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Mechanisms for the Effects of Acetylcholine on Sodium Transport in Frog Skin

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Summary. In frog skin (*Rana temporaria*) acetylcholine applied to the serosal surface produces either a sustained inhibiton or sustained stimulation of short-circuit current (SCC). The former effect is accompanied by a reduction and the latter by an increase in total tissue conductance. Both effects of acetylcholine can be accounted for, within experimental error, by changes in net sodium flux across the tissue. By use of selective agonists and antagonists it is concluded that acetylcholine interacts with muscarinic receptors in the serosal membrane. The effects of cholinoceptor agents are also seen with isolated epithelium.

The stimulatory effect of acetylcholine is potentiated by theophylline and blocked by inhibitors of prostaglandin synthetase and by mepacrine. It is suggested that acetylcholine stimulates transport by liberating prostaglandins which may then activate adenylcyclase. The inhibitory effect of acetylcholine is correlated with a reduction in cyclic AMP content of the epithelium. Calcium appears to be an important determinant of the type of response seen with acetylcholine, but the mechanism is not known.

Brief reports showing that acetylcholine can affect sodium transport in frog skin have been made (Barnes, 1940; McAfee, 1964, 1968; McAfee & Locke, 1967), but no detailed study of mechanism has been undertaken until now. The effects of acetylcholine in nonepithelial tissues are particularly well documented, yet the mechanisms involved, such as increased calcium permeability (Bolton, 1979), guanyl cyclase activation (Goldberg et al., 1975) and increased phosphatidylinositol turnover (Michell, 1975), are not those usually associated with modifications of sodium transport in frog skin. Possibly novel mechanisms triggered by cholinoceptor activation might provide unique insights into epithelial processes.

While these studies were in progress the inhibitory effect of acetylcholine on sodium transport in toad urinary bladder was reported (Sahib, Schwartz & Handler, 1978; Wiesmann, Sinha, Yates & Klahr, 1978). In skin, or the isolated epithelium prepared from whole skin, acetylcholine can cause either stimulation or inhibition of sodium transport. We show that the stimulatory response to acetylcholine is related to endogenous prostaglandin synthesis and, incidentally, that angiotensin shares a similar mechanism. Circumstantial evidence implicates cyclic adenosine 3′-5′ monophosphate in both the stimulatory and inhibitory responses to acetylcholine, while calcium ions may have a modifying influence by an unknown mechanism.

Materials and Methods

All experiments were performed on the abdominal skin, or epithelium derived therefrom, of frogs, *Rana temporaria*. The animals were kept in running water at 20 °C. Epithelia were prepared from skins by methods similar to those of Aceves and Erlij (1971). Sodium transport was measured as a short-circuit current (SCC) by conventional methods as first described by Ussing and Zerahn (1951).

Sodium fluxes were measured in double chambers using a single piece of tissue. Both halves were short circuited and trace amounts of ^{22}Na were added to the mucosal solution of one half and the serosal solution bathing the other half. Net flux was taken as the difference between forward (mucosal-to-serosal) and backward fluxes.

Tissue content of cyclic adenosine 3′-5′ monophosphate (cAMP) was measured with a protein binding assay similar to that described by Brown et al. (1971). At the end of the incubation period tissues were transferred to boiling Brown's buffer containing 8 mM theophylline, 6 mM 2-mercaptoethanol, and 50 mM Tris HCl, pH 7.4, to stop all enzymic processes and to preserve the cyclic nucleotide. Subsequently the tissue was homogenized in the buffer and the whole centrifuged. The cAMP content of aliquots of supernatant were, in all instances, compared to a standard curve constructed using known amounts of cAMP processed in an identical

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way. The tissue content of cyclic guanosine 3′-5′ monophosphate was also measured by a similar protein binding assay using assay kits supplied by the Radiochemical Centre, Amersham.

Muscarinic receptors in epithelia and intestinal muscle were measured using the irreversible ligand propylbenzilylcholine mustard with methods similar to those used by Cuthbert and Young (1973) for amniotic membranes. The label was cyclized in 10 mM phosphate buffer, at pH 7.6 for 1 hr.

Ringer solution used in these experiments contained (mM): NaCl, 111; KCl, 2; $CaCl_2$, 2; glucose, 11, and either Tris HCl, 5, pH 7.6, or sodium bicarbonate, 2.4, pH 8.0. Unless otherwise stated, the Tris-containing Ringer was used. The chloride-free Ringer contained (mM) sodium isethionate, 111; potassium sulphate, 2; calcium nitrate, 2; glucose, 11, and Tris HCl, 5, pH 7.6. All sulutions were gassed by bubbling with air.

Results

Effects on Short-Circuit Current in Isolated Skin

Acetylcholine and its analogue, carbachol, caused SCC changes at low concentration (0.05–10 μM) when applied to the serosal surface. Five types of response were seen as follows. A sustained stimulation (SS) or a sustained inhibition (SI) of SCC were the major types of response. Minor responses consisted of transitory (lasting a minute or two) increases or decreases in SCC and a mixed response, for example, stimulation followed by inhibition. The bulk of the experiments described here were made with the major types of response, although transitory responses recorded incidentally are shown in some of the records.

Sustained stimulation responses were obtained in 67% of skins taken from 237 animals during one year. In general the responses reached a peak within 5 min and then declined during 30 min or so. Repeated responses could be obtained at about 45-min intervals. Maximal SS responses were obtained with acetylcholine concentrations between 1–10 μM. Sustained inhibition responses were observed in 63% of tissues from the same population, the overlap indicating that

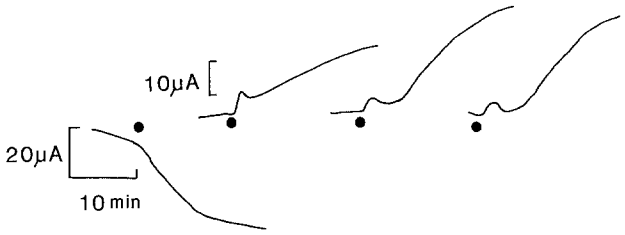

Fig. 1. Consecutive SCC responses to acetylcholine (2 μM) applied to the serosal side (at filled circles). Skin area was 3.2 cm^2; initial SCC's were 105, 71, 71 and 69 μA

some skins showed both types of response at different times. A transition from SI to SS type responses with repeated applications of acetylcholine is shown in Fig. 1. Note too the appearance of a transient stimulatory response in this experiment. Maximal sustained inhibitory responses were obtained over the same concentration range as for SS responses (1–10 μM) but, in general, the upper end of the concentration range was required.

Changes in transepithelial potential followed, but not in proportion, those in SCC. In 10 preparations showing SS responses to acetylcholine (1–20 μM) the mean PD increased by 27% from 48 ± 7 to 61 ± 7 mV ($P < 0.02$, paired t-test) while SCC increased by 63% from 20.3 ± 2.8 to 33.1 ± 3.4 μA/cm^2 ($P < 0.001$). In 15 preparations showing SI responses to acetylcholine (2–20 μM) the PD fell by 27% from 52 ± 6 to 38 ± 6 mV ($P < 0.01$, paired t-test) while the SCC fell from 44.7 ± 6.3 to 25.9 ± 3.8 μA/cm^2 ($P < 0.01$), i.e. by 42%. These results suggest the SS responses are associated with increases, and the SI responses are associated with decreases, in tissue conductance. Both these implications were confirmed by direct measurements of tissue conductance as shown in Fig. 2, where it can be seen that the conductance effects develop alongside the changes in SCC. The conductance increases during SS responses to acetylcholine (or carbachol) were

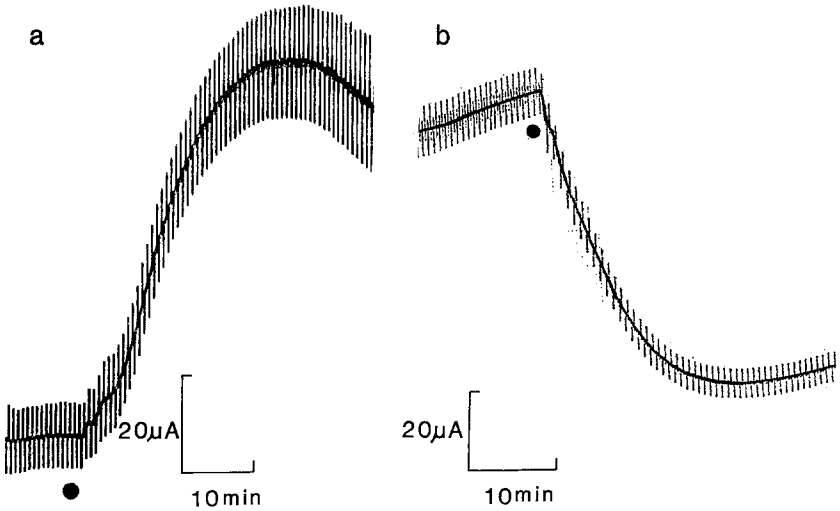

Fig. 2. Conductance changes caused by acetylcholine (10 μM, added to the serosal side at the closed circles) during SS and SI responses. The transepithelial voltage was clamped periodically and alternately at ± 5 mV. The currents required to clamp at these potentials are shown. The skin areas were 8 cm^2, and the initial currents were (*a*) 51 μA and (*b*) 187 μA

from 0.78 ± 0.09 to 0.94 ± 0.09 mmho/cm^2 ($P < 0.001$, paired t-test, 26 preparations), while the SCC increased from 19.4 ± 1.9 to 30.0 ± 2.2 μA/cm^2 ($P < 0.001$). Similarly during SI responses the mean conductance decreased from 1.10 ± 0.16 to 0.93 ± 0.13 mmho/cm^2 ($P < 0.001$, paired t-test, 27 preparations), while the current decreased from 40 ± 3.8 to 25.3 ± 2.5 μA/cm^2 ($P < 0.001$).

Effects on SCC in Isolated Epithelium

It seemed important to show that the effects of acetylcholine-like drugs were on the epithelium itself and not the underlying tela subcutanea. In addition, the connective tissue contains cholinesterase (Koblick, Goldman & Page, 1962), which might limit the action of acetylcholine but not, of course, of carbachol. With the exception of transient stimulation, all the types of response reported above were obtained in the isolated epithelium. However, the frequency with which each effect occurred was changed in the epithelium. In only one preparation of ten bathed in Tris Ringer was a SS response obtained, the others showing SI responses. In 21 further preparations bathed, this time, in bicarbonate-Ringer only three showed SS responses while the rest were inhibited by acetylcholine (or carbachol).

We consider that the SS response is sensitive to collagenase, for in skins exposed to collagenase but without removal of the connective tissue layer the SS response to carbachol is impaired (Fig. 3.). It seems unlikely therefore that the effects of acetylcholine-like drugs are due to indirect actions by release of substances from the connective tissue layer.

Ionic Nature of the SCC Responses to Acetylcholine

Sustained stimulation responses to acetylcholine were abolished by amiloride in a concentration (10^{-4} M) sufficient to inhibit sodium transport, as illustrated in the crossover type experiment shown in Fig. 4. Similarly, if amiloride (10^{-4} M) is added to the mucosal bathing solution at the time of the maximal response to acetylcholine (or carbachol), whether it be an SS or SI response, the SCC immediately falls to around zero. These results suggest that both the SS and SI responses are modifications of sodium transport or, alternatively, depend on the presence of normal sodium transport.

Unidirectional ^{22}Na fluxes were made in paired pieces of skin from the same animal under short-circuit conditions. The net sodium flux, obtained by subtraction of the backflux from the forward flux, was compared to the SCC measured in the skin used to measure forward flux. Six experiments in which either acetylcholine or carbachol caused a SS response

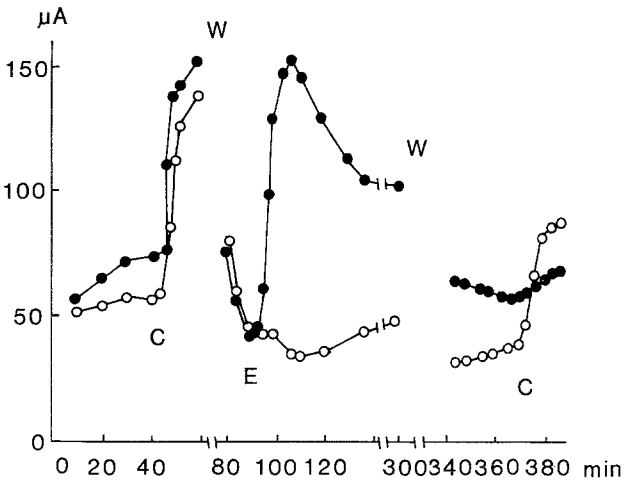

Fig. 3. Effect of collagenase on SCC in whole skin. Paired skins (each 3.2 cm^2) from a single animal were used. Responses to carbachol, 5 μM (at *C*) were obtained both before and after one skin (filled circles) was exposed to collagenase (at *E*) (28 U · ml^{-1} applied to serosal side) for 3½ hr. At *W* the solution on both sides of the skins was changed. Note that collagenase alone produces a marked stimulation of SCC

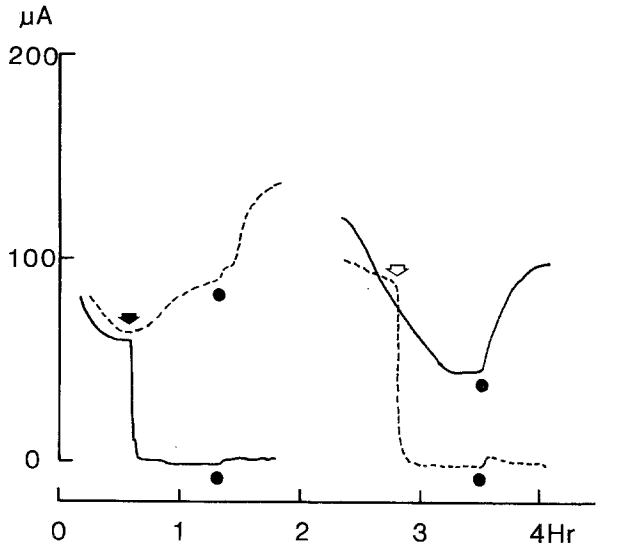

Fig. 4. Effect of amiloride on SCC responses to acetylcholine in paired skins (each 3.2 cm^2). Amiloride (0.1 mM) was added (broad arrow) to the mucosal solution of one preparation. Afterwards acetylcholine, 1 μM was added to the serosal solution bathing both preparations (filled circles). The crossover experiment was carried out after washing away the drugs from both sides of the skins

were carried out and the results are presented in Table 1. There was a significant increase in the forward flux, net flux, and SCC following drug addition, while the backflux and the ratio of SCC to net flux was unaltered. In this series the net flux increased from 0.77 ± 0.07 to 1.16 ± 0.10 μEq cm^{-2} hr^{-1}, values which correspond well with those for SCC of 0.79 ± 0.09 and 1.10 ± 0.09 μEq cm^{-2} hr^{-1}. The slight, non-significant disparity between SCC and flux values dur-

Table 1. Sodium fluxes in skins stimulated with ACh-like drugs

Expt.	Drug (μM)	SCC (μA 3.2 cm^{-2})		^{22}Na fluxes (μEq 3.2 cm^{-2} hr^{-1})							
		Control	Test	Control				Test			
				Forward	Back	Net	SCC/Flux	Forward	Back	Net	SCC/Flux
1	ACh, 1.0	36.0	75.0	1.77	0.21	1.56	0.86	3.48	0.21	3.27	0.86
2	ACh, 1.0	75.0	103.0	2.61	0.15	2.46	1.14	4.77	0.21	4.56	0.84
3	ACh, 1.0	65.0	73.0	2.79	0.24	2.55	0.95	3.36	0.24	3.12	0.87
4	ACh, 1.0	76.0	111.0	2.94	0.09	2.85	0.99	4.02	0.09	3.93	1.05
5	ACh, 1.0	87.0	115.0	3.03	0.21	2.82	1.15	4.83	0.30	4.53	0.95
6	CCh, 10.0	67.0	85.0	2.70	0.15	2.55	0.98	3.12	0.24	2.88	1.10
		68.0	94.0	2.64	0.18	2.47	1.01	3.93	0.22	3.72	0.95
		±8.0	±8.0	±0.20	±0.02	±0.21	±0.05	±0.33	±0.03	±0.33	±0.05

ACH indicates acetylcholine, CCh indicates carbamylcholine. SCC values were mean values obtained by integrating the area under the curve relating SCC to time. Flux values were the mean values of the fluxes determined consecutively throughout the period under study.

Table 2. Sodium fluxes in skins inhibited by ACh-like drugs

Expt.	Drug (μM)	SCC (μA 3.2 cm^{-2})		^{22}Na fluxes (μEq 3.2 cm^{-2} hr^{-1})							
		Control	Test	Control				Test			
				Forward	Back	Net	SCC/Flux	Forward	Back	Net	SCC/Flux
1	ACh, 10.0	108.0	68.0	4.02	0.20	3.82	1.05	2.88	0.41	2.47	1.03
2	CCh, 10.0	122.0	107.0	5.16	0.29	4.87	0.93	3.96	0.41	3.55	1.12
3	CCh, 10.0	121.0	88.0	4.56	0.31	4.25	1.06	3.60	0.41	3.19	1.03
4	CCh, 10.0	153.0	123.0	6.96	0.20	6.76	0.84	4.92	0.22	4.70	0.98
5	CCh, 10.0	101.0	75.0	4.32	–	–	0.87	3.54	–	–	0.79
6	CCh, 10.0	80.0	72.0	3.30	0.23	3.07	0.97	2.76	0.31	2.45	0.97
		114.0	89.0	4.72	0.25	4.55	0.95	3.61	0.35	3.27	0.99
		±11.0	±10.0	±0.56	±0.03	±0.70	±0.04	±0.35	±0.04	±0.46	±0.05

ACh indicates acetylcholine, CCh indicates carbamylcholine. SCC and flux values were measured as described in Table 1. In the fifth experiment backflux was not measured and the SCC/flux ratio is given with respect to the forward flux.

ing cholinoceptor stimulation might be due to the minor participation of ions other than sodium.

In six other similar experiments in which acetylcholine or carbachol caused a sustained inhibitory response, the analysis suggested again that only the transport of sodium was modified. Details of these experiments are presented in Table 2. There was a significant decrease in SCC, forward flux, and net flux in these experiments. There was also a small but significant increase in the backflux. Careful examination of the time course of this effect showed there was a transient increase in backflux following addition of acetylcholine which then declined to control values. The mean control and inhibited net fluxes, 1.42 ± 0.22 and 1.02 ± 0.14 μEq $cm^{-2}hr^{-1}$, respectively, were in reasonable agreement with the values for SCC, 1.33 ± 0.13 and 1.04 ± 0.12 μEq $cm^{-2}hr^{-1}$. Again it is not possible to eliminate minor participation of other ions in the inhibitory response to cholinoceptor agents.

It was possible to demonstrate SS and SI responses to acetylcholine in skins bathed in Ringer solution in which isethionate was substituted for chloride, implying that chloride ions are not importantly involved in either type of response.

Receptor Types Involved in SS and SI Responses

Acetylcholine and carbachol can activate both muscarinic and nicotinic receptors (Barlow, 1964) and it was necessary to turn to more selective agonists and antagonists to discover the receptor types involved. (±) Methacholine (acetyl-β-methylcholine) and methylfurmethide also caused SS and SI responses and were approximately equipotent to acetylcholine when judged on paired preparations. Oxotremorine and pilocarpine were considerably less effective than acetylcholine and often failed to cause any response at all. In this latter situation these compounds were able to antagonize the effects of acetyl-

choline. Thus oxotremorine and pilocarpine behave as partial agonists. Specifically acting nicotinic agents, succinylcholine, decamethonium, and tetraethylammonium had no effect in concentrations up to 200 μM.

When the two optical isomers of methacholine were applied separately to tissues it was found that the (+) isomer was more effective than the (−) isomer, with a potency ratio of between 100 and 1,000 (Fig. 5). All these data suggest that both SS and SI responses are mediated by muscarinic receptors.

When using antagonists, it proved impossible to obtain precise quantitative data with respect to affinity. To do this, repeated responses to a standard concentration of agonist must remain constant. However, the transition of SI and SS responses (*see* Fig. 1) together with tachyphyllaxis confound this requirement. Consequently, we have had to be content with semi-quantitative data such as shown in Fig. 6. It can be seen that atropine blocks both SS and SI responses and also the transitory stimulatory and inhibitory responses. Further, it is irrelevant whether acetylcholine or carbachol is used or whether these experiments are made with whole skin or isolated epithelium. The antagonism caused by atropine can be overcome by increasing the agonist concentration. The data again is consistent with the receptors being classified as muscarinic. It was shown in other experiments that the effects of acetylcholine were not modified by the β-receptor antagonist propranalol, whereas the stimulant effect of isoprenaline was completely inhibited. This suggests that the cholinoceptor agents do not act by releasing catecholamines from sites in the skin or the underlying corium.

Propylbenzylylcholine mustard (PrBCM) is an irreversible antagonist at muscarinic receptors (Cuthbert & Young, 1973). Using discs of isolated epithelium and ^{3}H-PrBCM, we measured the binding of the ligand in the presence and absence of a competitive inhibitor (atropine, 1 μM). With 0.25 nM ^{3}H-PrBCM applied for periods of between 15 and 60 min, we were not able to show any significant difference between the amount of irreversibly bound tritium in the presence or absence of atropine. With guinea-pig longitudinal muscle strips peeled from the ileum and exposed to 0.25 nM ^{3}H-PrBCM for 30 min in bicarbonate-Ringer solution 17.80 ± 2.83 pmol ^{3}H-PrBCM · mg wet wt^{-1} was bound irreversibly while only 1.64 ± 0.19 pmol ^{3}H-PrBCM · mg wet wt^{-1} was bound irreversibly in the presence of atropine, 1 μM, but with otherwise identical conditions. Thus under conditions in which significant ($P < 0.05$) amounts of binding were detected in ileum no significant binding was detected in epithelium. This suggests the epithelial receptors are very sparse.

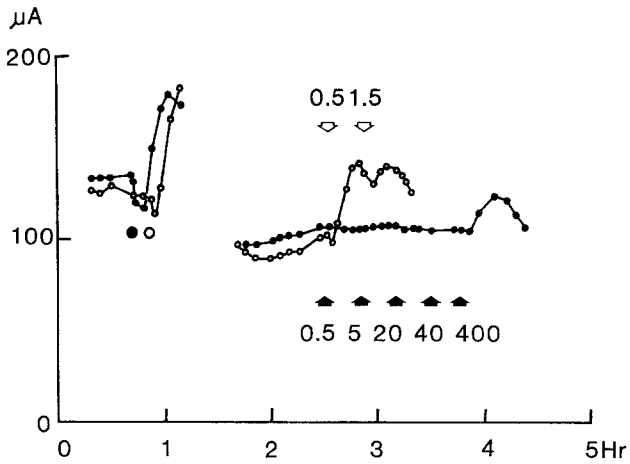

Fig. 5. Responses to the stereoisomers of methacholine in paired skins (each 3.2 cm^2). Initially comparable responses in both preparations were obtained to acetylcholine, 1 μM (circles). (+) methacholine (open arrows) and (−) methacholine (filled arrows) were added to serosal bathing solutions as indicated. Figures indicate the concentrations in μM

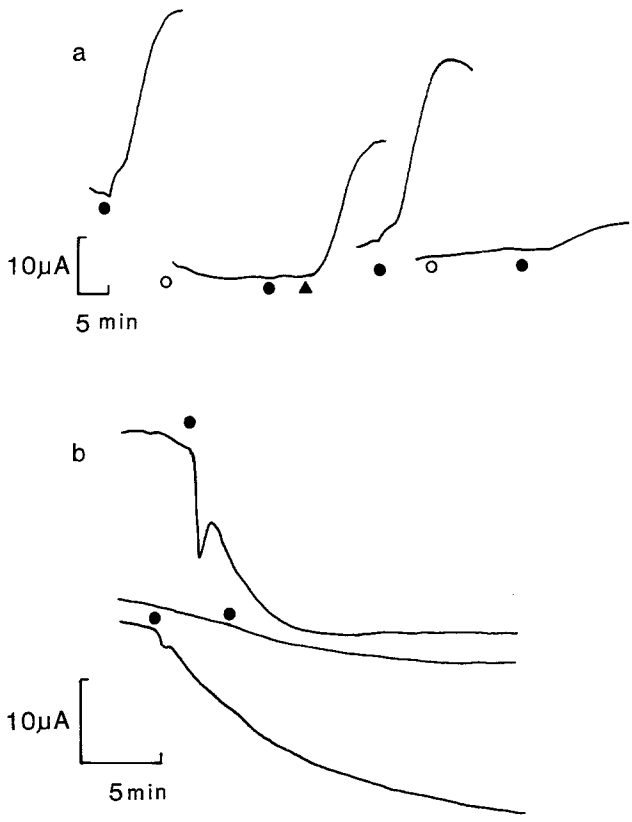

Fig. 6. Effects of atropine on responses to cholinoceptor agonists. (*a*): Responses to acetylcholine, 10 μM (filled circles) or 200 μM (filled triangle) were obtained before or after addition of atropine, 0.1 μM (open circles). (*b*): Responses of isolated epithelium to carbachol, 10 μM (filled circles), are shown. Atropine, 20 nM, was added 15 min before carbachol in the response illustrated by the middle trace. Tissue areas were 3.2 cm^2 in *a* and *b*. All drugs were added to the serosal side and initial SCCs were 40 μA in *a* and 48 μA in *b*

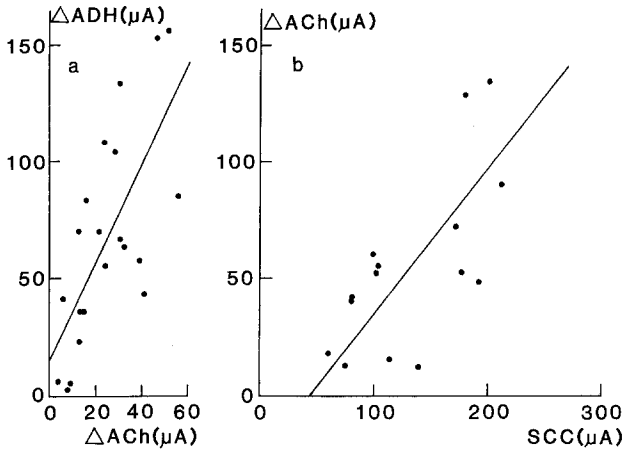

Fig. 7. (*a*): Relation between the response to ADH (20 mU ml^{-1}) and ACh (1 μM) applied to the serosal side of 21 skins. The responses to the two agents were consecutive, but the order was random. The regression line has the characteristics

$\Delta ADH = 2.05 \Delta ACh + 16$

where ΔADH and ΔACh are the increments in SCC (in μA) caused by ADH and ACh, respectively. 95% confidence limits of the intercept were 3.0 and 30.0 μA. Correlation coefficient was 0.7 ($P < 0.001$). Skin area was 3.2 cm^2. (*b*): Relation between SI response to ACh, 10 μM (in μA), *vs.* the basal SCC existing before addition of ACh in 15 skins. The regressive line has the characteristics

$\Delta ACh = 0.63\ SCC - 29$

where ΔACh and SCC are in μA; skin area is 3.2 cm^2. 95% confidence limits of intercept were −11.0 and −47.0 μA. Correlation coefficient was 0.84 ($P < 0.001$)

The Role of Nucleotides in the Responses to Acetylcholine

Sustained stimulation responses to acetylcholine were similar in time course to those produced by ADH, and a final common mechanism seemed possible. By comparing the peak responses to acetylcholine and ADH, given consecutively, using respective concentrations of 1 μM and 20 mU ml^{-1}, a significant correlation was found between the responses of the two agents ($r = 0.71$ $P < 0.001$ (Fig. 7)). There was no correlation between the size of the SI responses and the response to ADH; however, the size of the SI responses was dependent on the basal SCC. The peak SI response to carbachol (10 μM) in relation to SCC is also shown in Fig. 7. Regression analysis gave a value of $r = 0.84$ ($P < 0.001$).

In the presence of a maximal SCC increase in response to ADH, acetylcholine caused no further additional increase, neither did it cause an SI response. Thus ADH appears to suppress both SS and SI responses to cholinoceptor agents.

Theophylline is known to potentiate the responses to ADH and is also able to potentiate the responses to acetylcholine. As theophylline alone increased SCC at concentrations below 1 mM and as the responses to acetylcholine were small in relation to those with ADH (Fig. 7), the choice of the theophylline concentration was a delicate matter. By keeping the theophylline concentration low (100 μM) it was possible to show potentiation of the response to acetylcholine or carbachol in about 50% of experiments. An example is shown in Fig. 8. No consistent effect of theophylline on SI responses was found.

Relation of Response to Tissue Cyclic Nucleotide Concentration

Tissue contents of cAMP and cGMP were measured as described under methods. Discs of skin or epithelium were prepared from single animals, and at least one disc was used to ascertain the SCC response to carbachol (10 μM). The area used for SCC recording was only 1.3 cm^2 and two or three responses to carbachol were obtained to gauge the type of response predominating. The responses were classified into three groups, SS, SI, or indefinite, the latter group including mixed responses or even failure to respond at all to carbachol. The remaining tissue discs were divided into three groups, either controls, carbachol treated (10 μM for 6 min), or isoprenaline treated (1 μM for 6 min). In designing the experiments in this way we were able to relate changes in cyclic nucleotide content with SCC responses.

In 16 experiments using whole skin, the cAMP content in controls was: 5.08 pmol cm^{-2} while after treatment with carbachol or isoprenaline tissue content increased to 6.28 pmol cm^{-2} and 22.75 pmol cm^{-2}, respectively. The increase above control values

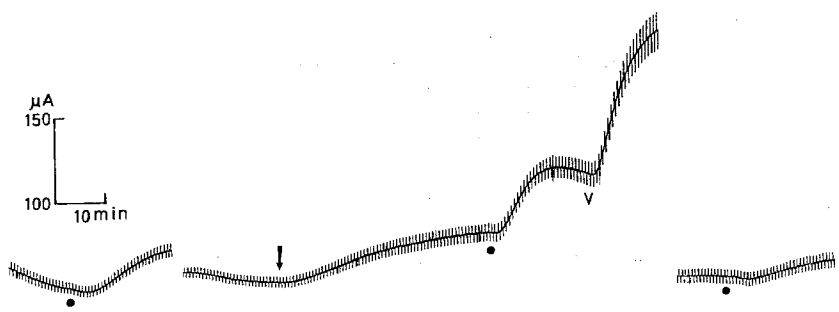

Fig. 8. Effect of theophylline (100 μM, applied both sides at arrow) on the response to carbachol, 2 μM (filled circles). At *V* antidiuretic hormone (100 mU ml^{-1}) was added to the serosal solution. Skin area was 3.2 cm^2; voltage commands were ±1 mV

Table 3. Effects of carbachol on cAMP content in skin and epithelium

Intact skin				Isolated epithelium			
Type	*n*	Control	Carbachol	Type	*n*	Control	Carbachol
SS	5	4.81±0.73	8.49±1.59*	SS	3	1.06±0.36	1.46±0.52
SI	5	5.34±1.59	6.17±0.95	SI	8	1.22±0.34	0.99±0.26*
I	6	5.08±0.59	4.54±0.50	I	2	1.35	1.70

Tissues were exposed to carbachol (10 μM) for 6 min before cAMP content was measured. All results are given as pmol cm^{-2}. Values with asterisks are significantly different from the appropriate control at $P<0.05$ using a paired *t*-test. *n* is the number of experiments.

SS indicates sustained stimulatory responses, SI sustained inhibitory responses, and I indeterminate responses.

reached significance ($P<0.05$) only in the case of isoprenaline. In these experiments the skins weighed 37.7 mg cm^{-2}, giving a cAMP content of 0.14 pmol mg wet wt^{-1}. This value is similar to that given by Hall, O'Donoghue, O'Regan and Penny (1976). In five experiments the skins responded to carbachol with stimulation, in five others there was clear inhibition with carbachol, while in the remaining six experiments the responses to carbachol were indefinite. When the effects of carbachol on cAMP content were grouped according to the responses obtained (Table 3), it was found that carbachol caused a significant increase in tissue cAMP in the group showing SS responses.

A similar set of 13 experiments was carried out using discs of prepared epithelium. The cAMP content was lower than that for whole skin, being 1.24± 0.25 pmol cm^{-2}. We found the epithelium had a wet wt of 4.02 mg cm^{-2} giving a cAMP content of 0.31 pmol mg wet wt^{-1}, a value similar to that of Johnsen and Nielsen (1978) for *R. temporaria* but five- to 10-fold higher than reported by Jard (1974) for *R. esculenta*. Treatment of epithelial discs with carbachol (10 μM for 6 min) or isoprenaline (1 μM for 6 min) gave, on analysis, values for cAMP content of 1.21±0.25 (13) and 4.54±0.55 (11) pmol cm^{-2}. The value for the isoprenaline-treated tissues was significantly greater than the control value ($P<0.05$).

As had been anticipated from the earlier results, most of the prepared epithelia responded to carbachol with an SI response and in these there was a small, but significant ($P<0.05$, paired *t*-test, 8 experiments) fall in cAMP content from 1.22±0.34 to 0.99± 0.26 pmol cm^{-2}. In only three experiments were SS responses obtained, and in each instance the cAMP content increased by between 32 and 42%. However, with so few experiments the increase was not statistically significant. The results for isolated epithelia are also shown in Table 3.

Our failure to detect a reduction in cAMP content in whole skins showing SI responses prompted us to examine the response of the corium with epithelium removed. In three experiments control and carbachol-stimulated values were measured for whole skin and corium alone. The values in whole skin were 5.29±2.6 and 7.88±0.77 pmol cm^{-2} for control and carbachol-treated tissues, these values being not significantly different. In corium alone the corresponding values were 1.90±1.3 and 4.80±1.46 pmol cm^{-2}, which were significantly different at the $P<0.05$ level, a rise which would obscure the reduction seen in isolated epithelium. Isoprenaline also increased the cAMP content of corium.

As it was known that ADH effects on cAMP content in epithelia were rather transient and that the fall-off in content could be prevented by removing sodium (Jard, 1974; Sapirstein & Scott, 1973), it was decided to investigate this possibility with acetylcholine. The choice of a sodium substitute presented some problems. Choline is a usual substitute, but as it also interacts weakly with cholinergic receptors, it could not be used. As an alternative we chose potassium chloride and potassium sulphate. Surprisingly, both of these salts produced large increases in cAMP content in both skin and epithelium so that the idea of sodium substitution was abandoned. However, in view of the interest there is in potassium-depolarized epithelia (*see*, e.g., Fuchs, Hviid Larsen & Lindemann, 1977), our values for a single experiment with whole skin bathed in potassium sulphate Ringer are given here. The cAMP contents were as follows: control with normal Ringer on both sides, 2.81; potassium sulphate Ringer both sides, 8.47; potassium sulphate Ringer on serosal side only, 7.53, and potassium sulphate on mucosal side only, 4.57 – all values given as pmol cm^{-2}.

Cyclic GMP content of skins and epithelia were also measured. The value for whole skin 1.28±0.11 (7) pmol cm^{-2} was four times smaller than for cAMP. Using isolated epithelia, the GMP content was not detectable in four out of five experiments and had a value of 0.061 pmol cm^{-2} in the fifth. No significant

effects of carbachol or theophylline on cGMP content were detected.

Role of Prostaglandins in the Response to Carbachol

The prostaglandin synthetase inhibitors indomethacin (40 nM to 1 μM) and aspirin (0.33 to 1.25 mM) caused a variable reduction in SCC in isolated skins bathed in bicarbonate Ringer. In skins in which SCC had been suppressed by indomethacin, the responses to low concentrations of prostaglandin E_1 (PGE_1) were enhanced compared to paired untreated tissues. Both of these findings are in accord with those of Hall et al. (1976).

Indomethacin (0.2–50 μM) reduced SS responses to carbachol by approximately 75%. With the lower concentrations of indomethacin the effect was reversible with prolonged washing, as shown in Fig. 9*a*. In some experiments an SS response to carbachol was converted to an SI response in the presence of indomethacin. This effect of indomethacin was mimicked by aspirin, but at higher concentrations (Fig. 9*b*). The phospholipase A_2 inhibitor mepacrine inhibited the responses to carbachol by 75% when used at 100 μM (2 experiments), 56% when used at 50 μM (1 experiment), and 34% when used at 25 μM (1 experiment) (Fig. 9*c*).

The dramatic effect of indomethacin on carbachol responses prompted us to test other stimulants of SCC in the presence of indomethacin. Low concentrations of isoprenaline (5–50 nM) and ADH (2–24 mU ml^{-1}) were each used on eight occasions in the presence of indomethacin. There was no consistent effect of indomethacin on the responses to either agent. Quite different results were obtained with angiotensin II. Responses to angiotensin (0.125 μM) were completely blocked by indomethacin (0.25 μM) (Fig. 9*d*). In other experiments responses to cAMP (1.25 mM) were unaffected by indomethacin (0.5 μM) while responses to carbachol (5 μM) were abolished in the same experiments.

The Effects of Calcium

Removal of calcium from the serosal bathing solution, with or without the presence of EGTA, had no consistent effect on SS or SI responses to carbachol either in whole skin or in isolated epithelia. Increasing the serosal calcium concentration to 10 or 20 mM from 2 mM had a variable effect on SCC. Interestingly, when the effect of increased calcium was to increase SCC, carbachol showed an SS response. The reverse was true for skins in which calcium inhibited SCC. There was a significant correlation between the responses to calcium and carbachol ($r=0.71$, $P<0.01$).

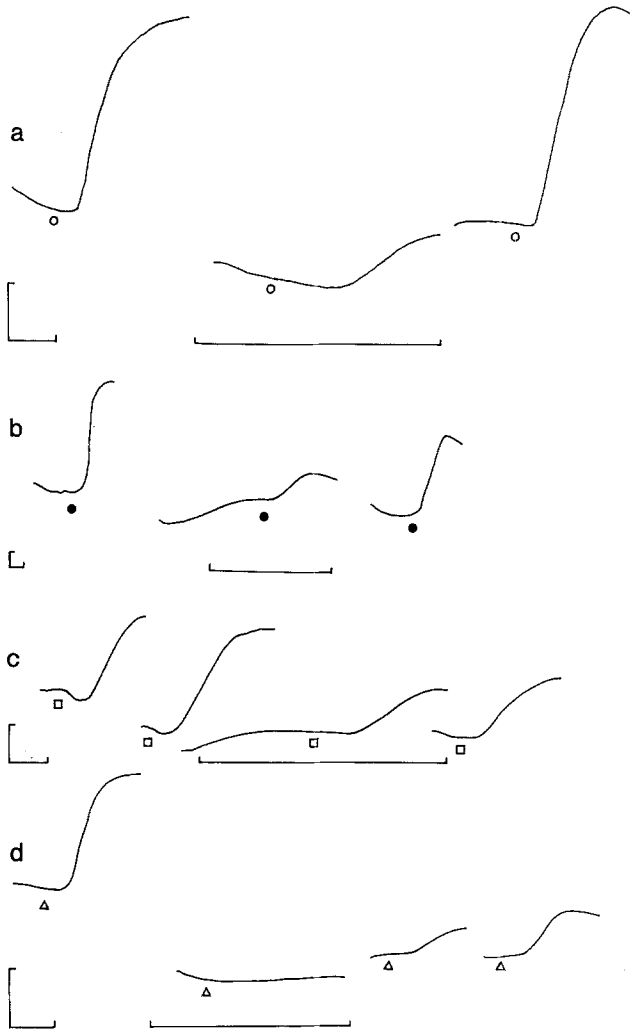

Fig. 9. Effect of agents affecting prostaglandin metabolism on responses to carbachol and angiotensin II. The agents were added to the solutions bathing both sides of the skin for the times indicated by the horizontal lines. Details of the four experiments are as follows. (*a*): Responses to carbachol, 5 μM (open circles), in the absence and presence of indomethacin, 0.25 μM. Initial current was 36 μA, area 1.33 cm^2. (*b*): Responses to carbachol, 10 μM (filled circles), in the absence and presence of aspirin, 0.7 mM. Initial current was 70 μA, area 3.2 cm^2. (*c*): Responses to carbachol, 2.5 μM (squares), in the presence and absence of mepacrine (50 μM). Initial current was 33 μA, area 1.33 cm^2. (*d*): Responses to angiotensin II, 0.125 μM (triangles), in the presence and absence of indomethacin (0.25 μM). Initial current was 33 μA, area 1.33 cm^2. Calibrations were 10 μA and 10 min

Discussion

Of the variety of hormonal effects on sodium transport which have been described for frog skin acetylcholine is the first hormone known to produce inhibition, although inhibitory effects have been described for α-receptor agonists in the presence of a β-receptor blocker (Watlington, 1968). The stimulatory effects of acetylcholine have been reported before (McAfee,

1964; Puppi, Szalay & Dely, 1975), but a detailed investigation of mechanisms has not been undertaken. Inhibitory responses to cholinoceptor agonists have been shown for toad urinary bladder (Sahib et al., 1978; Wiesmann et al., 1978) and turtle bladder (Schilb, 1969). We have shown that SS and particularly SI responses are present in the isolated epithelium, and this constitutes the firmest proof yet that acetylcholine can have a direct effect on epithelial transport, since the urinary bladders contain a layer of nonepithelial cells.

Stimulatory responses are associated with an increase in total tissue conductance, while inhibitory responses are associated with a conductance decrease. As the apical surface is the major resistive barrier in frog skin (Nagel, 1976), it seems likely that both effects are finally mediated by changes at the apical barrier, although the drugs are applied to the serosal side. However, it must be remembered that acetylcholine caused no measurable change in conductance in toad or turtle bladder (Sahib et al., 1978; Wiesmann et al., 1978; Schilb, 1969).

Both SS and SI responses are associated with changes in sodium transport across the epithelium, although the possible involvement of other ions in a minor way cannot be excluded. Puppi and Dely (1978) claimed chloride was required for the full effect of high concentrations (250 μM) of acetylcholine on the skin. Throughout this work our concentrations of cholinoceptor agonists have been modest by comparison, and no effects of chloride removal were found.

The spectrum of agonist activity together with the blocking effects of atropine at low concentration leave little doubt that both SS and SI responses are mediated by muscarinic type receptors. Previously McAfee and Locke (1967) have shown that acetylcholine stimulation of SCC was blocked by atropine but the concentration used (4 μM) left room for doubt that this was a specific muscarinic effect. That both the major and minor responses to cholinoceptor drugs are blocked by atropine suggests that activation of muscarinic receptors is the first event in the generation of the various types of response. The relatively low sensitivity of these tissues to acetylcholine compared to intestinal muscle might be expected if the muscarinic receptor population is small, i.e., there are no spare receptors (*see* Cuthbert & Young, 1973). Nevertheless, it is important to ask if these receptors are important physiologically. Schoeffeniels and Salee (1965) showed that stimulation of the brachial plexus affected transport parameters in frog skin, and although the effect was modified by atropine the amount used (1 mM) does not allow the conclusion that the effect was due to acetylcholine.

We have obtained evidence to implicate cAMP, prostaglandins, and possibly calcium in the responses to cholinoceptor drugs in frog skin. The correlation between the responses to ADH and carbachol and the potentiation of SS responses to carbachol by theophylline suggests cAMP may be involved. The results obtained by measuring cAMP content are more complex because, although carbachol causes an increase in skins showing SS responses, part or all of this may be generated in the corium. Unfortunately, when isolated epithelia are prepared nearly all responses are of the SI type, although there was an indication that cAMP was increased in a few instances with SS responses. It is possible, therefore, that acetylcholine increases SCC through the agency of cAMP.

Acetylcholine-like drugs are known to increase PG synthesis in a variety of tissues including adrenal glands (Ramwell, Shaw, Douglas & Poisner, 1966), adipose tissue (Ramwell & Shaw, 1970), intestine (Sanders & Ross, 1978), heart (Junstad & Wennmalm, 1974), and bronchial tissue (Stoner, Manganiello & Vaughan, 1973). The effects of indomethacin and aspirin on the stimulatory response to acetylcholine indicate that prostaglandins may be involved. Studies on other systems suggest that release of arachidonic acid rather than activation of prostaglandin synthetase is the rate limiting step in synthesis (Kunze & Vogt, 1971). Increased phosphatidyl inositol turnover is almost universally associated with activation of muscarinic receptors (Michell, 1975) and this effect may well generate the supply of arachidonic acid for prostaglandin synthesis (Lapetina, 1979). Mepacrine reversibly inhibited the SS response to acetylcholine, its effective concentration being similar to that reported by Blackwell, Flower, Nijkamp and Vane (1978) required for the inhibition of phospholipase A_2 activity.

Prostaglandin E_1 is known to stimulate sodium transport and to raise cAMP content in frog skin and toad bladder (Albert & Handler, 1974; Lipson & Sharp, 1971; Wong, Bedwani & Cuthbert, 1972; Hall et al., 1976) but also antagonizes the effect of low concentrations of ADH (Wooster, 1970) and isoprenaline (S. Wilson, *unpublished*) on sodium transport.

The reduction of SS responses by prostaglandin synthetase inhibition makes it likely that endogenous prostaglandin release is involved in the response. Whether the released prostaglandin activates adenylcyclase or acts in another way to increase SCC is unclear.

Like acetylcholine, angiotensin II can release prostaglandins in spleen (Diekmann, Jobke, Peskar & Hertting, 1977), vascular endothelium and smooth muscle (Gimbrone & Alexander, 1977), kidney (Zus-

man & Keiser, 1977), and heart (Isakson et al., 1977). Further, Spinelli and Walther (1979) have shown that the effects of angiotensin on sodium transport in the proximal tubule is inhibited by indomethacin. Thus the mechanisms of acetylcholine and angiotensin actions on sodium transport may be rather similar and distinctly different from those of isoprenaline and ADH. Although ADH can stimulate prostaglandin release in toad bladder (Orloff & Zusman, 1978), the combination of a PG releaser plus a powerful adenylcyclase activator ensures a substantial increase in cAMP content following hormone. ADH can also stimulate phosphatidyl inositol turnover in tissues like liver (Michell, Kirk & Billah, 1979) which allows the possibility for ADH to act in one way similar to that proposed here for acetylcholine.

Inhibitory responses to acetylcholine were associated with a small, but significant, fall in cAMP content. Even though acetylcholine-like drugs are known to activate guanylcyclase, giving a rise in cGMP content in some tissues, including the toad urinary bladder (Sahib et al., 1978; Wiesmann et al., 1978), we were unable to detect any change in isolated epithelium. Johnsen and Nielsen (1980) have measured nonstimulated levels of cGMP in isolated epithelium, finding only 0.014 pmol/mg dry wt which is below the amount we are able to detect with our system. They concluded from their experiments that cGMP had no effect on sodium transport by frog skin. In our experiments we were unable to detect any changes in SCC by addition of cGMP or 8 Br cGMP (*unpublished*), as was also found for toad bladder (Sahib et al., 1978), supporting the idea that cGMP is not involved in sodium transport mechanisms. The only biochemical correlate of SI responses we have discovered is a fall in cAMP content. Acetylcholine has been shown to lower cAMP content in heart (Murad, Chi, Rall & Sutherland, 1962; Watanabe et al., 1978) and neuroblastoma cells (Matsuzawa & Nirenberg, 1975). Against this, however, a fall in cAMP content was not found in toad bladder with cholinergic agonists (Sahib et al., 1978).

Unlike the toad bladder the SI responses in skin were not dependent on calcium in the external medium. The calcium ionophore A23187 also causes inhibition in toad bladder (Wiesmann, Sinha & Klahr, 1977; Ludens, 1978; Taylor & Windhager, 1979), and together with the increased calcium uptake associated with the acetylcholine response (Wiesmann et al., 1978) it is reasonable to suggest that transport inhibition was caused by increased intracellular calcium. However, in frog skin both stimulation (Nielsen, 1978; Balaban & Mandel, 1979) and inhibition (Grinstein & Erlij, 1978) with A23187 have been reported, and it is possible that calcium might be involved in both SI and SS responses as suggested by our results although the mechanisms are unclear.

In summary, we have evidence that cAMP and prostaglandins may be involved as mediators in the responses of frog skin to cholinoceptor agents and, additionally, a possible role for calcium cannot be excluded.

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