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Electron Microprobe Analysis of Intracellular Electrolytes in Resting and Isoproterenol-Stimulated Exocrine Glands of Frog Skin

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Summary. In the intact, in vitro frog skin, isoproterenol (ISO) stimulates an amiloride-insensitive increase in short-circuit current (SCC) that can be localized to the exocrine glands and is associated with secretion of chloride. To determine which cells in the glands respond to stimulation we measured the intracellular electrolyte concentrations of the various cell types of the mucous and seromucous glands of the skin using freeze-dried cryosections and electron microprobe analysis. In the resting state, the various cell types of the glands have intracellular electrolyte concentrations similar to the epithelial cells of the skin. Exposure to amiloride (10^{-4} M) has little effect on the concentration of Na and Cl in the cells of the glands. The effect of isoproterenol has two distinct phases. Analysis of glands in tissues frozen at the peak of the SCC response (13 min after addition of isoproterenol) shows that the only significant change is an increase in Na and Ca in a group of cells at the ductal pole of the acini of both gland types. These are termed "gland" cells. The duct cells and cells that secrete macromolecules did not show any significant changes at this timepoint. In the gland cells, after a one-hour exposure to isoproterenol the Na concentration is at prestimulation levels while Cl drops. There is also a smaller drop in Cl in the duct and skin epithelial cells. Ouabain, which can completely block the isoproterenol SCC response, has little short-term effect on Na and Cl in the control gland but accentuates the gain of Na and drop in Cl in the isoproterenol-treated condition. Bumetanide and, to a lesser extent, furosemide, also blocks the isoproterenol SCC response and causes a further drop in Cl. The results provide indirect evidence that a major portion of the ionic component of the gland secretion is produced by a distinct group of cells separate from those producing the macromolecular component and that the mechanism of secretion involves a Na : Cl coupled transport system linked to the activity of the basolateral Na pump.

Key Words exocrine glands · ion secretion · electron microprobe analysis

Introduction

It has long been established that the glands of frog skin respond to catecholamines [10]. Two types of responses can be elicited, depending on the molecular species. α-adrenergic stimulation results in the pulsed extrusion of material from the glands [4]. This secretion is not accompanied by any significant change in the transepithelial electrical characteristics of the isolated skin [24]. β-adrenergic stimulation also results in secretion of fluid on the skin surface. This relatively long-lived secretory response is accompanied by an increase in short-circuit current, transepithelial conductance, and net chloride secretion from serosa to mucosa [10, 22, 23]. The α-adrenergic stimulus appears to affect the granular (poison) gland and is manifested by a contraction of the smooth muscle cells which surround this gland [4, 8]. The mucous glands appear to be the site of action of the β-adrenergic stimulus. High doses result in contraction of the myoepithelial cells which surround these glands [19]. However, a lower dose (10^{-6} M) results in fluid secretion in parallel with changes in chloride transport with no distinct changes in gland cell morphology. This β-adrenergic stimulus of ion transport can be blocked by furosemide or ouabain and removal of Na or Cl from the serosal bath [22, 23]. Thus it seems to be driven, at least in part, by a transport mechanism similar to what has been described for other chloride secreting cells [6, 18].

The mucous glands of frog skin can be divided into two distinct morphological types: mucous and seromucous glands [11]. Within these glands are several different cell types, some containing no formed secretory product. Thus the cellular source of the ion transport response to β-stimulation may be to a distinct subset of cells within the gland different from those which release the macromolecular component. In order to answer this question and gain some understanding of the secretory mechanism we analyzed the ionic shifts that occur in the individual cell types using energy-dispersive electron microprobe analysis. The results show that

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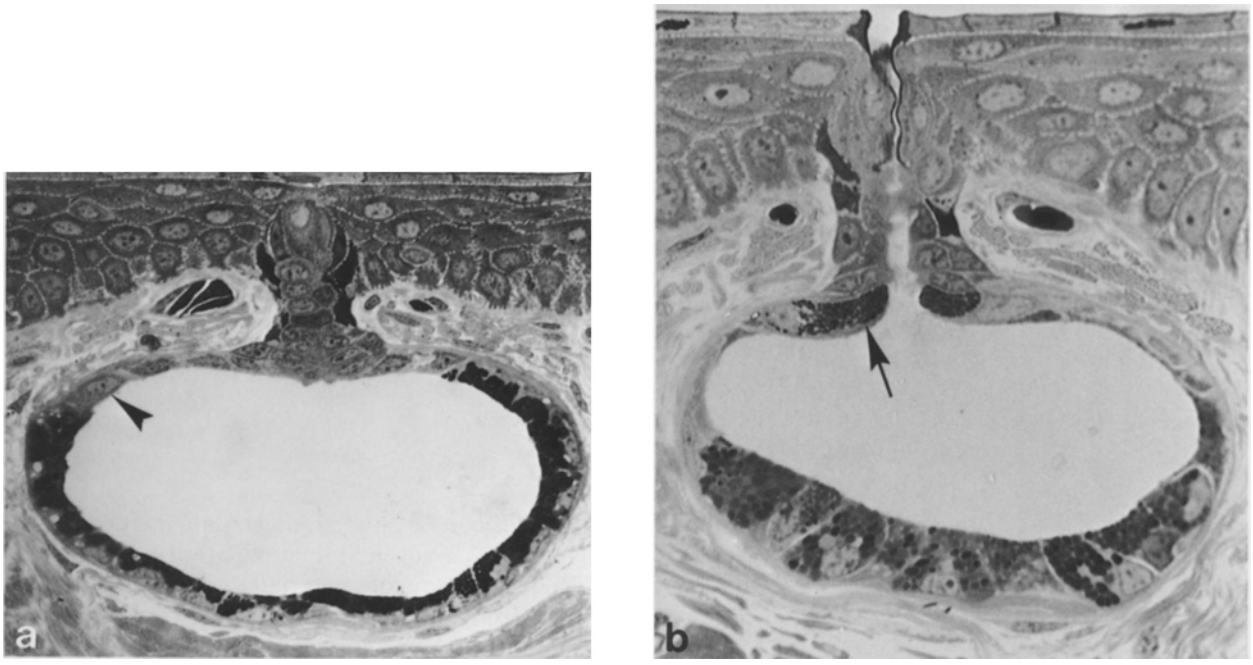

Fig. 1. Light micrographs of skin glands of *R. temporaria*. In both gland types cells containing secretory product are situated in a semilunar arrangement opposite the duct. Towards the ductal pole the cells do not appear to have secretory product. (*a*) Mucous gland. The arrowhead identifies the mitochondria-rich cell, which is usually located in this position. (*b*) Seromucous gland. The arrow points to the granule-containing cell that is found at the junction between duct and acinus. The morphology and position of this cell type is used as a key for identifying the gland types in freeze-dried cryosections. ×450

Table. Element concentrations and dry weight content of the various epithelial cell types of frog skin glands in the resting state and at the peak of isoproterenol-induced SCC response (in the presence of amiloride)

	Na	K	Cl	P	Mg	Ca	dry wt (g/100g)
	mmole/kg wet wt						
Gland cells							
Amiloride (n = 81)	9.4 ± 6.7	126.2 ± 19.4	45.2 ± 9.5	136.9 ± 22.4	7.7 ± 4.7	0.3 ± 0.3	18.6 ± 2.6
Amiloride + isoproterenol (n = 67)	16.0 ± 11.8[a]	118.9 ± 21.4	43.6 ± 12.3	130.1 ± 27.2	7.0 ± 4.0	1.1 ± 1.3[a]	19.0 ± 3.1
Mucous cells							
Amiloride (n = 41)	11.5 ± 7.4	127.2 ± 25.2	40.3 ± 8.7	133.6 ± 28.8	6.6 ± 4.4	0.6 ± 0.9	19.5 ± 4.6
Amiloride + isoproterenol (n = 36)	11.4 ± 9.7	129.0 ± 26.5	38.5 ± 8.7	130.3 ± 30.3	7.2 ± 6.1	0.9 ± 1.5	19.1 ± 4.2
Duct cells							
Amiloride (n = 63)	6.1 ± 4.3	131.4 ± 19.9	38.2 ± 8.0	153.8 ± 48.5	7.2 ± 2.5	0.3 ± 0.4	21.5 ± 4.1
Amiloride + isoproterenol (n = 85)	7.2 ± 6.0	128.6 ± 27.2	36.2 ± 8.1	149.4 ± 42.8	7.9 ± 4.1	0.4 ± 1.1	21.6 ± 3.9
MRC							
Amiloride (n = 9)	13.2 ± 7.0	131.4 ± 36.9	14.2 ± 3.4	182.7 ± 61.9	7.0 ± 3.7	0.1 ± 0.1	23.9 ± 4.8
Amiloride + isoproterenol (n = 10)	10.2 ± 6.7	136.8 ± 35.6	13.3 ± 3.1	183.2 ± 57.9	7.2 ± 4.1	0.1 ± 0.1	23.9 ± 6.1

Pooled data from 7 individual experiments. Combined values from mucous and seromucous glands.
[a] Statistically different from the amiloride value ($2P < 0.01$). Means ± SD.

during the early stages of secretion, when the ion transport response is at a maximum, as well as during later stages, the major ion shifts occur within cells located at the ductal pole of each of the gland types. Thus, there appears to be a functional heterogeneity within the glands that could provide the mechanism for modification of the final secretory product, depending on the type or duration of stimulus.

Materials and Methods

All skins were from *Rana temporaria*. The procedures for incubation and short circuiting are identical to previous descriptions [14]. Experiments were performed simultaneously on 2 or 4 pieces of the same skin. For each skin there was a preincubation period until the short-circuit current (SCC) reached a steady-state value. Incubation was then continued according to the experimental protocol described in Results. The composition of the incubation solution was (in mM): 110 NaCl, 2.5 $KHCO_3$, and 1

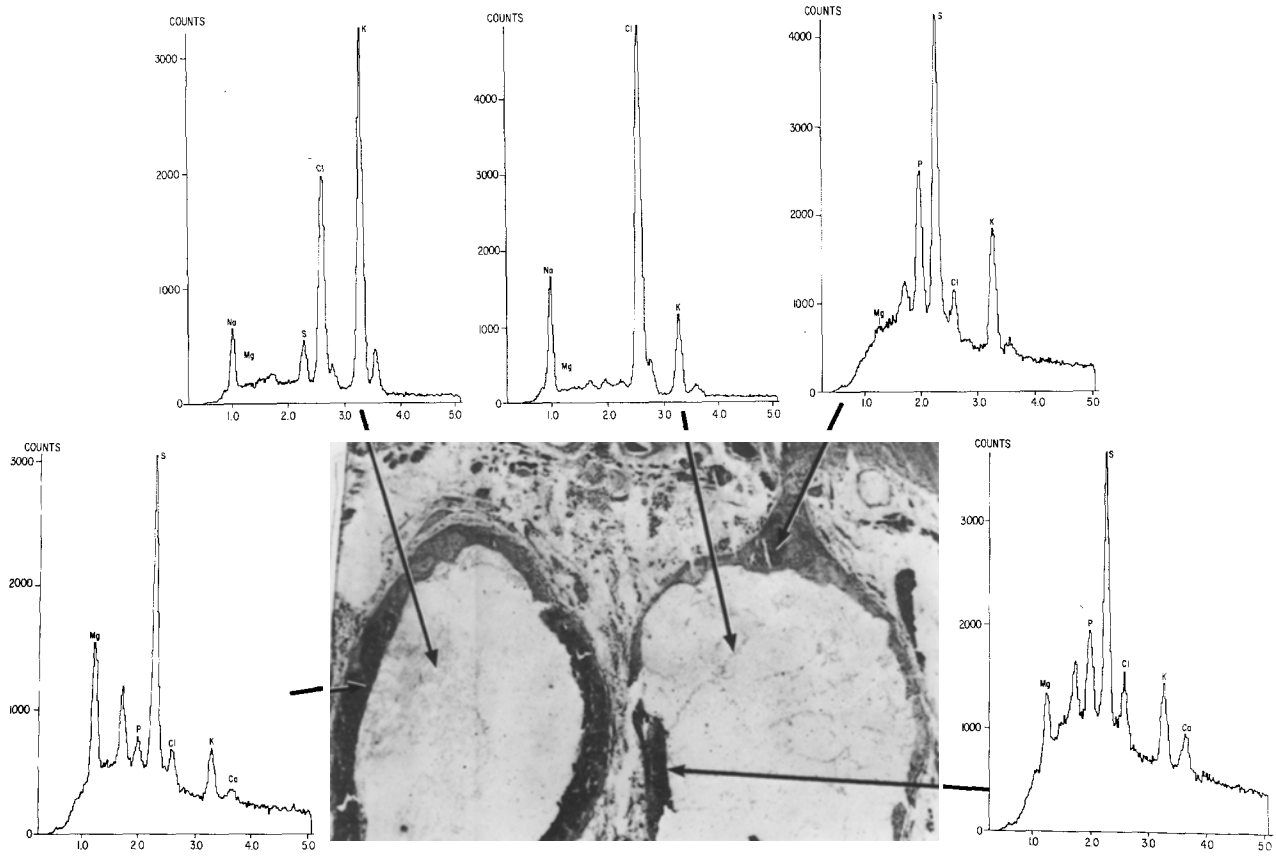

Fig. 2. Scanning electron microscopic image of freeze-dried cryosection and energy-dispersive X-ray spectra of individual measurements. Notice the dramatic difference in peaks between lumenal mucous and mucous within the secretory cells. Also notice that in the seromucous gland (right side of figure) a granule-containing cell in the duct region has a high sulfur signal but very low Mg and Ca peaks

$CaCl_2$. In Cl-free media NaCl was replaced by 55 Na_2SO_4 plus 55 sucrose. Amiloride (a gift from Merck, Sharp and Dohme) was used in 10^{-4} M concentration on the mucosal side. Isoproterenol (10^{-6} M), furosemide (5×10^{-4} M), bumetanide (10^{-4} M) and ouabain (10^{-4} M) were applied to the serosal side.

The preparation of the tissue for electron microprobe analysis is essentially similar to what has been described previously [5, 14] and in summary is as follows: Skin pieces were rapidly removed from the chambers, coated with an albumin standard solution (20g/100g Ringer's solution) and frozen in an isopentane/propane mixture [9] cooled to −196°C by liquid nitrogen. 1-μm cryosections were cut at −80°C, picked up on formvar-coated slot grids, and freeze-dried at −80°C and 10^{-6} mbar. Sections were analyzed in a scanning electron microscope with an energy dispersive X-ray detector. The accelerating voltage was 20 kV and the probe current 0.4 nA. Areas (1–2 μm^2) were scanned for 100 sec, and the X-rays in the energy range from 0.6 to 5 keV were analyzed. Quantification of elements was as previously described [16].

The applied deconvolution method [1] can resolve 0.4 mM Ca in an individual cellular X-ray spectrum. The calculated Ca concentration is independent of the K concentration as established in standard sections with K concentrations varying between 0 and 150 mM.

Identification of the various gland types and cells within the glands could be readily made in the scanning electron microscope (Figs. 1 to 3). The cellular measurements listed in the Table and displayed in the Figures are from nuclei. Where possible, additional measurements were made in cytoplasmic regions free of visible organelles. With the exception of lower P and higher Ca values those measurements provided essentially the same results. Since the cells toward the ductal pole were squamous, measurements were usually made in sections cut slightly tangential to the gland axis, thus providing a greater cellular profile which allows the scanning beam to be placed away from the cell membrane. Identification of the two layers of duct cells were difficult to make unless the section passed through the lumen of the duct. Thus most measurements of the duct cells were done on that portion of the duct that continued from the base of the skin epithelium to the acinus. However, enough sections were obtained showing the lumen of the duct to confirm that the cells along the length of the duct responded in a similar manner.

In each gland, where possible, measurements were taken of the product in the lumen of the gland and of the apical cytoplasm of the mucous or seromucous-secreting cells containing the secretory product. This was done as an aid and confirmation as to gland type. Occasionally, the mucous and seromucous secretory cells and lumenal contents were lost during sectioning. In some cases the gland type could not be definitely confirmed morphologically. In other cases positive identification could be made as long as the section included the duct region since the seromucous gland contained a distinct cell type in this region (*see* Results). The results in Figs. 7–10 are typical of between 4 to 10 experiments for the individual protocols. Points from the same experimental condition are connected by lines to

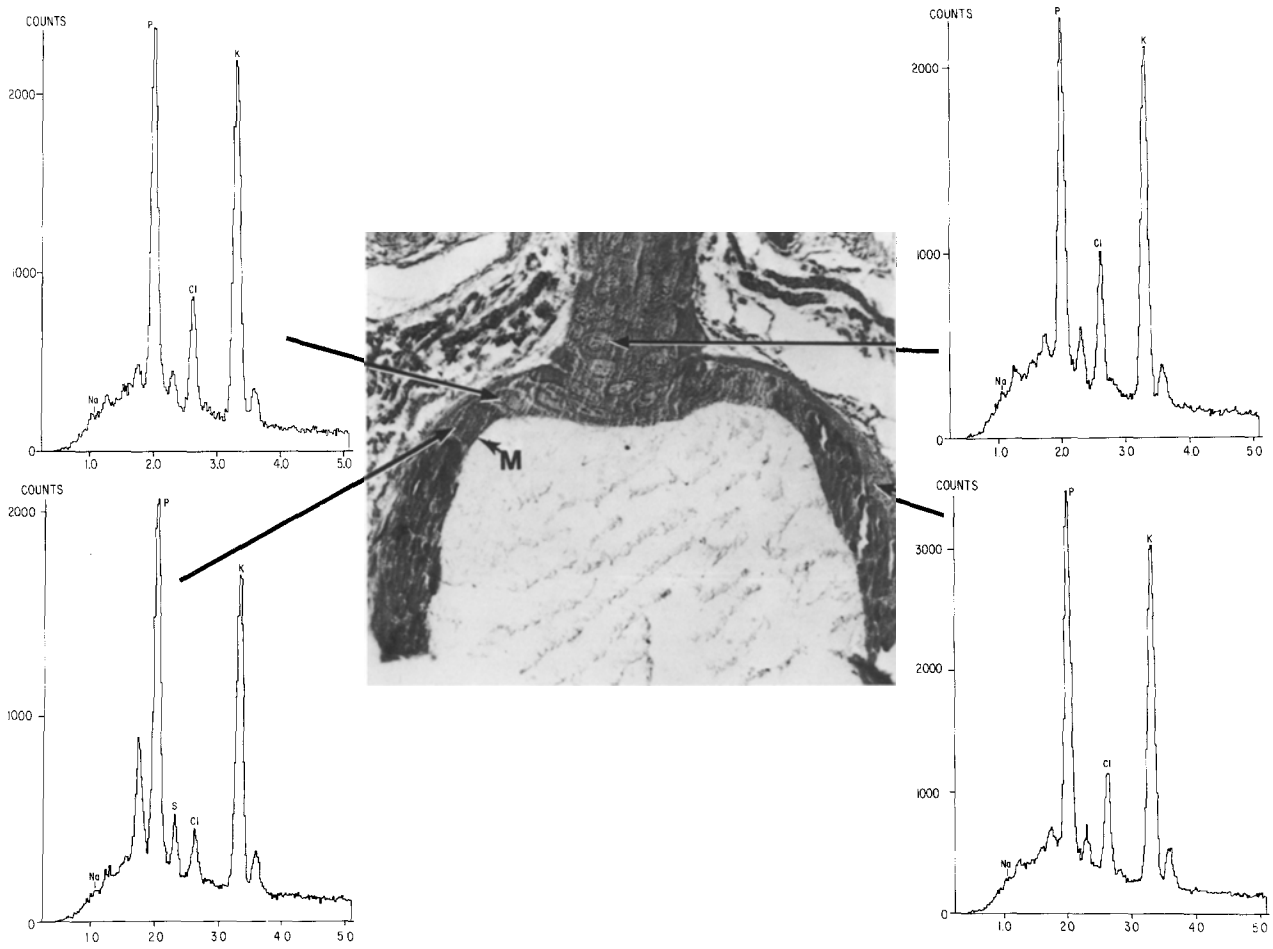

Fig. 3. Scanning electron microscopic image of freeze-dried cryosection and energy-dispersive X-ray spectra of individual measurements. Spectra are from the different cell types of the mucous gland. In general the spectra of the individual cells are identical except for the mitochondria-rich cell (*M*). In this cell type the Cl signal is approximately half what is seen in the other cell types

facilitate detection of parallel concentration changes in different cell types.

Results

Figure 1 shows the light microscopic view of the two types of mucous gland in frog skin. These have been tentatively classified as mucous and seromucous [11] based on standard criteria of morphology and staining characteristics [27]. It was also possible to identify this gland type in the scanning transmission electron microscopic image obtained from freeze-dried cryosections. Furthermore, when a section passed through the duct region of a seromucous gland there was a readily identifiable cell type which contained dark granules (Figs. 1 and 2). The granules in this cell have a high S signal and a low Mg and Ca signal (Fig. 2). The apical cytoplasm of the other secretory cells, in both the mucous and seromucous glands, have high Mg and Ca peaks as well as the prominent S signal (Fig. 2).

For purposes of classification, the cell types in the glands are arranged as follows: the cells that contain secretory product packaged in vesicles are called mucous cells (for both gland types); the cells that are in the acinus of the gland and do not appear to contain secretory product are called gland cells; the two layers of cells making up the duct are duct cells. The one exception to this was the mitochondria-rich (MRC) cell in the mucous gland. This cell is known from detailed morphological studies to be located adjacent to the last mucous cell in the acinus and makes up a discontinuous ring at the ductal pole of the cell [11]. This cell could usually be identified and thus was classified separately as MRC. The MRC had distinct intracellular ion concentrations (Fig. 3 and the Table). Since we found no systematic differences in the element concentrations between the analogous cells types in the two glands, we have grouped the data except for the MRC of the mucous gland.

The typical SCC response of *R. temporaria* to

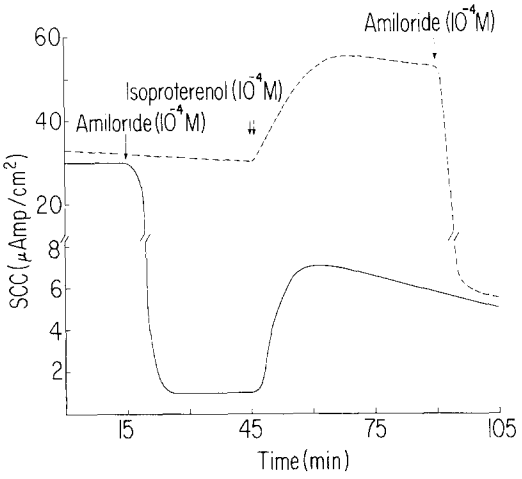

Fig. 4. Typical response of *R. temporaria* skin to isoproterenol. If isoproterenol (10^{-6} M) is added to the serosal solution, there is a dramatic increase in current. If amiloride (10^{-4} M) is added, the current is reduced but does not reach zero. In all cases the remaining current is between 4 and 10 μA cm^{-2}. If amiloride is added first, the current is reduced to less than one. Subsequent addition of isoproterenol results in a sustained increase in current that is equal to that remaining in the skin exposed to the opposite sequence

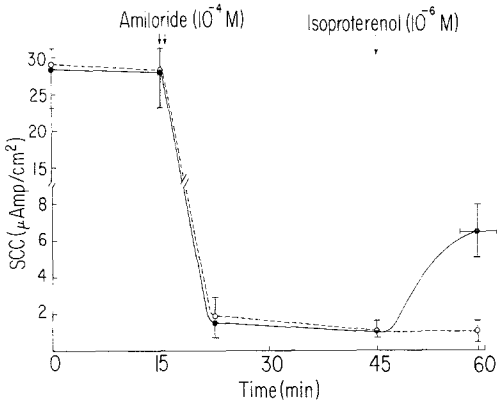

Fig. 5. Short-circuit current response of *R. temporaria* exposed to isoproterenol after amiloride. The peak response for the seven skins is 6.2 μA cm^{-2}. Skins were frozen at the peak

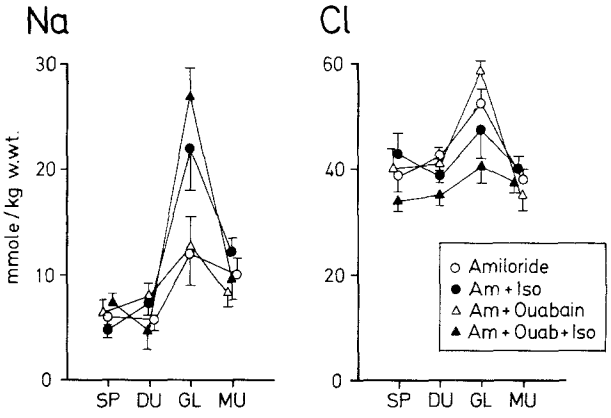

Fig. 6. Effect of ouabain on the intracellular Na and Cl concentration changes induced by short-term isoproterenol stimulation (11 min) in the presence of amiloride. *SP* = spinosum cells; *DU* = duct cells; *GL* = gland cells; *MU* = mucous cells; *Am* = amiloride; *Iso* = isoproterenol; *Ouab* = ouabain. Each point represents the mean of n=5 to n=12 individual measurements, ± SE

isoproterenol is shown in Fig. 4. Whether amiloride is added before isoproterenol or after, the final current that remains is identical. This amiloride-insensitive current is identical in magnitude to that previously demonstrated in *Rana catesbeiana* [22]. As with *R. catesbeiana* [22] this amiloride-insensitive current change does not occur in split skins devoid of gland acini (data not shown).

Figure 5 shows the combined SCC data for the *R. temporaria* skin used to obtain the microprobe data. After isoproterenol the SCC increased from 1.0 ± 0.6 to 6.2 ± 1.5 μA/cm^2 (n = 7). The average time to reach the peak was 13.0 ± 4.5 min (n = 7). The results of the electron microprobe analysis on glands frozen at the peak of the SCC response are shown in the Table. The only significant change is an increase in the Na and Ca concentration in the gland cells. The rise in Na is matched by a fall in the K concentration. In addition, a small decrease in the Cl concentration is apparent in all cell types, which, however, does not attain statistical significance.

Figure 6 shows the Na and Cl concentrations in one of the seven individual experiments underlying the Table. In this particular experiment two further pieces of the skin were exposed to 10^{-4} M ouabain. After 20 min, one of these skin pieces was exposed to isoproterenol. This treatment results in a complete blunting of the SCC response to isoproterenol (data not shown). It is evident that, despite this effect of ouabain on the SCC, the Na increase and Cl decrease in the gland cells is present. In fact, the drop in the Cl concentration seems to be enhanced by ouabain. In addition to the three major glandular cell types, Fig. 6 also depicts the Na and Cl values for one cell type of the surface epithelium, the stratum spinosum cells, exhibiting no significant variations under the experimental conditions tested.

In further experiments the glandular SCC response to isoproterenol was blocked by 10^{-4} M furosemide added 20 min before isoproterenol (no amiloride present). In the experiment underlying Fig. 7, the current rose from 21.2 to 35.6 μA/cm^2 at the peak (12 min), whereas the skin pretreated with furosemide rose from 17.5 to a peak of 21.9 μA/cm^2 (12 min). These current changes represent increased Na transport by the skin epithelium as well as Cl transport by the glandular epithelium since

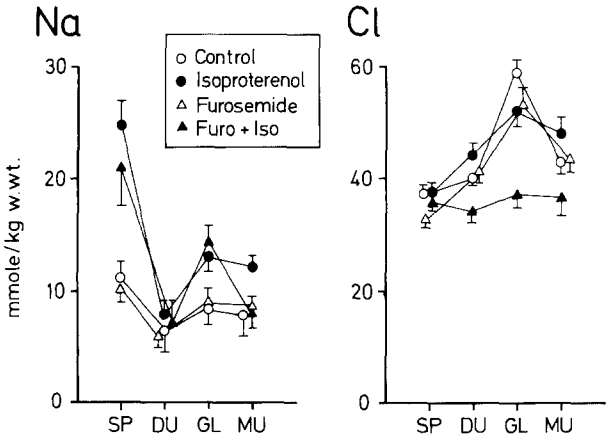

Fig. 7. Effect of furosemide on the intracellular Na and Cl concentration changes induced by short-term isoproterenol stimulation. *Furo* = furosemide. For further abbreviations *see* Fig. 6. Each point represents the mean of $n=5$ to $n=13$ individual measurements, ± SE

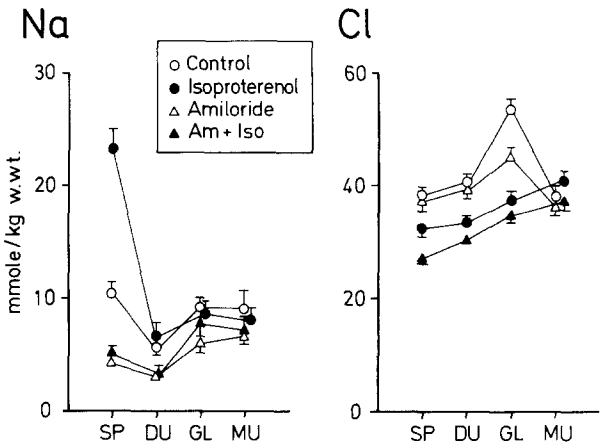

Fig. 8. Effect of amiloride on the intracellular Na and Cl concentration changes induced by long-term isoproterenol stimulation (60 min). For abbreviations *see* Fig. 6. Each point represents the mean of $n=8$ to $n=19$ individual measurements, ± SE

amiloride was not present. The same partial sensitivity of the current response to furosemide has been demonstrated in amiloride-inhibited skins [23]. As shown in Fig. 7, despite the significant reduction in the current response the Na increase as well as the Cl decrease in the gland cells is still present. Again, the drop in the Cl concentration seems to be amplified when the SCC response is inhibited. In the spinosum cells a large Na increase can be observed after isoproterenol, which is apparently insensitive to furosemide. A similar experiment performed in the presence of amiloride provided essentially the same results for the glandular epithelium, while the increase in Na in the spinosum cells after isoproterenol was prevented (*see also* Figs. 6 and 9).

In two further groups of skins the effect of isoproterenol was studied at longer exposure times (60 min), when in *R. temporaria* the amiloride-insensitive SCC is already reduced to about 70–80% of its peak value. The rationale behind these experiments was to amplify the isoproterenol-induced ion shifts, assuming that new steady-state concentrations might not have been achieved within the short time to reach the peak SCC. Figure 8 shows such an experiment in which isoproterenol was added either in the presence or in the absence of amiloride. In either case, isoproterenol stimulation results in a significant decrease in the Cl concentration of the gland cells. In addition, a significant though somewhat smaller drop in the Cl concentration of the duct cells and spinosum cells is evident, whereas the mucous cells show no significant changes. In spinosum cells a large Na concentration increase can be observed after isoproterenol which is abolished by amiloride. Apart from spinosum cells a small amiloride-sensitive component of the intracellular Na concentration can also be observed in duct cells.

Figure 9 shows an experiment in which the glandular secretion was inhibited by bumetanide. Like the Cl-dependent SCC in the frog cornea [3] the amiloride-insensitive SCC response to isoproterenol in the skin of *R. temporaria* is fully inhibitable by this drug. Compared to furosemide, bumetanide seems to be a more selective inhibitor of the Na/K/2Cl transporter [13]. As already seen in the previous experiment, isoproterenol (60 min) leads to a significant decrease in the Cl concentrations in all cell types except in mucous cells. In the nonstimulated skin addition of bumetanide (60 min) results in a statistically significant decrease of the Cl concentration in the gland cells and spinosum cells. A further marked reduction in the Cl concentration of the gland cells is seen after additional application of isoproterenol. No systematic changes are detectable in the intracellular Na concentrations.

In a last series of experiments the exchangeability of the intracellular Cl with the outer or inner bathing medium was investigated. Figure 10 shows an experiment in which Cl was removed from the serosal bath. After 60 min incubation in all cell types a drastic fall in the Cl concentration can be observed. In contrast, when Cl is removed from the mucosal bath no significant change is detectable (data not shown). In the gland cell, Cl removal resulted in a large drop in Na as well.

Discussion

One interpretation of the results of this study is that the β-adrenergic stimulatable ion secretory response of the frog skin glands is primarily localized

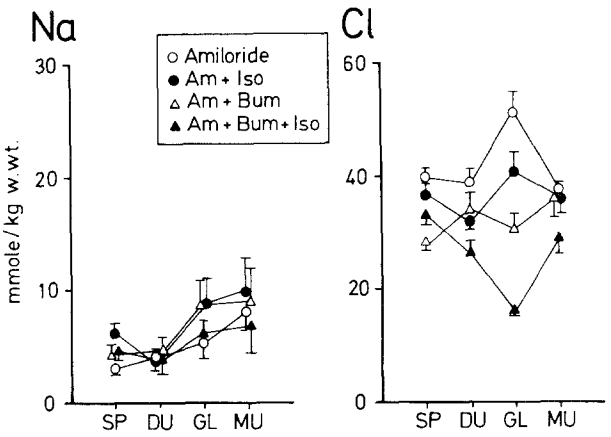

Fig. 9. Effect of bumetanide on the intracellular Na and Cl concentration changes induced by long-term isoproterenol stimulation (60 min) in the presence of amiloride. *Bum* = bumetanide. For further abbreviations *see* Fig. 6. Each point represents the mean of $n=6$ to $n=11$ individual measurements, ± SE

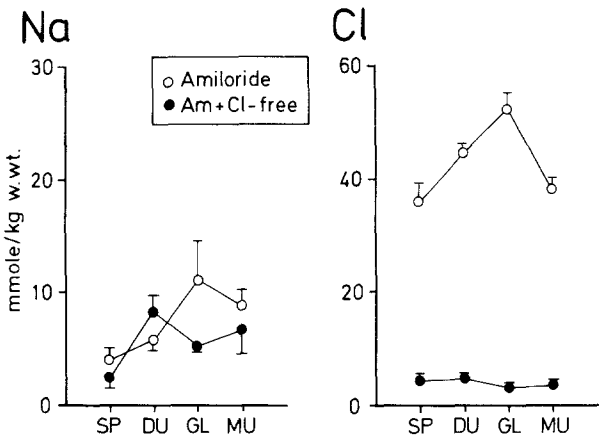

Fig. 10. Effect of removal of Cl from the serosal bath (60 min) on the intracellular Na and Cl concentrations. For abbreviations *see* Fig. 6. Each point represents the mean of $n=5$ to $n=7$ individual measurements, ± SE

to a distinct subset of cells situated at the ductal pole of the acinus of both the mucous and seromucous gland types. This is based on the observations that these cells, termed "gland" cells to distinguish them from mucous or seromucous granule-containing cells of the acinus, are the only cells to demonstrate consistent and significant shifts in Na and Cl concentrations after various manipulations that effect the secretory response of the glands. The best support for this argument comes from comparing the Table and Fig. 6 with Fig. 8. After a short-term exposure to isoproterenol, at a time when the secretory response is at a maximum (Fig. 5, also Fig. 2 of ref 22), the only cell to show a significant ion shift is the gland cell with an increase in Na and Ca. When a relative new steady state is attained (60 min after exposure to isoproterenol) the most dramatic change within the cells of the gland is the drop in Cl in the gland cells (Fig. 8). After the secretory response is blocked by prior exposure to ouabain, the gland cells show a large increase in Na and decrease in Cl whereas there is little change in the other cells of the gland.

The method for recording the secretory response of the gland requires the presence of amiloride in the outer bathing solution [22]. Without amiloride the Na transport response of the epithelial cells of the skin predominates (Fig. 2). It is possible then that the data in the Table does not represent the complete response of all the cells in the gland because of amiloride's potential effect on a Na channel or Na : H antiport in any of the cells analyzed [21]. The dramatic effect of amiloride on the Na channel can be seen when one compares the Na levels in the spinosum cells in the presence of isoproterenol (Figs. 6 and 7). The spinosum Na concentration more than doubles after isoproterenol when amiloride is absent but is unaltered in the presence of amiloride. A more detailed account of the effects of isoproterenol and amiloride on the cells of the different epithelial layers has been given previously [15]. A comparison of Figs. 6–8 indicates that the amiloride exposure did have an effect on the Na and Cl concentrations of several of the cell types. The most noticeable effect, besides the expected reduction in Na in the skin epithelial cells, was a reduction in Cl in the gland cells both in the skins exposed to amiloride for approximately 45 min (30 min preincubation plus time to isoproterenol-induced current peak) or 180 min (Fig. 8). This may reflect a secondary inhibition of an anion exchange via a blockage of a Na : H antiporter by amiloride [21]. No amiloride effect on Cl flux across the skin could be determined in previous studies [23].

The only other effect that appeared to reflect a direct action of amiloride was a small drop in the Na concentration in the cells of the duct. This was not a consistent finding but the lack of consistency may be more related to the anatomical organization of the duct rather than variability in the responsiveness of the duct cells. Since the duct is bilayered and sections only rarely would pass through the lumen it was very difficult to determine which cell layer of the duct was being analyzed. It is possible that the cell layer facing the lumen may have an amiloride-sensitive Na entry step since this layer is directly continuous with the outer-living cell layer of the skin epithelium. Likewise, the second layer of the duct may be insensitive to amiloride. Thus the variable results possibly reflect the relative population of each cell layer analyzed in each experiment.

The duct is an obvious candidate to possess a

Na-reabsorptive mechanism, as is the case for the duct of sweat glands [17], and could have an ion secretory mechanism, as proposed for pancreatic ducts [7]. No change in Na concentration was seen in duct cells exposed to isoproterenol for short or long periods in the absence of amiloride. In the frog skin epithelium, stimulation of Na uptake by isoproterenol leads to a dramatic increase in Na concentration. Considering the problem raised above about effectively analyzing the two layers of the duct, we are tentatively concluding that the duct does not respond to isoproterenol by an increased Na reabsorption.

The duct cells did show a drop in Cl concentration in long term exposures to isoproterenol and this occurred whether amiloride was present or not (Figs. 8 and 9). There was also a slight reduction in the duct cell analyzed at peak response, but this was not significant (Table). This could be taken as evidence that the duct contributes to the increase in SCC and Cl transport that occurs after β-adrenergic stimulation via a Cl-secretory mechanism. This possibility cannot be ruled out by the present study.

The mucous and seromucous cells are by far the most abundant cell type in the acinus and would be logical candidates for the site of the ion transport response stimulated by isoproterenol. The mechanism could be by active ion secretion across the cell membrane or be driven by exocytotic release of granules. It does not appear, however, that these cells take part in the isoproterenol response. We found no change in Na and Cl in these cells when analyzed at either the peak of the response or after a long-term exposure. Since the cytoplasm of these cells is filled with secretion product all of the probe measurements in the Table and the figures are from areas in the nucleus. Although it is possible that the nuclear measurements do not reflect cytoplasmic values, it has been demonstrated that in frog skin, at least, there is no reproducible difference in Na, K and Cl between the cytoplasm and nucleus [14]. We did measure cytoplasm (secretion granules) in every case, as well. The results for the granule measurements are quite variable with no consistent trend detectable in any condition. The large variation is probably due to variable amounts of granule-free cytoplasm included in individual measurements [11] as well as degrees of maturation and dehydration of the secretion granules [12]. Separate morphological studies indicate that β-adrenergic stimulation does not cause a change in mucous or seromucous cell morpology, has no effect on the release of granules, and produces no discernible change in structure and content of the secretory product stored in the lumen (data not shown). This is in agreement with previous reports by Benson and Hadley [2] and Dockray and Hopkins [4]. Thus β-adrenergic stimulation of fluid secretion does not appear to proceed by an exocytotic mechanism of release of macromolecules from the mucous or seromucous cells.

The only other cell type that could be identified was the MRC of the mucous gland. This cell type has both a consistent location within the acinus and a relatively low nuclear Cl concentration, which aided identification. Individual cytoplasmic measurements indicated similarly low Cl values. We were unable to detect any ion shifts in the MRC under the various conditions employed. Thus it does not appear to be involved in the isoproterenol-induced Cl secretion across the gland epithelium. Transport of an undetectable ion (H^+, HCO_3^-) cannot be ruled out.

Mechanism of Ion Secretion

Although detailed studies of the ionic dependency of the secretory response have not been done, the results presented here and previously [22, 23] allow some tentative conclusions about the mechanism of this β-adrenergic stimulated ion transport system. Since removal of Na and/or Cl from the serosal medium blocks the response, it was proposed earlier that a cotransport mechanism for these ions exists on the basolateral membrane of the isoproterenol-sensitive cells of the gland [22] and that net transepithelial ionic movement occurs via a mechanism similar to that proposed earlier by Frizzell et al. [6]. Some of the data presented here provides further support for this concept.

Short Term Alterations

At the peak of SCC response to isoproterenol the major ion shifts are a gain of Na and Ca in the gland cells. The Na response is similar to what is seen in the spinosum cells exposed to isoproterenol in the absence of amiloride and thus could be viewed as the result of stimulation of a Na absorptive mechanism. Dual isotope flux measurements demonstrate, however, that in amiloride inhibited skins exposed to isoproterenol there is actually a small but highly significant increase in net Na flux from serosa to mucosa that is about one-third the net Cl serosa to mucosa flux [23]. Thus the early increase in Na, with no change in Cl, more likely reflects the result of a rapid increase in Na entry across the basolateral membrane, due to the stimulation of the ion secretory mechanism, rather than Na entering across the apical membrane.

In the presence of ouabain and isoproterenol

the Na increase is amplified (Fig. 6). In this case the pump may be unable to keep up with the increased basolateral Na influx. Surprisingly, the gland cells show no increase in Na during application of ouabain alone. This result is consistent with the view that the gland cells are quiescent until exposed to isoproterenol and that in the resting state the Na leak is very small.

If isoproterenol is acting at the apical membrane to increase Cl conductance, a drop in Cl would be expected after stimulation [20]. Likewise, an effect on the entry step could result in an increase. Obviously, a dual effect could result in increased Cl flux with no change in intracellular Cl. The observation that the Cl concentration did not change significantly at a time when the SCC response is at a peak is similar to recent results on canine tracheal epithelium and dogfish rectal gland [25, 26]. In these tissues secretagogues enhanced Cl-dependent secretion, but no change in intracellular Cl activities, as measured with Cl-sensitive microelectrodes, was detected. Thus, it may be that isoproterenol affects both the basolateral entry and apical exit step for Cl.

The significance of the change in nuclear Ca in the gland cells is difficult to assess. The microprobe measures total Ca, and this includes Ca bound in intracellular stores as well as free. So the actual change in freely exchangeable Ca cannot be determined. The results could mean that the elevated Ca, after stimulation by isoproterenol, is due to entry from the external medium. However, since we do not have sufficient data on cytoplasmic Ca we cannot rule out the possibility that the increase in nuclear Ca is actually due to redistribution between the two cellular compartments. The possibility that changes in cytoplasmic Ca levels mediate hormone-stimulated Cl secretion in the tracheal epithelium has been proposed [20].

Long Term Effects

After a 60-min exposure to isoproterenol the Na concentration in the gland cells has returned to control levels, whereas the Cl concentration has decreased by 15 to 20 mmol (Fig. 8). A smaller reduction in cell Cl is also seen in the spinosum and duct cells at this point. The change in the spinosum cells is probably not related to Cl secretion since no change in SCC is seen in amiloride-inhibited split skins [22].

Bumetanide, a more potent inhibitor of Na-Cl coupled transport than furosemide [13], completely blocks the SCC response to isoproterenol. In the experiment shown in Fig. 9 Na is slightly but not significantly altered by bumetanide or bumetanide followed by isoproterenol. However, Cl drops dramatically both in the presence of bumetanide or bumetanide followed by isoproterenol. This indicates a blockage of the Cl entry step at the basolateral membrane. It also indicates that the Cl entry mechanism involved in isoproterenol-stimulated secretion is to some extent active in the quiescent state of the glands (*see also* Fig. 10). The fact that this drop is accentuated in the presence of isoproterenol suggests that the Cl exit step is independently stimulated.

In summary, the results presented here indicate that the primary location of isoproterenol-stimulated ion shifts in the exocrine glands of the frog skin is to a group of cells located at the ductal pole of the gland acinus. The mucous and seromucous cells do not appear to respond to isoproterenol. Duct cells may be involved in ion secretion over a long time course. Thus β-adrenergic-mediated ion secretion appears to be highly cell specific and could provide the basis for production of qualitatively different secretions from the same gland.

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