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Failure of Thapsigargin to Alter Ion Transport in Human Sweat Gland Epithelia while Intracellular Ca^{2+} Concentration Is Raised*

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Ca_i in cultured human sweat gland epithelial monolayers was measured using Fura-2 fluorescence. Thapsigargin (Tg) caused a sustained increase in Ca_i, the rate of rise being slower but the magnitude greater than with the agonists lysylbradykinin and ATP.

Tg caused an irreversible change such that even after it was removed Ca_i was dependent on the ambient calcium concentration, consistent with the hypothesis that Ca^{2+} entry is controlled by the state of the intracellular stores. Calcium entry after Tg was not modified by nimodipine, ω-conotoxin, or BAY K8644 but could be blocked by low concentrations (0.5 mM) of La^{3+}. High concentrations of La^{3+} (2 mM) caused an increase in the response to Tg, suggesting that membrane ATPase exerts a major Ca_i lowering effect. Intracellular Ca^{2+} ion chelation with 1,2-bis(2-aminophenoxy) ethane-*N,N,N′,N′*-tetraacetic acid significantly blunted the response to Tg. Finally, Mn^{2+} entry rate into epithelial cells was doubled by Tg.

In spite of the evidence that Tg raises Ca_i to values greater and for longer than calcium requiring agonists only the latter affected transepithelial transport processes. It is shown that Tg neither affects transepithelial sodium transport nor chloride conductance, both of which increase in response to lysylbradykinin or ATP. It is concluded that spatio-temporal patterns of Ca_i increase after Tg and other agonists are different.

Many calcium-dependent agonists, following interaction with their receptors, activate phosphatidylinositol hydrolysis liberating inositol triphosphate which in turn liberates calcium ions from an intracellular store (1, 2). In some unknown way depleted intracellular stores signal a further increase in intracellular calcium (Ca_i), derived from the extracellular fluid. Calcium influx does not appear to pass directly into the store but along a pathway in which the external source, the cytosol, and the stores are in series (3). Thapsigargin, a sesquiterpene from *Thapsia garganica* L (4) is thought to be a useful tool in Ca_i signaling mechanisms since it appears to bypass membrane receptors. Thapsigargin (Tg)[1] is considered to increase Ca_i by disturbing the pump-leak system of the intracellular stores (5). The Ca-ATPase responsible for calcium sequestration is inhibited, while the leak continues, a process apparently not involving inositol phosphates (6).

This report is concerned with the role of Ca_i in ion-transporting epithelia. In a human colonic epithelium, Tg causes electrogenic chloride secretion accompanied by an increase in Ca_i (7). As with other chloride secretory responses to other agonists on this epithelium, secretion is accompanied by an increase in Ca_i, the extent of which is dependent upon the external calcium concentration (8). Presumably, this indicates that emptying of the stores by Tg triggers calcium influx.

Here we report on the effects of Tg on calcium handling by cultured monolayers of human sweat gland epithelia (SGE). These epithelia are sodium absorbing and the stimulation of this activity by agonists such as lysylbradykinin, histamine, carbachol, and ATP is dependent upon an increase in Ca_i (9–11). While Tg causes an increase in Ca_i in suspensions of human sweat gland epithelial cells, equivalent or greater than those in response to agonists, no effect on sodium absorption has been detected (10). For example in SGE monolayers lysylbradykinin increased sodium transport by 15.7 ± 1.7 μA cm^{-2} (n = 15) and Ca_i by 184 ± 109 nM (n = 6) in cell suspensions. In contrast Tg (0.17 μM) altered sodium transport by −0.3 ± 0.4 μA cm^{-2} (n = 5) but increased Ca_i by 531 ± 110 nM (n = 4). Here we report detailed studies on the effects of Tg on human SGE monolayers. Although the paradox remains unsolved, we conclude that either (i) Ca_i elevation alone is not sufficient to stimulate transport, (ii) the rate at which Ca_i is increased is insufficient to trigger crucial events, or (iii) that Ca_i is released in a topologically unfavorable way.

EXPERIMENTAL PROCEDURES

All experiments were performed using epithelial monolayers grown from human sweat glands. Non-cauterized skin samples were obtained at surgery from normal and cystic fibrosis patients. Permission for the procedure was obtained from the Huntingdon Health Authority Ethics Committee. Sweat glands were isolated by a previously described procedure (12) and primary cultures grown as given before (9). After 3 weeks the cells were harvested from the primary cultures and used as described below. In other situations whole glands were explanted directly in a way which did not require harvesting before use.

Three types of procedure with epithelial monolayers have been employed in this study. They are measurements of transepithelial sodium transport (measured electrically as short circuit current (SCC)), of intracellular Ca^{2+} (Ca_i) using Fura-2 fluorescence and apical membrane anion conductance, using efflux of $^{125}I^-$ from preloaded cells to monitor this.

Short Circuit Current Recording

Short circuit current recording was made using a WP dual voltage clamp by methods described in detail elsewhere (7–10). With human sweat gland epithelia grown on Millipore filters as described above,

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[1] The abbreviations used are: Tg, thapsigargin; BAPTA, 1,2-bis (2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; Fura-2, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid; SCC, short circuit current; SGE, sweat gland epithelia; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis (oxyethylenenitrilo)]tetraacetic acid; CF, cystic fibrosis.

the SCC is a measure of the transepithelial sodium transport in the apical to basolateral direction. To do this harvested cells were used to seed small wells (0.2 cm^2), using 0.2–0.5 × 10^6 cells, the base of which consisted of a Millipore filter coated in Matrigel. Full details of the procedures have been given elsewhere (9, 10). On some occasions whole sweat glands were cultured directly upon the Millipore-well assemblies as given in Ref. 13.

Measurements of Ca_i

Whole sweat glands were seeded onto the plastic slips in Leighton tubes (Costar), one gland in the center of each half-slip. The medium (2 ml) consisted of Williams E containing L-glutamine (1 mM), penicillin-streptomycin (100 units ml^{-1}-100 $\mu g\ ml^{-1}$), bovine insulin (10 $\mu g\ ml^{-1}$), hydrocortisone (10 ng ml^{-1}), transferrin (10 $\mu g\ ml^{-1}$), epidermal growth factor (20 ng ml^{-1}), tracer element mix (0.5%), and fetal calf serum (1%). After 4–5 weeks each gland had grown into a circular monolayer approaching 1 cm in diameter. During this time the tubes were incubated at 37 °C in an atmosphere of air containing 5% CO_2 and the medium was changed every 3–4 days. To measure Ca_i in the cultured monolayers medium was removed and the plastic slips exposed to Fura-2-AM, 1 μM, in a medium containing (mM): NaCl, 135; KCl, 5.4; $CaCl_2$, 1.0; KH_2PO_4, 0.4; $MgSO_4$ 0.3; Hepes buffer (pH 7.4), 10.0; glucose, 1.2; and bovine serum albumin (0.1%). This solution was previously bubbled with O_2. Exposure to Fura-2-AM was for 60 min at 37 °C with gentle agitation. After removal of the plastic slips the monolayers were washed gently in the above buffer without Fura-2-AM and cut into two to give two virtually identical preparations. Each in turn was placed in a cuvette and held in a vertical position as described previously (8). The cuvette could be perfused with drug solutions made up in the same buffer as above at 37 °C at 2 ml min^{-1}. A Perkin-Elmer LS5B luminescence spectrometer, in conjunction with a 640 kByte PC and Perkin Elmer software package was used to capture fluorescence emission at 510 nm. The monolayer was irradiated alternately at 340 and 380 nm every 2 s so that 15 340/380 ratios were obtained every minute. To calculate Ca_i the following equation (14)

$$Ca_i = K_d \frac{R - R_{min}}{R_{max} - R} \cdot \frac{Sf_2}{Sb_2}$$

was used, where K_d is the dissociation constant of the Fura-2-Ca^{2+} complex (224 nM at 37 °C), R is the 340:380 ratio, R_{max} is the same ratio in the presence of excess Ca^{2+}, whereas R_{min} is the same ratio in the absence of Ca^{2+}. Sf_2/Sb_2 is the ratio of the 380-nm signals measured in the absence and presence of excess Ca^{2+}. Details of the measurement of R_{max} and R_{min} for epithelial monolayers is given elsewhere (8).

Measurement of Apical Chloride Conductances

To measure apical chloride conductance of human sweat gland epithelial cells, a procedure measuring the rate constant for $^{125}I^-$ efflux was used. Cells harvested from primary cultures were used to seed 3.5-cm diameter well plates. Cells were grown to 70% confluence in the supplemented Williams E medium (given above). When ready the monolayers were washed three times in buffer solution and the cells loaded with $^{125}I^-$ (2.5 $\mu Ci\ ml^{-1}$) for 90 min at 37 °C in the same buffer solution. Afterward the monolayers were washed three times with buffer to remove excess label and efflux of $^{125}I^-$ measured by adding and removing 3 ml of buffer to each well every 30 s for 10 min. Finally, the tracer remaining in each well was eluted with 3 ml of 0.1 N HNO_3 for 30 min. Effects of agents on $^{125}I^-$ efflux were measured by including these in the buffer from 3.5 min onward. Instantaneous rate constants for efflux were calculated after counting the samples. The method described is essentially similar to that given elsewhere (16, 17). The buffer used throughout had the following composition (mM): NaCl, 135; $CaCl_2$, 1.2; $MgCl_2$, 1.2; K_2HPO_4, 2.4; KH_2PO_4, 0.6; glucose, 10' and Hepes 10 (pH 7.2).

Solutions and Chemicals

Solutions—The solution used for the measurement of Ca_i in monolayers was the same as that used for loading Fura-2-AM except that bovine serum albumin was omitted. This same solution was modified in various ways for different procedures. Nominally calcium-free solution was obtained simply by omitting $CaCl_2$. The Ca^{2+} concentration of this solution was around 1 μM. Calcium-free solution was obtained by adding EGTA, 1 mM, (neutralized to pH 7.4) to nominally calcium-free solution. The basic solution needed to be modified when La^{3+} was used to prevent precipitation. This now had the following composition (mM): NaCl, 116; KCl, 5.4; $MgCl_2$, 0.8; $CaCl_2$, 1.0; Hepes, 20; glucose, 1.2. Again the solution was adjusted to pH 7.4 and gassed with O_2. This solution was used to mount tissues for short circuit current recording. As before this solution could be made nominally calcium free or calcium free by omitting $CaCl_2$ with or without EGTA.

Chemicals—BAPTA-AM (2 mM) dissolved in dimethyl sulfoxide was mixed with an equal volume of Pluronic F127 (20%) in dimethyl sulfoxide. This was added to the solution bathing the cells to give a final BAPTA-AM concentration of 5 μM. Fura-2-AM was similarly dissolved in dimethyl sulfoxide and used to give a final concentration of 1 μM in the loading solution. Sources of the drugs and chemicals used in this study were as follows: thapsigargin and Fura-2-AM from Calbiochem, La Jolla, CA; BAPTA-AM from Molecular Probes, Eugene, OR; sodium nitroprusside from Sigma, ω-conotoxin from Peninsula Laboratories, Belmont, CA; nimodipine and BAY-K-8644 were from Bayer, Germany.

RESULTS

In all experiments Tg was applied only once to epithelial monolayers, at different concentrations and for different times (Table I), and was then removed at the first solution change. This single exposure produced an irreversible change in the way the intracellular calcium concentration (Ca_i) responded to changes in extracellular calcium, monitored from Fura-2 fluorescence. Addition and removal of external calcium was achieved by changing from a calcium-containing solution to one without calcium and containing EGTA. This maneuver we call the calcium switch response and have used it extensively to characterize the mechanisms by which Tg elevates Ca_i in SGE.

An example of the calcium switch response following a single exposure to Tg (170 nM) is illustrated in Fig. 1. In this example the terpene caused the value of Ca_i to rise from around 100 nM by 375 nM during 10 min, following which Tg was washed away by changing alternately between a calcium-containing and calcium-free solution, the latter containing EGTA. The calcium switch caused a Ca_i increase of between 450–500 nM with five consecutive exposures to Ca^{2+}, 1 mM. To show that Tg was responsible for this effect and that similar data could not be achieved after repeated exposure to calcium-containing and calcium-free solutions alone, five experiments were carried out without Tg (Table I). One of these is illustrated in Fig. 1 and shows that the calcium switch causes very little change in Ca_i without prior exposure to Tg. It also indicates that Ca_i is resistant to external EGTA and in this and other figures (*e.g.* Figs. 2 and 4), even after Tg, that exposure to calcium-free solution containing EGTA did little to lower Ca_i below approximately 100 nM.

TABLE I

Effect of Tg on Ca_i on epithelial monolayers

The effect of thapsigargin (Tg) on the response to the calcium switch. The table shows both the concentration and time for which Tg was applied. The increase in Ca_i at the moment Tg was removed is also given. Preparations were then cycled between conditions where they were exposed to calcium (1 mM) or the same solution without added calcium plus EGTA. Consecutive responses to the switch are given in most instances.

[Tg]	Time	Tg response	Ca^{2+} switch response					n
			1	2	3	4	5	
nM	*min*	*nM*	*nM*					
0			31 ± 16					5
170	10	187 ± 21	291 ± 40					13
10	12	25	25	45				1
10	25	90	103	140				1
85	18	240	345	370	355			1
170	10	180	231	266	257	249	231	1
170	10	375	430	445	505	445		1
850	10	170	240	295	320			1

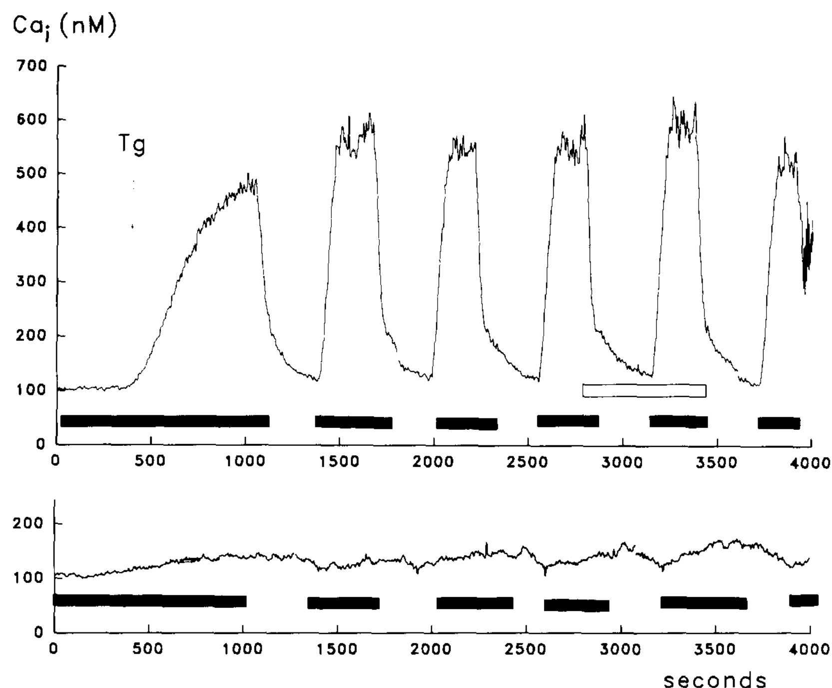

FIG. 1. **Effect of Tg (170 nM) on the response to calcium switching in a SGE monolayer.** The *closed horizontal bars* indicate when Ca^{2+}, 1 mM, was present in the bathing solution; at other times Ca^{2+} was absent and EGTA (1 mM) was present. For the duration shown by the *open bar*, nimodipine (10 μM) was present in the solutions. Below is shown an identical experiment with another SGE monolayer, except on this occasion pre-exposure to Tg was not used before calcium switching. Tg was present only from when added (*arrow*) to the first solution change.

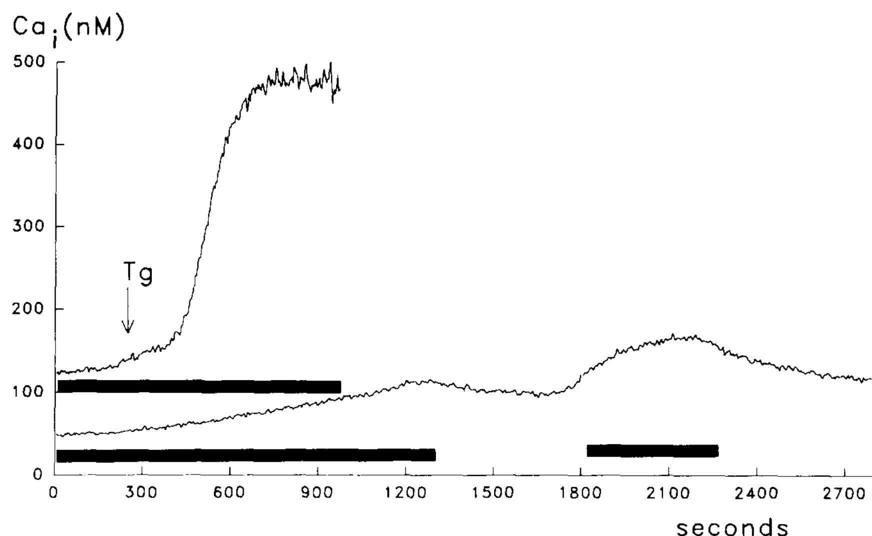

FIG. 2. **Effect of BAPTA on the response to Tg and to calcium switching.** An SGE monolayer was preincubated with BAPTA-AM (5 μM) for 60 min after which it was exposed to Tg (170 nM) followed by calcium switching (*lower trace*). Ca^{2+} ions (1 mM) were present during the time shown by the *horizontal closed bars*. For comparison a monolayer from the same batch was exposed to Tg (170 nM) without preincubation with BAPTA-AM (*upper trace*).

The calcium switch was repeated in 13 experiments using Tg at different concentrations or for different exposure times (see Table I). Several important features can be discerned from the data. First the Tg effect is seen with a concentration as low as 10 nM, if exposure was prolonged to 25 min. Second, the Ca_i responses to Tg were variable even when the same concentration was used, yet the calcium switch responses bore a close relation to the preceding Tg responses, the average first calcium switch response being 1.15 ± 0.1 (n = 13) times the Tg response, when allowance was made for the size of the switch response in the absence of Tg.

The easy repeatability of the calcium switch response made it possible to test a variety of substances for their ability to modify Ca^{2+} entry after Tg exposure. For example the calcium channel antagonist, nimodipine, was included in the solutions for part of the experiment illustrated in Fig. 1. Clearly, this agent was without any significant effect on the calcium switch response. Similarly negative results were obtained with ω-conotoxin (1 μM) and with sodium nitroprusside (5 μM), the latter to activate guanylate cyclase. The calcium channel agonist BAY K 8644 (5 μM) was investigated in three experiments, with the expectation that the size of the response to the calcium switch might increase. However, the responses were marginally inhibited by this agent, although not significantly so. The change in Ca_i following the calcium switch in three experiments was 140, 515, and 287 nM. Then in the presence of BAY K 8644 the responses were 121, 83, and 76% of their respective controls. After removal of the drug, the mean value of the responses was 90% of the original control values. There was no change in the form of the responses in the presence of BAY K 8644, and this result should be compared to the effects of La^{3+} given later.

It was crucial to find other evidence that the calcium switch response reflected changes in Ca_i. To do this a monolayer was preincubated with BAPTA-AM, endogenous esterase activity releasing BAPTA trapped in the cells. If Tg increases Ca_i and if the calcium switch is reflecting changes in Ca_i then both these parameters should be attenuated by chelation of Ca^{2+} by BAPTA. Inclusion of BAPTA in the cells reduced the basal value of Ca_i to approximately 40 nM compared to 120 in the

paired control (Fig. 2). Tg (170 nM) caused an increase of Ca_i of only 59 nM over a period of 15 min, while the paired control showed an increase of 350 nM in only 5 min. In the presence of BAPTA, calcium switch responses produced Ca_i swings of less than 100 nM, compared to an expected value of 300 nM (Table I).

The size of the first calcium switch response was 1.15 times (*i.e.* roughly equal) the increase in Ca_i caused by Tg. If Tg causes an increase in Ca_i both by releasing from intracellular stores and by influx from outside them this ratio should be altered if influx from the exterior is prevented during the action of Tg. This was achieved by using nominally calcium-free (≈1 μM) solution but not containing EGTA. Two examples are shown in Fig. 3 where the responses were 110 and 25 nM, yet when Ca^{2+} was increased to 1 mM there was a substantial increase in Ca_i and the size of the calcium switch was 2.8 and 6.2 times the Tg response.

To further characterize the effects of Tg on SGE monolayers, interaction with agonists and effects of calcium entry and exit from the cell were investigated. The failure of Tg to elicit a sodium transporting effect in SGEs (10) was the stimulus for asking whether Tg and other agonists affect the same intracellular calcium stores. If Tg liberates Ca^{2+} from a different intracellular store than agonists then the Ca_i response in SGE monolayers should not be antagonized by Tg. ATP has proved to be a reliable Ca_i elevating agonist in SGEs and was chosen for this test. The three experiments shown in Fig. 4 were all carried with a single batch of SGE monolayers. ATP was added before, during, or after the response to Tg. In the latter condition ATP was added either in the absence or presence of external calcium. The figure shows clearly that Tg produces a very slow increase in Ca_i compared to the agonist ATP. The latter is unexceptional in causing a fast response as this property is shared by all other effective agonists (10). After Tg was removed and the calcium switch response established, ATP caused no effect in the absence of external calcium (or in its presence, although this is not shown in Fig. 4). In other experiments (not shown) ATP was able to cause a transient response in the absence of external Ca^{2+} and in the presence of EGTA, but without Tg pretreatment. This together with the data of Fig. 6 strongly suggests that Tg empties the intracellular stores available to ATP. One interpretation of this result is that the store(s) which is usually released by ATP is empty after Tg, even when the presence of external calcium allows the possibility of repletion. Finally, when ATP was added toward the peak of the Tg response small transient responses were seen, possibly indicating that, at this stage, there are subcompartments which still have a ATP releasable store.

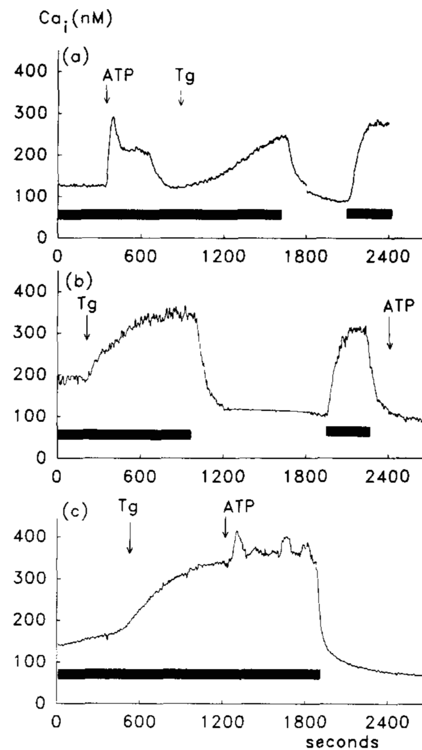

FIG. 4. **Interaction ATP (100 μM) and Tg (170 nM) on Ca_i in SGE monolayers.** ATP was added before (*a*), after (*b*), or during (*c*) the response to Tg. After Tg had been applied, the calcium switch response was used to examine the response to ATP in the presence and absence of Ca^{2+} (data for the latter not shown). The *horizontal closed bars* indicate when Ca^{2+}, 1 mM, was present. Otherwise there was no Ca^{2+} present and EGTA was added.

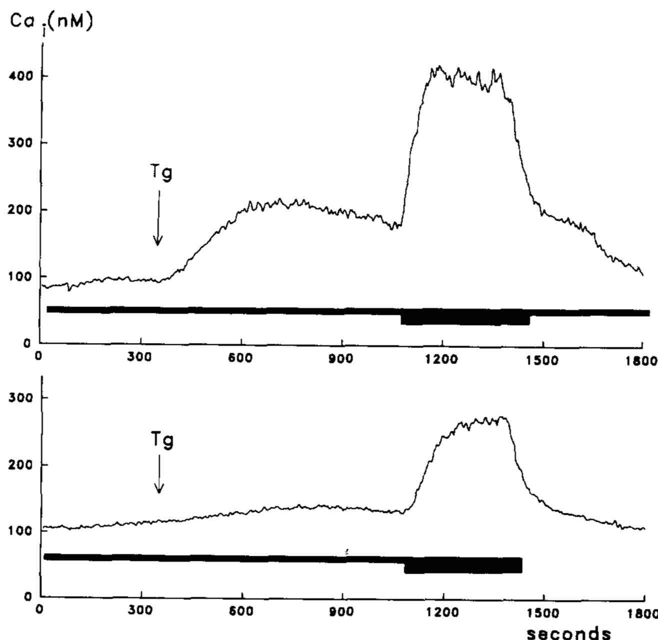

FIG. 3. **Response to Tg (170 nM) in low external Ca^{2+} (≈1 μM) (*narrow horizontal bar*).** After the response to Tg had developed, the external Ca^{2+} concentration was raised to 1 mM (*wide bar*), after which the preparation was returned to low Ca^{2+} (*upper trace*) or low Ca^{2+} with EGTA (*lower trace*) (no bar).

To explore the effects of Tg on calcium entry into SGE cells, while a major effect of nimodipine, nitroprusside, or BAY K 8644 was lacking, attention was turned to lanthanum ions, La^{3+}. Under appropriate conditions La^{3+} can block both the response to Tg and the calcium switch response, while under other conditions the response to Tg is potentiated.

If La^{3+} ions are added to an SGE monolayer during the plateau of the Ca_i response, then graded inhibition of Ca_i occurs with increasing La^{3+} concentrations, inhibition being apparent on concentrations as low as 5 μM but not complete with concentrations of 500 μM (data not shown). Presumably La^{3+} by blocking Ca^{2+} entry alters the balance between entry and exit from the cell, resulting in a lower value in the presence of Tg. More informative findings can be obtained by using the calcium switch response following Tg pretreatment as in Fig. 5. Inhibition of the calcium switch response depended crucially on the time at which La^{3+} and Ca^{2+} were readmitted. For example, as shown in Fig. 5*a*, when La^{3+} was added ahead of Ca^{2+} but in the presence of EGTA, no La^{3+} ions were available until the moment Ca^{2+} was added. Thus,

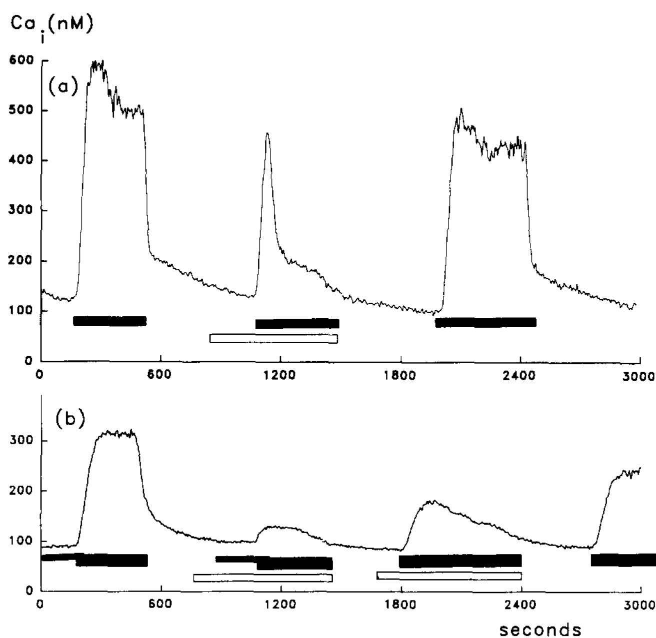

FIG. 5. **The effect of La^{3+} ions (0.5 mM) on the calcium switch response following Tg (170 nM).** SGE monolayers were exposed to Tg and then subjected to the normal calcium switching protocol. (This part of the experiment is not shown.) The presence of Ca^{2+} (1 mM) is shown by the *horizontal closed bars* and Ca^{2+} (≈1 μM) by the *narrow closed bars*. La^{3+} (0.5 mM) was present as indicated by the *open bars*. In the intervals between the *closed bars*, tissues were exposed to low calcium solution containing EGTA, 1 mM. In *a*, La^{3+} and Ca^{2+} are admitted simultaneously since La^{3+} was unavailable in the presence of the chelator. In *b*,) La^{3+} was admitted before the Ca^{2+} was added.

when La^{3+} and Ca^{2+} were added effectively simultaneously substantial responses to the switch were obtained, but unlike the normal responses only a transient with a low plateau value was seen. To get round the problem of La^{3+} chelation in the presence of EGTA, the switch was made first to low Ca^{2+} (μM range) and then from low Ca^{2+} to high Ca^{2+}. This allowed La^{3+} ions to be present before the switch from low to high Ca^{2+} was made. In this situation (Fig. 5*b*), the response to the calcium switch was reduced to a modest increase without the rapid transient.

The concentration of La^{3+} ions used in the blocking experiments was 0.5 mM, but with higher concentrations of 2 mM an unusual potentiating response to Tg was recorded, provided Tg was added after the lanthanide. It was noted earlier that Tg responses were rather variable, but in younger epithelial monolayers the response was much smaller. We used these monolayers to show the potentiating effect. The six separate responses shown in Fig. 6 were derived from six monolayers all in the same batch and the experiments completed within 1 day. Responses to Tg were relatively small unless La^{3+}, 2 mM, was added first (Fig. 6, *a*, *c*, and *e*). Potentiation did not occur when La^{3+} was added afterward at either 2 or 0.5 mM (Fig. 6, *b* and *d*). With La^{3+}, 0.5 mM added before Tg the response was either unaffected or marginally increased (Fig. 6*f*). At the higher concentration of La^{3+}, the ion sometimes caused a minor effect on Ca_i even before Tg was added (Fig. 6, *a* and *c*).

In many systems Mn^{2+} can enter cells apparently by the same route as Ca^{2+} (18), but since Mn^{2+} quenches Fura-2 fluorescence the rate of quenching can be used as a measure of Mn^{2+} influx, in turn providing an estimate of the effects of Tg on Ca^{2+} entry. To do these experiments fluorescence is measured at the isobestic point (360 nm), since at the usual wavelengths the resulting signal would represent a combination of quenching by Mn^{2+} and the response of unquenched Fura-2 to changes in the ambient Ca_i. Therefore, in all the Mn^{2+} experiments fluorescence was measured at 510 nm following alternate irradiation at 340 and 360 nm. The 340-nm signal therefore gives an indication of Ca_i changes provided the 360-nm signal is constant. Fig. 7*a* shows results of this type for Tg application followed by the calcium switch, while the early part of Fig. 7*c* shows the effect of removing calcium with EGTA. In both instances the 360-nm signal remains satisfactorily steady, the slow decline presumably representing the loss of Fura-2 from the cells. By contrast the 340-nm signal responds predictably to changes in Ca_i.

Mn^{2+} (0.1 mM) was applied to monolayers in the absence of Ca^{2+} (low Ca^{2+} solution plus 1 mM EGTA), in the presence of low Ca^{2+} (μM range) or high Ca^{2+} (1 mM). Monolayers were previously treated with Tg or were untreated. Fig. 7*b* shows the result from a Tg-treated monolayer where Mn^{2+} was added in the absence of Ca^{2+}, whereas Fig. 7*c* shows a similar result for a monolayer untreated by Tg.

The composite data for this series of experiments is given in Table II. While the table represents few experiments there is a persuasive internal consistency about the results. The reduced apparent rate constants for entry which occur with increasing external Ca^{2+} indicate a Mn^{2+}-Ca^{2+} competition. In the presence of Ca^{2+} (1 mM), and allowing for the rate of Fura-2 loss from the cells, Tg increases Mn^{2+} entry by 2.5 times. A similar pattern was seen using Mn^{2+} (10 μM) to those in Table II with the higher Mn^{2+} concentration. However, in this situation the rate constants were only marginally greater than the value which could be ascribed to loss of Fura-2.

Although, as given in the Introduction, Tg failed to stimulate SCC in SGE monolayers (10), the data accumulated in this study suggested a way in which Tg may be made effective. This is to use La^{3+}, 2 mM, ahead of Tg, a device already shown to increase the Ca_i response to Tg (Fig. 6). In six experiments the effects of Tg (170 nM) on SCC in human SGE monolayers were investigated. On three occasions La^{3+} (2 mM) was added to the fluid bathing both sides of the epithelium 5–6 min before Tg was added to the apical side. At least 30 min was allowed for the effects of Tg to develop. Finally, amiloride (10 μM) was added to the apical side to assess the extent of sodium transport in the epithelium. This concentration of amiloride is sufficient to abolish sodium transport in SGE monolayers (10). The mean ± standard error ($n = 3$) for the increase in SCC following Tg (170 nM) in the absence of La^{3+} was 2.7 ± 2.1 μA cm^{-2}, while after La^{3+} (1 mM) the current decreased by −2.4 ± 2.7 μAcm^{-2}. The two groups of epithelia were entirely comparable in transporting capability since amiloride, 10 μM, reduced SCC by −16.3 ± 4.7 μM cm^{-2} in the absence of La^{3+}, while in those preparations to which La^{3+} had been added amiloride reduced SCC by −14.0 ± 7.4 μA cm^{-2}. While these two latter values are not significantly different, La^{3+} itself caused a fall in SCC of 2.4 μA cm^{-2}, indicating the initial currents in both sets of epithelia were identical at 16.3 μA cm^{-2}. Thus, although the maneuver used increases the Ca_i response to Tg there is still no stimulation of sodium current, as reported earlier (10).

All the SGE monolayers used herein were grown from whole human sweat glands and are derived, therefore, from cells of the secretory coil and reabsorptive duct. Under the conditions used for culture, monolayers take on ductal characteristics, that is they exhibit transepithelial sodium absorption (9, 10). No differences in the sodium transporting responses in normal and cystic fibrosis (CF) epithelia were found when exposed to calcium requiring secretagogues (10). Apical anion

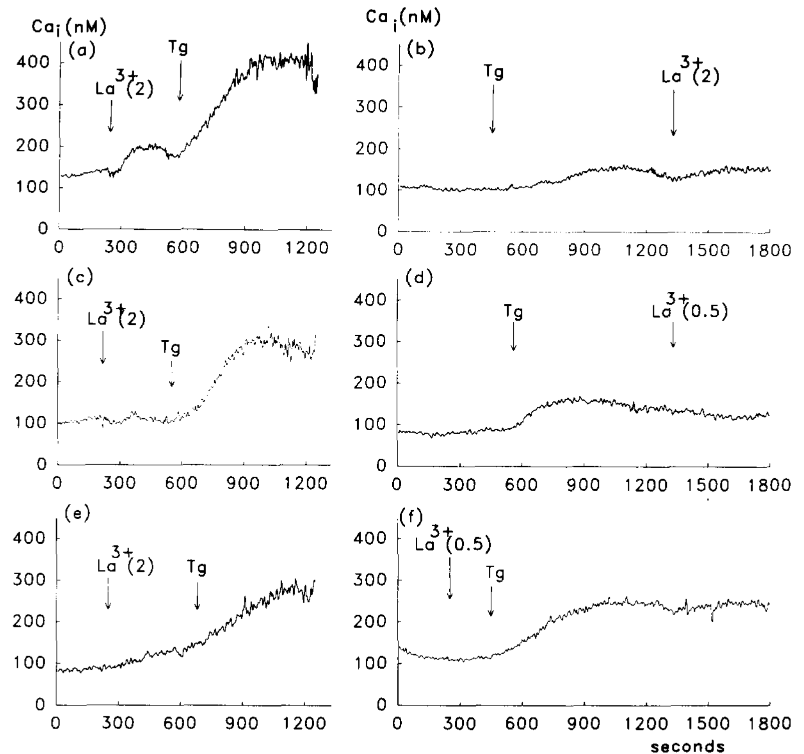

FIG. 6. **La^{3+}-Tg interactions in SGE monolayers.** The figure illustrates data from six SGE monolayers from same batch. Tg (170 nM) was added once to all monolayers. In *a*, *c*, and *e*, Tg was added after La^{3+} (2 mM), while in *b* La^{3+} (2 mM) was added afterward. The effects of La^{3+} (0.5 mM) added after and before Tg is shown in *d* and *f*, respectively. All experiments were made in the presence of 1 mM Ca^{2+}.

conductance in SGE monolayers was measured as the rate constant for $^{125}I^-$ efflux, using monolayers derived from CF sweat glands. Four agents which increase sodium transport, namely ATP, lysylbradykin, carbachol, and A23187 (10) also increased the rate constant for $^{125}I^-$ efflux. Data for lysylbradykinin is given in Fig. 8. By contrast, Tg produced no definitive change in anion efflux, indicating that neither of the transporting effects characteristic of sweat glands can be evinced by Tg.

DISCUSSION

The low rate of Fura-2 loss from SGE monolayers (Table II) together with the repeatability of the calcium switch response has allowed the mechanism of Tg action to be explored in intact SGE epithelium, whereas most other studies have used cell suspensions. It is worth considering at the outset if the effects of Tg we have found are consonant with the general mode of action proposed for this terpene, *i.e.* inhibition of a Ca-ATPase associated with the intracellular Ca^{2+} store. For example, could the calcium switch response be due to ionophoric properties of Tg partitioned into the plasma membrane? This seems unlikely since La^{3+} applied after Tg inhibits its effect (Fig. 5), whereas when applied before Tg the effect of the terpene is enhanced (Fig. 6). La^{3+} effects are rapidly reversible (Fig. 5) and the ion unexpected to enter the cell. Taken together this suggests Tg acts at a site other than the cell membrane, although a minor effect at this site is not precluded. Alternatively, the effects of Tg might result from inhibition of plasma membrane Ca-ATPase. In a recent study Tg was shown to inhibit all the sarcoplasmic or endoplasmic reticulum family of Ca-ATPases, but had no effect on plasma membrane Ca-ATPase or Na,K-ATPase (19). However, high concentrations of La^{3+} which inhibit membrane Ca-ATPase (see later) does not inhibit the effect of Tg, again suggesting a predominant intracellular mode of action.

Here we show that Tg allows cells to respond reversibly to the ambient Ca^{2+} concentration, even though the terpene is removed. This is consistent with one proposed mode of action of Tg, that is it acts as an irreversible (20) Ca-ATPase inhibitor of membranes bounding intracellular calcium stores (21) some of which, at least, are agonist-sensitive, shown here for ATP (20–22). Furthermore, since re-exposure to Ca^{2+} following EGTA alone does not promote a calcium switch response it seems that calcium entry following Tg is dependent on the emptiness of the store, consistent with capacitative models for calcium entry (23–25). Here ATP was unable to promote Ca^{2+} entry after Tg, either in the presence or absence of Ca^{2+} which argues that the inositol triphosphate-sensitive store is not *en route* to the cytosol, favoring the modified version of the capacitance model for Ca^{2+} entry (22). In low external Ca^{2+} ($\approx 1\ \mu M$), Tg produced only a modest elevation of Ca_i, presumably since the extrusion mechanisms in the plasma membrane are intact, yet subsequently a full size calcium switch response was obtained, indicating that Tg had emptied the stores (Fig. 3). Tg was potent, producing effects at concentrations as low as 10 nM, but both the rise in Ca_i and the associated calcium switch responses were smaller than with high concentrations (Table I). Whether with low Tg concentrations all the compartments of the stores are partially emptied or, alternatively, a fraction of the stores are completely emptied the results imply a close connection between entry and the state of the store. In a recent study using a non-hydrolyzable inositol triphosphate analogue, it is argued the emptiness of the store is the only condition needed to promote Ca^{2+} entry (26). We may conclude from experiments with BAPTA that Ca^{2+} enters the cytosolic space during the calcium switch, assuming as is reasonable that BAPTA and Fura-2 report on the same compartment.

These data with nimodipine, ω-conotoxin, and Bay K 8644 effectively eliminate L-type or N-type calcium channels as a route of Ca^{2+} entry following Tg. Cyclic GMP has been implicated in the activation of plasma membrane Ca-ATPase serving as a feedback control of Ca_i in pancreatic acinar cells

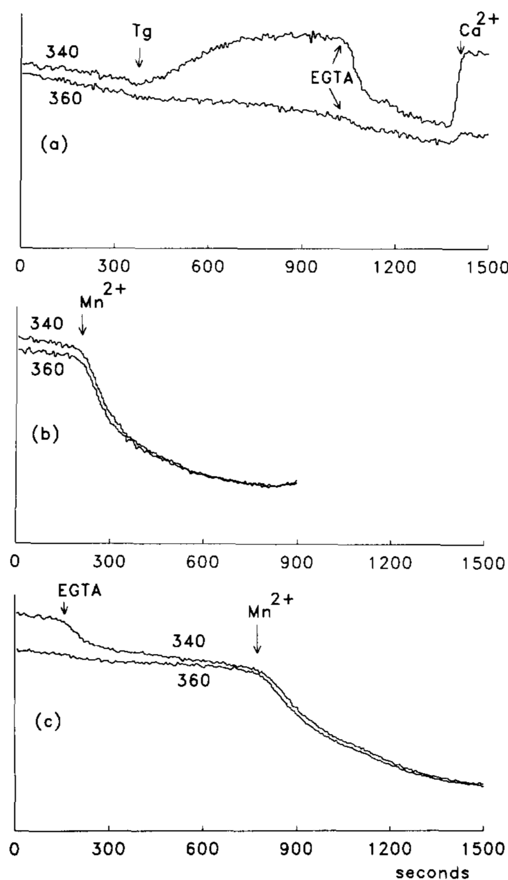

FIG. 7. **Fluorescence intensity (*ordinate*) of Fura-2 irradiated at 340 and 360 nm in SGE monolayers.** In *a* is shown the data following treatment with Tg (170 nM) and application of the calcium switch. In *b*, the addition of Mn^{2+} (100 μM) in the absence of Ca^{2+} is shown following Tg and EGTA treatment. In *c*, the result of adding Mn^{2+} (100 μM) in the absence of Ca^{2+} but without Tg pretreatment is illustrated.

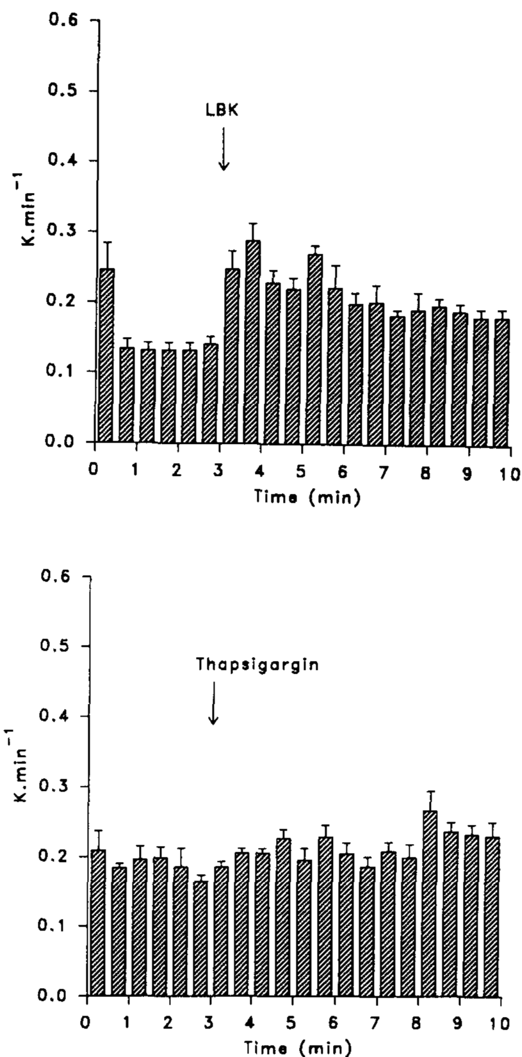

FIG. 8. **Efflux rate constants for loss of $^{125}I^-$ from SGE monolayers grown from CF skin.** Lysylbradykinin (*LBK*) (0.1 μM) caused a sustained increase in efflux while Tg (0.17 μM) caused no definitive effect although occasional values were significantly greater than those in the control period. Each column gives the mean value ± standard error for six measurements.

TABLE II
Mn^{2+} entry into SGE monolayers

Mn^{2+} (100 μM) was added to preparations in the presence of zero Ca^{2+} (EGTA), 1 μM Ca^{2+}, or 1 mM Ca^{2+}, with or without pretreatment with Tg (170 nM). The control value (no Mn^{2+} present) represents Fura-2 loss from the monolayers. Rate constants were estimated by fitting an exponential to the decay curves at 360 nm.

	Rate constants (s^{-1}) × 10^{-3}	
	Tg	No Tg
Control (no Mn)		0.25 ± 0.03 (6)
EGTA	2.56	2.01
Low Ca^{2+} (1 μM)	1.56	1.21
Ca^{2+} (1 mM)	1.01	0.54

(27), smooth muscle (28), and macrophages (29). However, using nitroprusside to activate guanylate cyclase no evidence for a similar effect was found for SGE monolayers.

In common with other cells Ca^{2+} entry into SGE monolayers was blocked by La^{3+}, 50% inhibition being achieved with less than 0.5 mM La^{3+}. Although La^{3+} can interact with Fura-2 (30), it is unlikely it enters the cells since its effects are readily reversible yet there is no mechanism to pump it from the cells. It is clearly shown that the block of the calcium switch response by La^{3+} was dependent upon the sequence in which Ca^{2+} and La^{3+} were added. When La^{3+} was added before Ca^{2+} inhibition was much greater than when both ions were added simultaneously, suggesting competition or interaction between these ions at the entry site. In isolated lacrimal cells (3), the response to methacholine was enhanced by a high concentration of La^{3+} (2 mM) as were smooth muscle contractions to agonists (31), both actions being attributed to blockade of the plasma membrane Ca-ATPase in a situation in which Ca^{2+} entry was also blocked. La^{3+} (2 mM) in SGE monolayers enhanced the responses to Tg, indicating that the Ca-ATPase in this tissue must be very active since a higher value of Ca_i is achieved with entry and exit blocked than is achieved with both processes intact and the stores emptied. As given earlier no evidence that Tg affects plasma membrane Ca-ATPase has been found (19, 32).

Mn^{2+}, unlike La^{3+}, can enter cells like Ca^{2+} and the entry can be monitored by quenching Fura-2 fluorescence. Agonists are shown to increase Mn^{2+} influx in some (18, 33) but not all cells (33). In endothelial cells Mn^{2+} appears to enter by the Ca^{2+} pathway in a rate determined inversely as the fullness of the store (18). The greatest rate of fluorescence quench measured in this study was when Ca^{2+} was absent. This is not surprising since when both ions entered together Mn^{2+} and Ca^{2+} compete for interaction with Fura-2 but the 360 nm fluorescence only will report interaction with Mn^{2+}. Never-

theless, pseudo-exponential quenching of Fura-2 by Mn^{2+} occurred at zero, low, and high Ca^{2+} concentrations. The important feature of these results is that under all three circumstances Tg increased the rate of Mn^{2+} (and presumably Ca^{2+}) influx, a feature reported before for this agent (22, 25) and consistent with the view that Mn^{2+} entry is controlled by the state of the store. For example, in the presence of a normal calcium concentration the rate of Mn^{2+} quench was doubled by Tg.

In summary, in SGE monolayers it appears that Tg empties the intracellular store, sensitive in part at least to the agonist ATP, and promotes Ca^{2+} entry across the plasma membrane through a La^{3+}-sensitive entry mechanism. Additionally, SGE monolayers are able to extrude Ca^{2+} so efficiently that Ca_i increases to higher values when Ca^{2+} extrusion is blocked, even though the procedure also blocks Ca^{2+} entry. Recently (15), it was shown that cardiac sarcoplasmic reticulum Ca-ATPase is relatively insensitive to Tg. The position of human SGE endoplasmic reticulum Ca-ATPase in the scale of sensitivity to Tg is unknown. However, considerations of this kind cannot explain the lack of effect of Tg on ion transport as Ca_i is elevated more than with other agonists and for longer. The primary stimulus for this study was that Tg failed to increase SCC in SGE monolayers, even though all other agonists which increased Ca_i also increased SCC (10). That study showed Tg increased Ca_i in sweat gland cells suspensions to values equivalent to those caused by other agonists. Here it is shown that Tg is equally effective in increasing Ca_i in SGE monolayers.

Even when the rate and extent of the Ca_i increase following Tg is increased by blocking plasma membrane Ca-ATPase with La^{3+} there was still no increase in SCC in SGE epithelia. Although it has been shown that other agonists which increase Ca_i also increase SCC (10), the stimulation of phosphatidylinositol hydrolysis sets in train a more complex array of second messenger events than does Tg. It can be argued that elevation of Ca_i alone is insufficient stimulus to affect transporting functions, yet this is refuted by the stimulation of SCC by the calcium ionophore, A23187 (10).

The increase in sodium transport and anion conductance in SGE monolayers caused by Ca^{2+} requiring agonists is considered to be due, at least in part, to the presence of Ca^{2+}-sensitive maxi K^+ channels. These have been shown to be present in the membranes of sweat glands of both normal and cystic fibrosis subjects (34). Elevation of Ca_i thus hyperpolarizes the cell by activation of these channels increasing the electrical gradient for either Na^+ influx or Cl^- efflux across the apical surface (10, 11). Additionally, there may be effects of Ca_i directly on chloride channels in the apical surface. The chloride secretory response in CF sweat gland is retained for Ca^{2+}-requiring agonists, while the cAMP-requiring agonists are ineffective (35). SGE monolayers from CF tissues were chosen deliberately to compare the effects of Tg with lysylbradykinin on anion efflux. The reason is that the kinin also stimulates prostanoid formation in addition to raising Ca_i. In turn, prostanoids may lead to cAMP formation giving increased anion efflux by opening cAMP-dependent chloride channels, a process which is lost in CF. It is shown that kinin was able to stimulate anion efflux in SGE monolayers grown from CF glands but that this property is not shared by Tg.

A distinct feature of the Tg effects on Ca_i in SGE monolayers is the extremely slow rise time compared to Ca^{2+}-requiring agonists. It may be that the rate of rise is insufficient to trigger crucial events or so slow that compensatory mechanisms serve to prevent the transporting events. This is not true for other epithelia as in cultured human colonic epithelia Tg is an effective chloride secretagogue (7). Finally, it is becoming clearer that both temporal and locational changes in Ca_i within cells are important features in determining the response of cells (23). In this respect data obtained from imaging single bovine adrenal chromaffin cells show that more than one agonist can increase Ca_i but not all cause catecholamine secretion (36, 37). Our data show that similar features are exhibited by ion secreting and absorbing epithelial cells. Recording from intact epithelial monolayers gives only an average value for the intracellular calcium concentration. It will be important to determine, with single cell imaging, the changing patterns of Ca_i following agonists such as ATP and lysylbradykinin and to compare these with those generated by thapsigargin.

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