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ISOPROTERENOL-INDUCED INTRAMEMBRANE PARTICLE AGGREGATION AND WATER FLUX IN TOAD EPIDERMIS

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Abstract

Stimulation of toad skin with isoproterenol resulted in a dramatic increase in water flow, and in the appearance of aggregates of intramembrane particles in the apical membrane of granular cells of the replacement layer, just beneath the stratum corneum. This membrane structural modification appears to be a general prerequisite for the change in water permeability of vasopressin-sensitive epithelia.

The pathways for transport of water across the plasma membrane are a central issue in cell biology. The problem is most acute in tight epithelia which undergo dramatic changes in water permeability (hydrosmotic response) upon exposure to osmoregulatory hormones such as neurohypophysial peptides. In amphibian urinary bladder, a tissue often used as a model for the mammalian collecting duct, a correlation has been found between increased water flow and the appearance of aggregates of intramembrane particles in the apical portion of the plasma membrane of freeze-fractured epithelial cells [1—5]. We present here, for the first time, evidence showing the appearance of similar structures in toad skins in which the transepithelial net water fluxes had been markedly increased by the β-adrenergic agonist, isoproterenol. This finding, in a non-urinary epithelium and with a different hydrosmotic agent, provides strong support for the theory that clustering of intramembrane particles has a physiological significance related to transmembrane water permeability.

Toads (*Bufo marinus*) were obtained from Charles P. Chase Inc., Miami. The pelvic skin was removed and divided in two segments, which

were mounted as diaphragms in glass chambers. These were designed for measuring water fluxes (J_{H_2O}) with an automatic technique [6,7]. An osmotic gradient across the skin was established using standard amphibian Ringers solution [8] on the internal side and ten-fold diluted Ringers to bathe the external surface. At the peak of the hydrosmotic response to isoproterenol, the skins were quickly removed from the chambers, fixed briefly in 2 % glutaraldehyde and processed for freeze-fracture electron microscopy as previously described [9].

Basal water flows were quite steady and varied between 0.36 and 0.87 $\mu l \cdot min^{-1} \cdot cm^{-2}$. Addition of isoproterenol (1 μM) to the internal side of the skin induced a conspicuous rise in J_{H_2O} as shown in Fig. 1. In some prep-

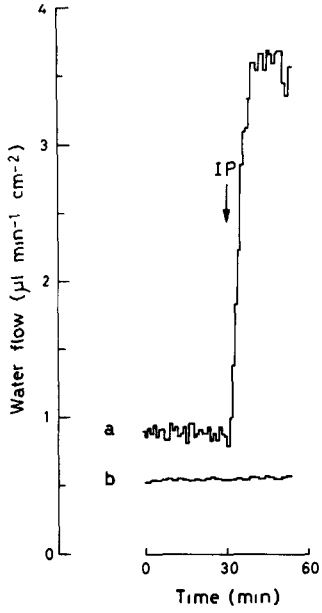
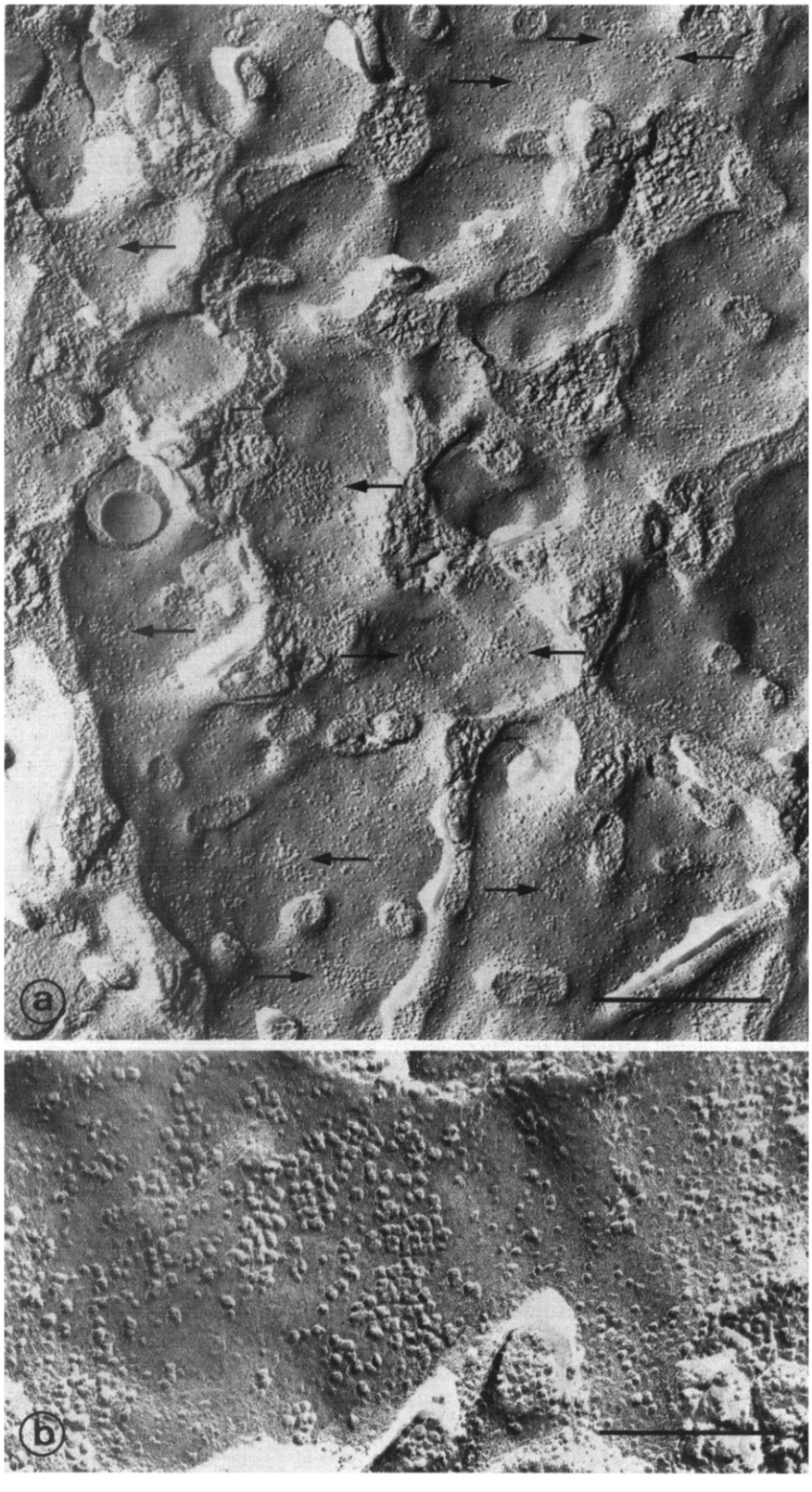


Fig. 1. Direct recording of water flux across two segments of the same pelvic skin of a toad *Bufo marinus*. The automatic technique employed was described elsewhere [6,7]. Note the dramatic increase in water flow following addition of isoproterenol (IP), 1μM (record a), in contrast to the steady flow observed in the control segment of the skin (record b). Percentage increases in water flow with isoproterenol in the other three skins examined with freeze-fracture were 217, 553 and 562%.

arations, the stimulated water flow reached values 600 % higher than basal flow. The hydrosmotic effect of isoproterenol was completely abolished by 10^{-4} M propanolol [10]. It has already been shown that the skin of amphibians possesses β-receptors to catecholamines and that in this tissue β-adrenergic agonists elicit a cyclic AMP-dependent hydrosmotic response similar to that induced by neurohypophysial hormones [10—13]. It has also been shown that isoproterenol stimulates water gain in vivo [14]. In *Bufo marinus*, the sensitivity to catecholamines was extremely high, and the J_{H_2O} values measured across the skin were comparable to those induced by vasopressin in the urinary bladder of the same species [10].

In view of the striking similarity between J_{H_2O} values following stimulation of the skin and bladder, we postulated that if particle aggregates


a
b

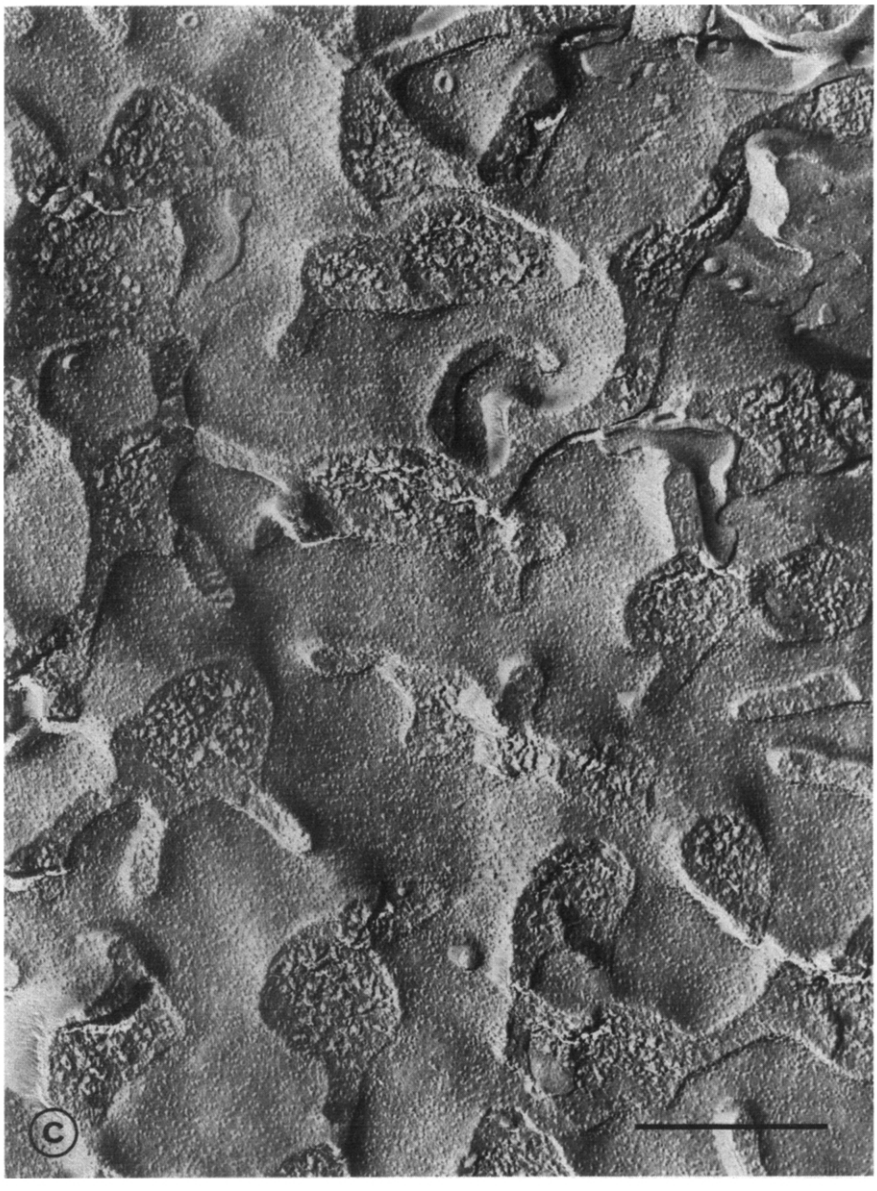


Fig. 2. (a) Replica of the apical P-face of the replacement cell layer from isoproterenol-treated *B. marinus* epidermis, showing many aggregates of intramembrane particles (arrows). The stimulatory effect of isoproterenol on water permeability of the same piece of skin is shown in Fig. 1. × 50 000 (Bar = 0.5 μm). (b) Higher magnification of an aggregate from the same isoproterenol-treated skin shown in (a). Many of the particles in the aggregate appear to be formed of smaller subunits, and some have a slightly flattened appearance. In the membrane surrounding the aggregate, some individual particles can be found which are larger than the majority of the particles in non-stimulated tissue (see c). × 100 000 (Bar = 0.25 μm). (c) An area of the replacement cell layer apical membrane P-face from the paired, non-stimulated control of the skin shown in (a) and (b). There are no particle aggregates. × 50 000 (Bar = 0.5 μm).

described in toad bladder were indeed a general phenomenon related to an increase in water permeability, then we ought to find a similar membrane feature following freeze-fracture of the stimulated epidermis. Having solved the problem of identifying the membranes of the different cell types present in the epidermis [15,16], we were able to find and localise intramembrane particle aggregates on the apical membrane of the cell layer immediately beneath the stratum corneum (Fig. 2 a,b). This cell layer is known as the replacement cell layer or the first reacting cell layer [17]. No aggregates were seen on the membranes of any other epidermal cell types, and they have not so far been found in non-stimulated paired control skins (Fig. 2c). The aggregates, present on the cytoplasmic leaflet of the membrane (P-face), were formed of intramembrane particles which appeared to be larger than those seen in the remaining regions of the same membrane. Complementary patterns on the extracellular leaflet (E-face) were not found in all replicas, but when present they appeared as numerous, geometrically-arranged shallow pits. For measurement of the number of aggregates per μm^2 of membrane, negatives of random areas of membrane were projected onto a graphics tablet (Tektronix, type 4953) at a final magnification of 57 000 ×. The outline of the membrane was followed with a pen connected to the graphic tablet and to an IMSAI microprocessing system, which calculated the surface area. The membrane outline traced by the pen was simultaneously transferred to a display terminal, so that no regions of membrane could be accidentally measured more than once. A total of 220 μm^2 of membrane was examined from 4 animals, and the total number of aggregates counted was 665. The values for the individual animals were 4.9, 2.7, 2.5 and 1.7 aggregates per μm^2. The area of membrane occupied by the aggregates was measured on negatives projected onto the graphics tablet at a magnification of 130 000 ×. The area of membrane was calculated first, followed by the area of the aggregates, and the results were expressed as a percentage of total membrane area. A total of 56 μm^2 of membrane was examined and the total area occupied by the aggregates in the 4 animals was 1.2 μm^2. Results for each individual skin were 2.6, 2.7, 1.7 and 1.4 % of the total membrane area, which is higher than that reported for the toad bladder [3]. The size of the individual aggregates varied considerably, up to a maximum of 0.04 μm^2, with a mean size of 0.007 μm^2.

The results presented here strongly suggest a morpho-functional correlation that might be of general significance in the physiology of epithelia. In fact, according to current concepts, cyclic AMP-induced hydrosmotic flows in tight epithelia follow a transcellular path, the rate-limiting barrier of which is the outward facing membrane [18,19]. In a multilayered epithelium like the epidermis, such a barrier corresponds to the RCL apical membrane which lies immediately beneath the stratum corneum. It is precisely in this membrane that the particle clusters were seen and they always appeared when the water permeability of the epidermis was increased. The aggregates may represent water permeable patches, containing aqueous pores or channels, which function as osmotic shunts across the apical membrane [19]. Similar structures, induced by oxytocin, were first described in frog urinary bladder [1] and, more recently, loose clusters of

particles have been detected in the collecting duct of rat kidney following injection of the animals with vasopressin [20]. Morphologically, the clusters described here in the epidermis are also reminiscent of the orthogonal arrays of intramembrane particles which have been described in basolateral membranes of light cells from the rat collecting duct [21] as well as in the plasma membrane of astrocytes [22], gastric oxyntic cells [23] and toad urinary bladder mesothelial cells [5]. However, the appearance of these square arrays of particles has not yet been shown to be hormone-dependent. It is still debatable whether the aggregates result from increased lateral mobility of membrane proteins* [24], or whether they are inserted in the apical membrane from pre-formed intracytoplasmic structures. However, no intracytoplasmic vesicles similar to those found in the toad bladder and whose membrane contains aggregated particles [4,5], have yet been found in the replacement cell layer. In contrast, we had the impression that the number of particles in the non-stimulated replacement cell layer apical membrane was greater than that of the non-aggregated regions of the stimulated membrane (compare Figs. 2a and 2c). This observation is currently being quantitatively evaluated. Whatever their mechanism of formation, the important point is that clustering of particles also occurred in the skin following the action of a non-polypeptidic, hydrosmotic agent, isoproterenol, the effects of which are also mediated by cyclic AMP. Since the epidermis, with respect to the urinary bladder epithelium, has a different structure and a different embryological origin, it is likely that clustering of intramembrane particles on the outward facing membrane is a general pre-requisite for changes in water permeability in osmoregulatory epithelia. Work now in progress in our laboratory should provide additional evidence to substantiate a specific relationship between the formation of aggregates and the increase in transepithelial water flow. Results obtained so far show that in toad skin, aggregate formation is also induced by vasopressin and that this effect is inhibited by methohexital, a specific blocker of vasopressin-induced water flow [10].

Furthermore, in skins exposed to amiloride (10^{-4} M) the subsequent exposure to isoproterenol or vasopressin failed to stimulate sodium transport; in contrast, the hydrosmotic response was normal, and aggregates were still found on the replacement cell layer apical membrane.

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*Although it is widely believed that intramembrane particles, as visualised by the freeze-fracture technique, represent proteins which are embedded in the lipid bilayer [25,26], it has recently been suggested that membrane lipids, in certain conditions, may also give rise to particle-like structures on freeze-fracture replicas [27,28].

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