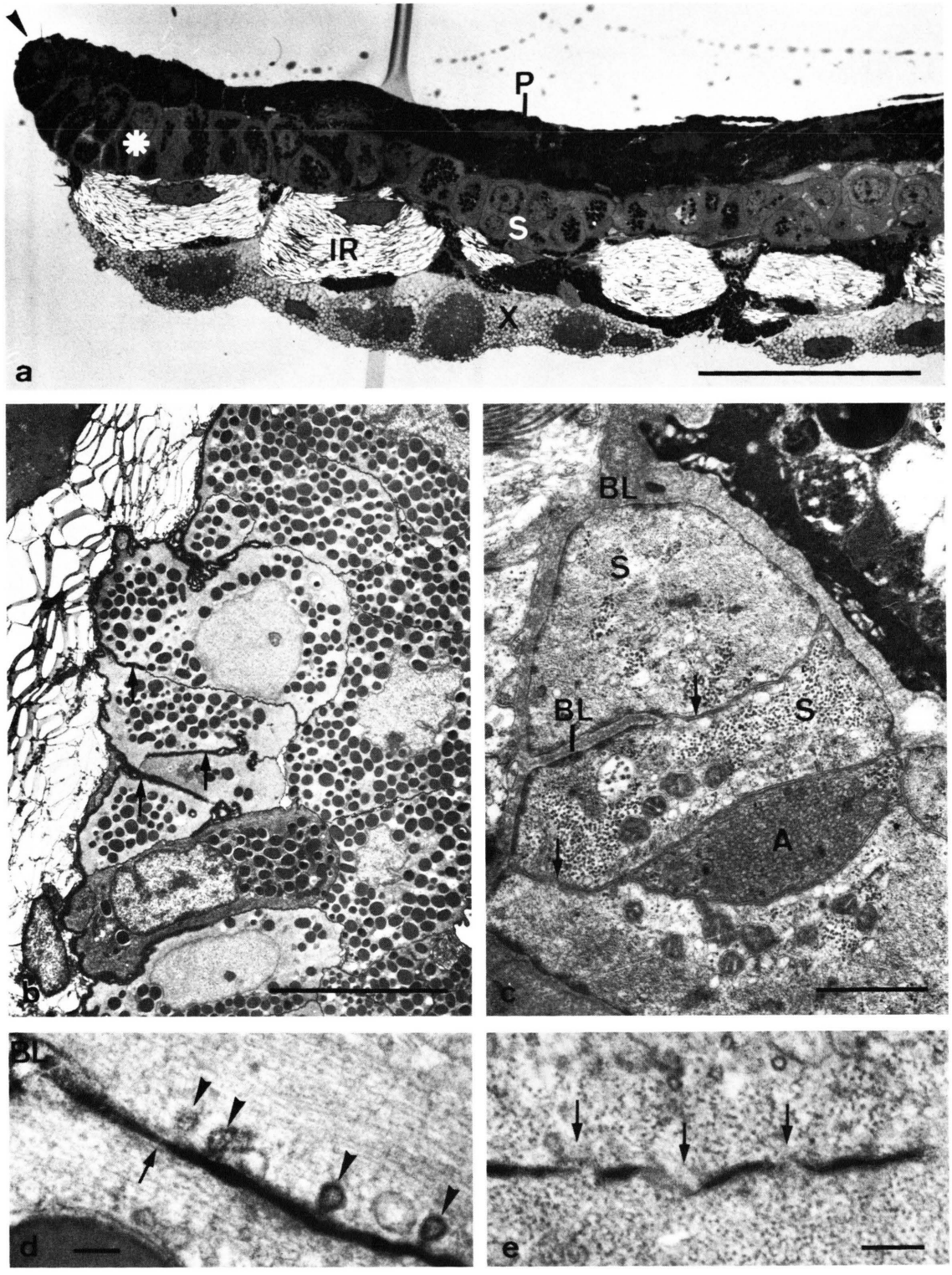

Fig. 2. Radial section of the iris showing transverse sections of sphincter cells (S), the posterior pigment epithelium (P), iridophores (IR), anterior xanthophores (X), and the pupillary margin (arrowhead). Asterisk, transition region between the pigment epithelium and the sphincter pupillae. – (b)–(e). Transverse sections of the sphincter pupillae. Arrows in (b), basal lamina. Arrows in (c), narrow course of the plasmalemmas. BL, basal lamina; S, smooth muscle cells; A, axon (cross section). Arrow in (d), focal tight junction. Arrowheads, caveolae. Arrows in (e), interruptions of the dense ruthenium red staining of the extracellular mucoid substances. The extracellular clefts in (b), (d), (e) are stained with ruthenium red. – (a) 350×. – (b) 2,850×. – (c) 18,000×. – (d) 72,000×. – (e) 100,000×. – Bars (a) 100 μm, (b) 10 μm, (c) 1 μm, (d), (e) 0.1 μm.

Over long distances the extracellular cleft between two neighbouring muscle cells has no basal lamina (Fig. 2c, arrows). Then the plasma membranes mostly run parallel to each other at a distance between 10 nm and 15 nm. This parallelism is interrupted by caveolae [16–18] (Fig. 2d, arrowheads), and by junctional membrane specializations as described below. Within that narrow extracellular cleft the ruthenium red adsorption by mucoid substances proved to be non uniform (Fig. 2e, arrows).

Innervation

In sections perpendicular to the long axis of the sphincter muscle cells (Fig. 2c) and in sections parallel to the long axis (not shown) numerous axons can be observed which lie in close proximity with the smooth muscle cells. By examining serial sections we were not able to find any muscle cell which is not tangent to at least one axon.

Focal tight junctions

Occasionally focal tight junctions (see [19]) can be observed (Fig. 2d, 3a, arrows). The term "focal tight junctions" is based on electron microscopy of thin sections. In membrane cross sections they appear as transparent bodies that span the narrowed intercellular space. Ruthenium red staining of the extracellular mucoid layer revealed that the transparent "bodies" consist of two subunits, as suggested by Raviola [20], each belonging to one plasma membrane. Together they exhibit a narrow electron dense contact zone (Fig. 3a).

The focal tight junctions are often situated near the interruptions of the basal lamina where the plasmalemmas of two muscle cells begin to follow a narrow and parallel course (Fig. 2d).

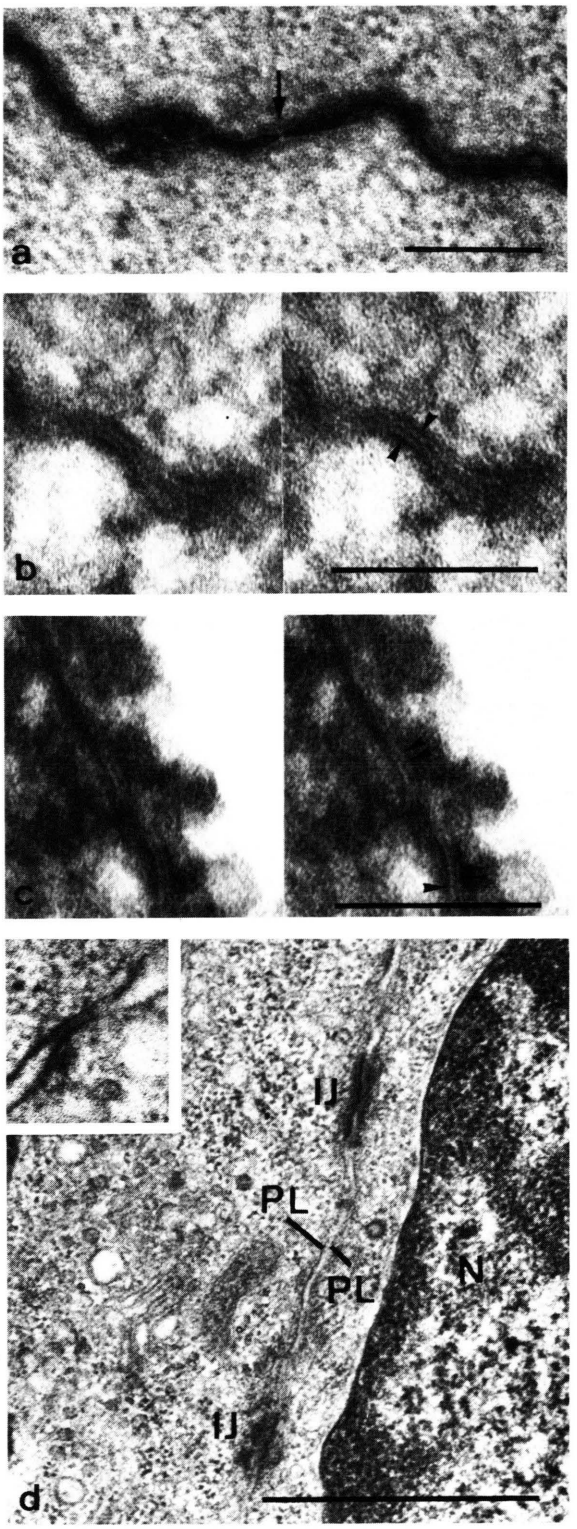

Fig. 3. Junctional plasmalemma specializations of cross sectioned neighbouring sphincter muscle cells. – (a) Focal tight junction (arrow). – (b), (c) Stereo pairs of gap junctions exhibiting electron lucent bridging structures (arrows) of the extracellular gap, and electron dense interruptions (arrowheads) of the light central cores of the individual membranes. – (d) Intermediate junctions (IJ, Inset). N, nucleus; PL, plasmalemmas. Ruthenium red staining of the extracellular space in (a)–(c). One should examine (b) and (c) with stereoviewer to discern the fine structural details. – (a) 180,000×. – (b), (c) 27,000×. – (d) 36,500×, Inset 110,000×. – Bars (a)–(c) 0.1 μm, (d) 1 μm.

Nexus (gap junctions)

Very infrequently we were able to detect areas of membrane narrowings with distances between the membranes of about 1–2 nm (Fig. 3b, c). At these points dense strata are associated with the cytoplasmic surfaces of both plasmalemmas. Although these membrane specializations are very short (20 nm in Fig. 3b), they can be identified as small gap junctions or nexus (for review see [21]).

During ruthenium red treatment the extracellular gap has been filled with electron dense stain so that the nexus exhibit only a pentalaminar structure (for review see [22]). The advantage of this staining procedure, on the other hand, is that it is easy to recognize electron lucent structures within the gap. They appear periodically and seem to bridge the central, ruthenium red impregnated zone (Fig. 3c, arrows). Those bridging structures have been described in detail in other tissues after lanthanum or uranyl acetate staining procedures (*cf.* [21, 23], for review see [22]). Furthermore, the ruthenium red stain interrupts the electron lucent central cores of the individual membranes (Fig. 3b, c, arrowheads). These features correspond to the apparent subunits of nexus membranes which have been detected in thin sections by others (*cf.* [23, 24]).

Intermediate junctions

Besides the membrane attachments described above, narrowings of the plasma membranes of the iris muscle cells can be observed which maintain a wider intracellular cleft than in gap junctions. (In Fig. 3d the minimum distances between the membranes are about 6–7 nm.) In these and in the accumulation of an electron dense material at both, the extracellular and cytoplasmic sides, these structures resemble the junction-like structures which have been described in double cones of the chick retina by Nishimura *et al.* [25]. In our opinion the term "intermediate junction", as defined by Townes-Anderson and Raviola [26], can be applied to these structures.

Freeze fracturing

According to the low number of focal tight junctions that could be seen between sphincter cells in thin sections, linear tight junctional strands [27–30] were occasionally found on frozen-cleaved membrane faces (Fig. 4). The strands appear on EF, rather than on PF (for review see [31]). It must be stressed that the tight junctional strands do not branch to form zonulae occludentes like in epithelial tissues (literature cited above) but are single, individual strands in our preparations. Since in thin sections the single strands are nearly always be cut more or less perpendicular to their long axis we correlate them to the focal tight junctions as described above. One can speculate whether they are rudimentary tight junctions.

A second type of junctions that appeared very sporadically in the replicas of sphincter cells can be identified as nexus (Fig. 5a, b). The nexus particles (connexons) appear on PF and are not as closely packed as in many other tissues (for review see [31]). Instead of the macular arrangements they show linear, circular, or patchy arrangements in our preparations. The distances between the connexons range from close contacts (Fig. 5b) up to 20 nm (Fig. 5a, arrowheads). The connexons are about 10–12 nm in

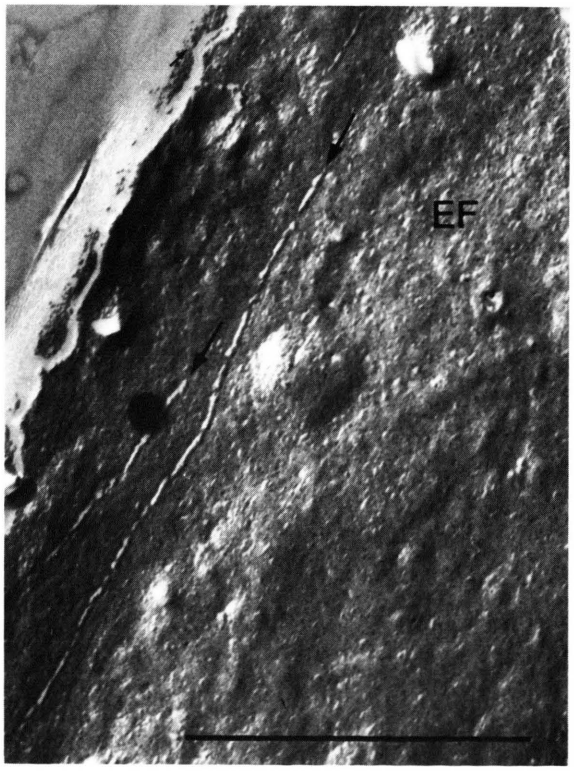

Fig. 4. Freeze fracture replica of the plasmalemma (EF) of a sphincter muscle cell. Arrows, single tight junctional strands. EF, exoplasmic fracture face. 92,000×. – Bar 0.5 μm.

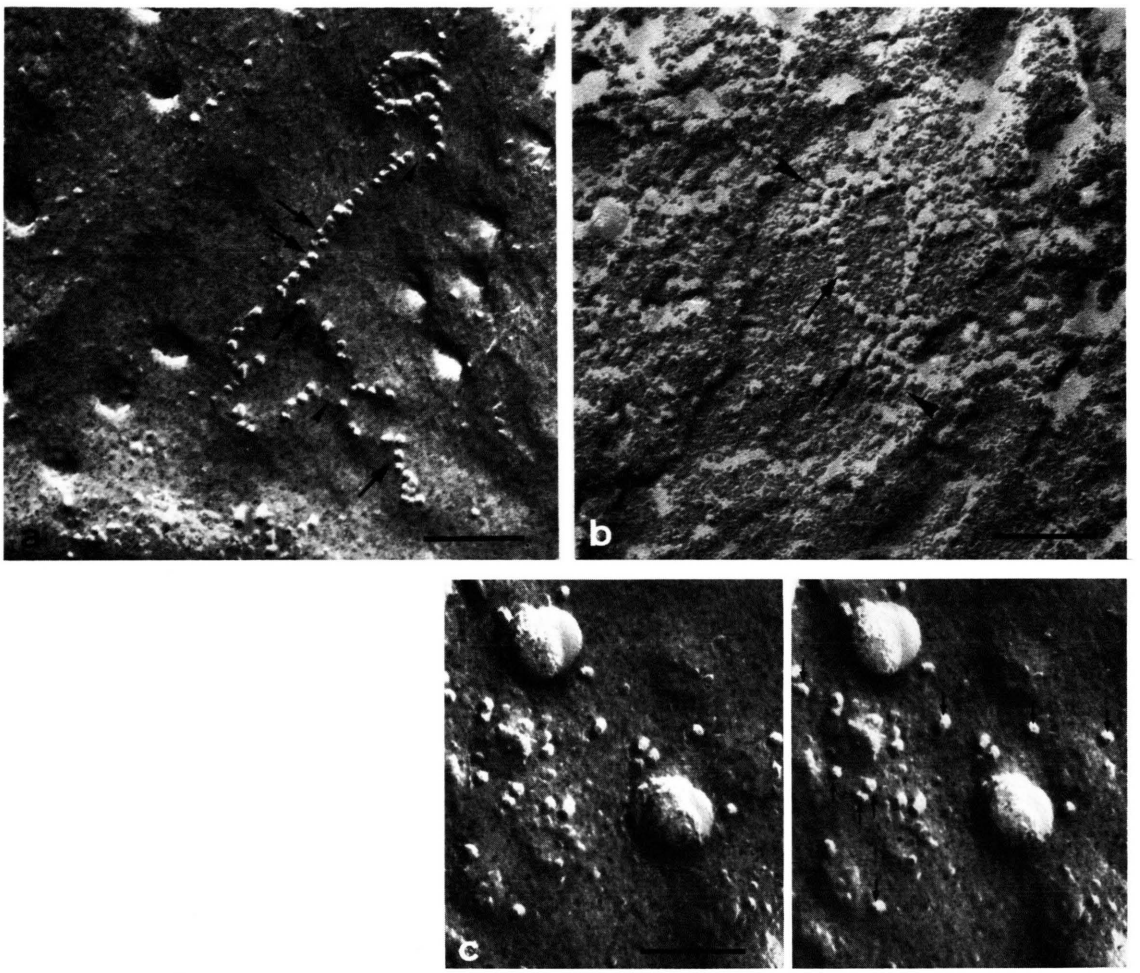

Fig. 5. (a), (b) Gap junctions on the protoplasmic fracture faces of the plasmalemmas of sphincter muscle cells. The connexons often exhibit a central pit (arrows). In (a) the arrowheads point to large interspace between neighboring connexons. In (b) the arrowheads point to patchy arrangements of the connexons. – (c) Area of a freeze-fractured smooth muscle plasmalemma (EF) containing increased numbers of IMP which are morphologically identical with connexons. The arrows point to pit containing particles. Stereo micrograph – (a), (b) 135,000×. – (c) 140,000×. – Bars 0.1 μm.

diameter and they often exhibit a central depression with 2 nm diameter (Fig. 5a, b, arrows). No indications arose that there were differences between irises fixed in the dark-adapted and light-adapted state. But this was difficult to judge because of the very low number of nexus which could be identified in this study.

In contrast to the very low number of gap junctions, which could be observed between iris sphincter cells, we were able to detect a great number of IMP which had the same diameter and fine structural appearence (*i.e.* a central depression) as the gap junctional connexons (Fig. 5c). These pit-containing particles appeared distributed over the entire plasmalemma. Sometimes, but not as a rule, a tendency for clustering of these IMP could be observed. In contrast to the connexons arranged in nexus, the connexon-like particles are found on EF as well as on PF. The stereo pair in Fig. 5c confirms that the depressions in the centre of the particles are real and not deceptions caused by the freeze fracture electron microscopy. Furthermore, only such IMP among

those of varying diameter, which were as large as connexons of nexus were found to possess central depressions.

Discussion

The present paper shows that there exist low numbers of tight junctions, intermediate junctions and gap junctions between smooth muscle cells of sphincter pupillae of the frog iris. Especially gap junctions are found very seldom. (This must be seen in relation to investigations of toads [6] and *Rana pipiens* [32] which gave no indications of gap junctions.)

The thin section investigations suggest that the sphincter cells and the posterior pigment epithelium of the iris originate from the same embryonic tissue (*cf.* [32]) and separate from each other only late during ontogenesis. There are more and larger gap junctions between pigment epithelium cells (not shown) than between sphincter muscle cells. Thus, it seems to be a valid hypothesis that either single gap junctions occur only as rudimentary formations between sphincter cells or a great number of gap junctions disappear during sphincter muscle differentiation. Such a decrease in the number of gap junctions between differentiating cells in the optic cup of monkeys has been described elsewhere [26].

The question arises whether there are fine structural details in sphincter membrane morphology which can be correlated to dynamic alterations of the gap junction *i.e.*, assembly and disassembly of connexons: It is under discussion whether gap junctions form during aggregation of solitary but complete connexons [33–36] or whether smaller precursor molecules appear first in clusters and then transform into connexons [36]. We have found both, non-aggregated IMP which are identical to connexons in their fine structural appearance, and clusters of particles which are smaller than connexons and which do not exhibit central impressions. One can speculate whether one of these features corresponds to gap junction formation and the other one to gap junction disassembly. We favour the idea that the gap junctions between sphincter muscle cells of the frog iris are in a state of disaggregation while the connexons themselves remain morphologically stable. This judgement is based on i) the developmental aspect as discussed above, ii) the relatively loose arrangement of the connexons in sphincter gap junctions and iii) the high number of solitary connexon-like particles in our replicas.

Are there indications for physiologically significant coupling of sphincter cells? Since the work of Barr *et al.* [37] it is well established that smooth muscle cells are coupled *via* gap junctions. On the other hand, Williams and De Haan [38] presented strong evidence that electrical coupling between cells *in vitro* can be maintained by low resistance cell-to-cell pathways without ultrastructurally defined gap junctions. This supports the idea that pit-containing IMP possibly represent free floating junctional structures *per se* – though pit-containing particles are also present in the plasmalemmas of cell types which are not electrically coupled (*e.g.* red blood cells [39]). Thus it is likely that it depends on the tissue whether cells are electrotonically coupled *via* gap junctions or single channels (solitary connexons). When gap junctions are involved in cell coupling, experimentally induced uncoupling can cause slight morphological changes in the connexon package (for review see [22], but *cf.* [40]) or a drastic disorganization of the nexus can occur until the connexons become dispersed over the membrane [42]. Thus, the question is open whether the connexon like IMP in the isolated frog iris sphincter function as cell coupling structures or represent residues of uncoupled gap junctions.

The physiological experiments described in this paper revealed that only that defined area of the sphincter muscle which was stimulated by a focused light bundle showed contraction. No visible reaction of the neighbouring muscle regions occurred. These results confirm similar observations from Weale [41] and v. Campenhausen [3] and show that the cells of the frog iris sphincter, or of larger parts of it, do not form a functional syncytium. If couplings between a few neighbouring cells yet occur, as it must be suggested in view of the morphological findings, only a very limited spread of the light-induced excitation exists. On the other hand, a rapid adaptation of the eye to changing light intensities requires a synchronous contractile response of each muscle cell with a minimum time delay. From this and from the observation that the frog iris can contract upon light even after isolation from nervous control it can be concluded that most of the sphincter cells have identical direct reactiveness to the light stimulus.

It must be realized that the innervation of the sphincter is so extensive that every single muscle cell seems to be supplied with an axon. Therefore it is undoubted that the light adaptation of the pupil is under nervous control *in vivo*. Similar results were

obtained from guinea-pigs [9] but in this species an extensive number of gap junctions was found. The iris of guinea-pigs, and of most other mammals, is not able to react directly upon light stimulation (*cf.* [8]). It is still unknown what functional significance the direct contraction of iris muscle cells of frogs and many other amphibians has *in vivo* and whether this capability is substituted by the physiological function of gap junctions during the phylogenesis of higher vertebrates.

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