J. Membrane Biol. 86, 157–166 (1985)

The Journal of
Membrane Biology

Indirect Effects of Adenosine Triphosphate on Chloride Secretion in Mammalian Colon

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Summary. The effects of adenosine triphosphate (ATP) on short-circuit current (SCC) in rat colonic epithelium are described. ATP caused a large increase in inward-going current and was considerably more potent in this respect than ADP, AMP or adenosine. The response to ATP was sided, there being only minor effects when the nucleotide was added to the apical side of the tissue. The effects of ATP were not modified by the cyclooxygenase inhibitor, indomethacin, eliminating eicosanoid formation as a mechanism. The effects of ATP were potentiated by theophylline and not blocked by α,β-methylene ATP. The data are consistent with the effect being dependent on the activation of adenylate cyclase, but it has not been possible to classify the receptors into P_1 or P_2 categories. Using inhibitors of NaCl cotransport (piretanide), carbonic anhydrase (acetazolamide), and chloride channels (diphenylamine-2-carboxylate), it was concluded that the SCC response to ATP was due to chloride secretion with, perhaps, a minor contribution from bicarbonate. Flux measurements with ^{22}Na and ^{36}Cl confirmed this view, there being approximate equivalence of chloride secretion with the SCC responses. Additionally, flux measurements revealed an inhibition of electroneutral NaCl absorption in response to ATP.

The effects of ATP were antagonized by tetrodotoxin (TTX), greater than 50% inhibition being achieved with 10 nM TTX. This result suggests that ATP does not act directly on receptors in the epithelial cells but rather on neuronal elements in the lamina propria. It will be necessary to re-examine other secretagogues for indirect effects of this kind and to search for the final effector neurotransmitter which evokes secretion.

Key Words ATP · ADP · AMP · adenosine · tetrodotoxin · chloride secretion · intramural plexus · rat colon epithelium

Introduction

The effects of adenine nucleosides and nucleotides on transepithelial ion transport in the mammalian gut have been reported previously (Kohn, Newey & Smyth, 1970; Grasl & Turnheim, 1984). While our results do not differ in many respects from the earlier reports, we have discovered an important major difference. We have found that tetrodotoxin can virtually abolish the effects of ATP on electrogenic chloride secretion. The data are consistent with the view that the effects of the nucleotide are indirect, via neural elements in the intramural plexus. This view contrasts with a direct action on epithelial cells reported previously. There are a great variety of secretagogues that stimulate chloride secretion in the mammalian gut, and it will be necessary to re-examine many of these to see if they too act indirectly. It is conceivable that many secretagogues act through a final, common, neuroeffector mechanism.

Materials and Methods

All experiments were made with the isolated colonic epithelium of male rats (Sprague Dawley). From each colon two pieces of tissue, about 1 cm long, were taken 4 to 5 cm from the most caudal Peyer's patch. These were opened, washed in Krebs Henseleit (K-H) solution, and pinned to a dissecting tray with the mucosa downwards. The muscle layers were dissected away under a microscope to leave the epithelium with its lamina propria. These two pices of epithelium were mounted in Ussing chambers, window 0.6 cm^2, and used as paired preparations. The tissues were bathed on each side with 20 ml K-H solution, gassed with 95% O_2: 5% CO_2 and maintained at 37°C. The pH was at 7.4. The tissues were short circuited using a standard, previously described, methodology (Cuthbert & Margolius, 1982).

The K-H solution had the following composition (in mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24.8, KH_2PO_4 1.2, and glucose 11.1. All drugs used were obtained from normal commercial sources, dissolved in distilled water, and added in small volume (maximal 200 μl) to the solution bathing one or the other side of the tissue. The exception was diphenylamine-2-carboxylate, which was a gift from Dr. R. Greger. This material was dissolved in a small volume of 0.1 NaOH to form the sodium salt. There was no change in pH when added to the fluid bathing the tissue.

Transepithelial flux studies were made with either ^{22}Na or ^{36}Cl. Paired preparations were used, and a trace of isotope (2–4 μCi) was added to the apical bathing solution of one preparation and the basolateral side of the other. Around 45 min was allowed for the isotope to achieve a constant specific activity within the tissue. Five samples (each 1 ml) were collected from the *trans* side at 20-min intervals. Smaller samples (100 μl) were taken

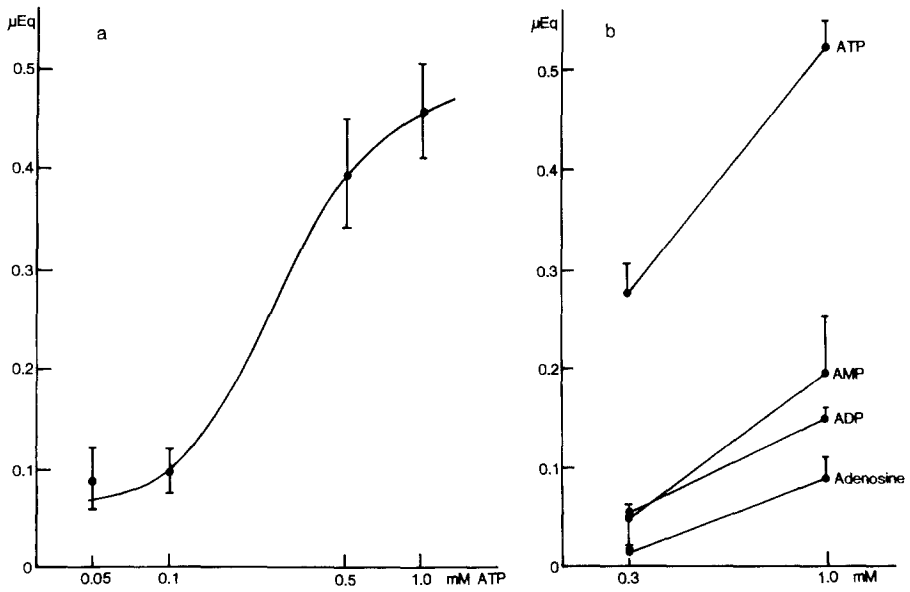

Fig. 1. Effects of adenosine analogues on SCC in isolated rat colon. In each experiment the epithelial area was 0.6 cm^2. The values of SCC increase (given in μeq) were measured during 10 min following addition of the drug. All drugs were added to solutions bathing the basolateral side of the tissue. (*a*) Concentration response curve for ATP. Each value shows the mean ±SE for thirteen separate preparations. Each concentration of ATP was applied once to each preparation, with washing and restoration of basal SCC between each application. (*b*) Partial concentration response curves for ATP, ADP, AMP and adenosine. A single preparation was exposed to the two concentrations of ATP and the two concentrations of one of the other analogues. After each response the drug was washed away and basal SCC restored before further drug exposure occurred. Mean ±SE values are given for 16(ATP), 4(ADP), 4(AMP) and 8(adenosine) separate values. The responses for ATP were significantly greater ($P < 0.001$) at both concentrations than the corresponding responses to the other analogues

from the *cis* side to allow specific activity to be calculated. Samples were replaced by equal volumes of K-H solution, and allowance was made for the resulting dilution in calculating fluxes. Samples were counted by standard liquid scintillation procedures. The areas under the SCC response curves were integrated by planimetry (using an Allbrit planimeter). In alternate experiments the most caudal of the two preparations was used to measure apical-to-basolateral flux.

Results

Effects of Adenosine Analogues on Epithelial Transport

The concentration response relationship for ATP on the colon was investigated by using four concentrations of the nucleotide, applied in a randomized order to the basolateral side of the tissue, in thirteen separate experiments. At concentrations above 0.5 mM the SCC response was maintained, while at lower concentrations the SCC usually declined from a peak value to give a sustained plateau. In every instance the response was terminated after 10 min and the area under the curve integrated to give the total charge transfer, calculated as μeq of univalent ion. The values of charge transfer were used to construct the concentration-response curve shown in Fig. 1*a*.

The activity of ATP relative to that of ADP, AMP and adenosine was investigated in a separate set of experiments using only two concentrations of each compound. In every instance ATP was compared with one of the other compounds in the same preparation and at the same concentration. The results of these measurements are given in Fig. 1*b*. It is clear that ADP, AMP and adenosine are relatively inactive compared to ATP. Adenosine appears to have the least activity, while ADP and AMP have similar activity.

The foregoing data were obtained by application of ATP and its analogoues to the basolateral side of the tissue. As it is not uncommon for epithelia to show asymmetry in response to drugs (Cuthbert, 1984), the sidedness of the response was investigated. The experimental protocol can be seen by reference to Fig. 2. Paired preparations taken from adjacent pieces of colon were prepared and exposed to two concentrations (300 μM and 1 mM) of ATP on

both the apical and basolateral sides, with washing between each application. These concentrations were chosen to give responses that were around 50% of maximal and maximal when added on the basolateral side. In one preparation the apical side responses were obtained first, while in the other they were obtained after the basolateral responses. Responses on the apical side were transient while from the other side the SCC were maintained during the 10-min period of measurement. Several things are apparent from the data presented at the foot of Fig. 2. First, the responses are significantly greater ($P < 0.001$) when ATP is added to the basolateral (serosal) bathing fluid than when added to the apical (mucosal) side, irrespective of the side to which the nucleotide is added first. Secondly, the responses from the apical side are somewhat larger after the nucleotide has been added first to the other side. We cannot be sure that any response, at all, is obtained by an interaction with the apical surface of the epithelium, as it is possible that there is some penetration of the tissue by ATP, which then alters SCC by interaction with receptors on the basolateral side. Furthermore, it is possible that prior exposure of the basolateral aspect increases the permeability of the tissue to ATP. Clearly, the colon shows a distinct sidedness to the actions of ATP.

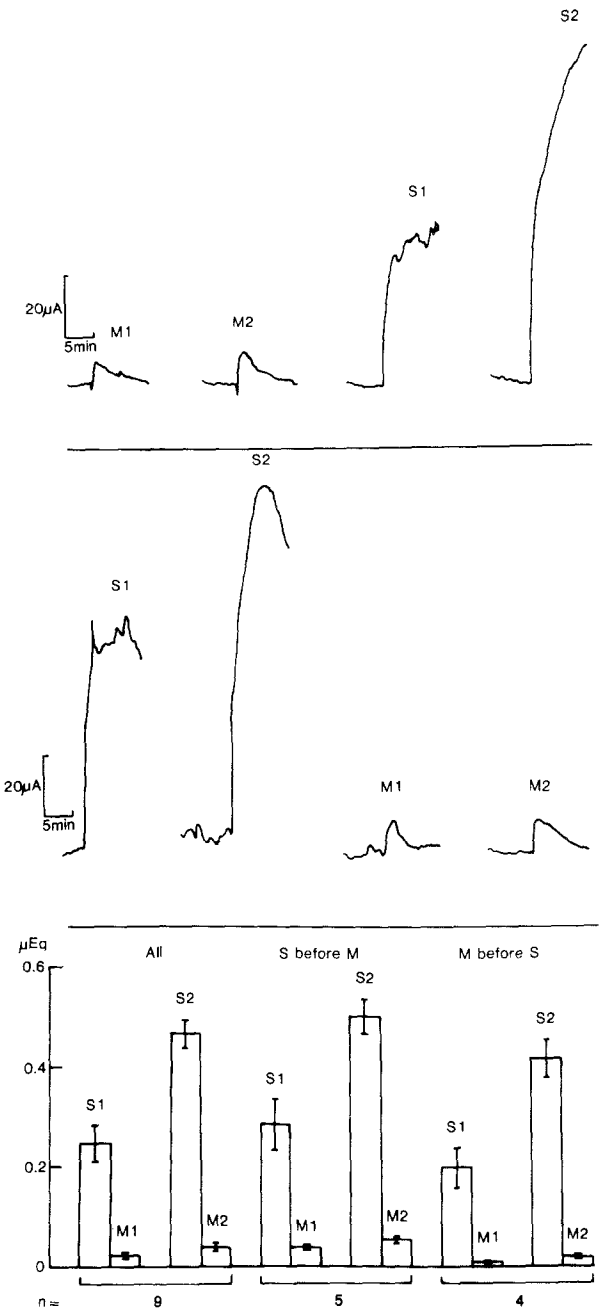

Fig. 2. Responses of colon to ATP at two concentrations, 300 μM (1) and 1 mM (2) applied either first to the apical (*M*) and then to the basolateral (*S*) side of the tissue or vice versa. Illustrated is one set of paired preparations, each 0.6 cm^2, in which apical application preceded (upper) or followed (lower) application to the basolateral side. The horizontal lines indicate zero SCC. Areas under the responses, each of which was terminated after 10 min, were integrated and converted to μeq. In the lower part of the figure the results for 9 paired preparations are shown. Mean values ±SE for 9 measurements (*All*), 5 measurements (*S before M*) and 4 measurements (*M before S*) are shown. All *S* values were significantly greater ($P < 0.001$) than the corresponding *M* value

Involvement of Neural Elements in the Response to ATP

Although the longitudinal and circular muscle layers have been removed from our preparations, some of the lamina propria remains. It seemed possible that the actions of ATP may be indirect, being exerted on some tissue elements which, in turn, affect epithelial function. Obvious candidates are the neural elements contained within the lamina propria. To test the hypothesis the specific neuronal blocking agent tetrodotoxin (TTX) was used. The results of one series of experiments is given in Fig. 3.

Again, paired preparations were used and to one was added 0.1 μM TTX. Ten minutes later 300 μM ATP was added to both preparations on the basolateral side. The responses to ATP were terminated after 10 min and the preparations thoroughly washed. After the basal SCC had been restored a crossover experiment was performed. Thus it was possible to compare the effects of TTX on the responses to ATP both in the same preparation and in a paired preparation. Eight paired experiments of this type were performed, and the data are given in

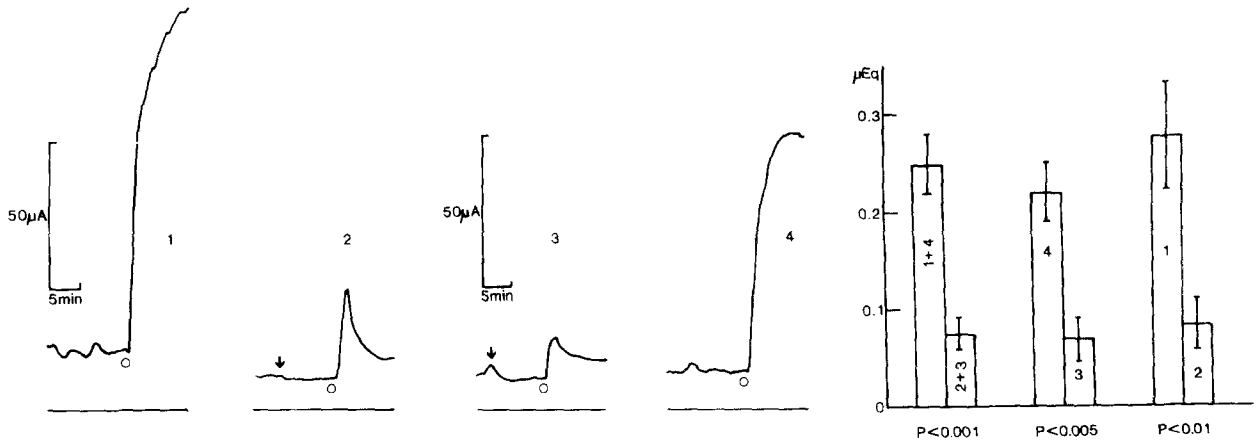

Fig. 3. Effects of TTX on the response to ATP. Illustrated are responses from paired preparations (0.6 cm^2) to ATP (300 μM) applied to the basolateral side. Each preparation was exposed twice to ATP in the presence and absence of TTX (0.1 μM). In the preparation illustrated on the left-hand side the control response without TTX was obtained first, while in the other preparation it was obtained after TTX had been removed. TTX was added at the time indicated by the arrow and ATP by the open circles. The horizontal line indicates zero SCC. The data at the right-hand side of the figure illustrates results from 8 pairs of preparations. Means ±SE are given either for 16 measurements (1 + 4 and 2 + 3) or for 8 measurements (single numbers). The numbers indicate the type of response as indicated in the left-hand part of the figure. TTX caused a significant reduction in the responses to ATP; *P* values are indicated

Fig. 3. TTX caused a significant reduction in the response to ATP, irrespective of whether the control response to ATP was obtained before or after the response in the presence of TTX. Further, there was a significant reduction in the response to ATP when combined control responses were compared with combined responses in the presence of TTX and irrespective of the order of addition.

During these experiments several other phenomena were noticed which are worthy of comment. For example, responses to ATP are rather variable, but we noticed that preparations showing minor flucutations in basal SCC (for example, the first response in Fig. 3) responded well. Other preparations in which the basal SCC showed no fluctuations gave, in general, poor responses. Preparations with an unsteady basal SCC responded to TTX with a minor reduction in current and loss of current fluctuations (*see* Fig. 3). It is possible that the minor fluctuations in basal SCC represent fluctuating activity in the neuronal plexus within the lamina propria. Finally, it was noticed that the responses to ATP were smaller, although not significantly so, after TTX had been removed when compared with those in the paired preparation untreated with TTX. This may indicate that it is difficult to remove, completely, TTX from its binding sites. Two other sets of experiments were performed similar to the first set using TTX concentrations of 10 nM and 1 μM. The inability to wash off TTX became more apparent at 1 μM, but did not appear to be a problem at 10 nM. Figure 4 gives the composite data for all three sets of experiments. From this it is clear that, at all three concentrations of TTX, there is a significant reduction of the response to ATP when all the data are considered. However, it appears there is some difficulty in removing TTX at the highest concentration resulting in a reduction in size of the control responses. To avoid this problem data are presented showing the effects of TTX measured only after the control responses were obtained; now the control responses are very comparable. The percentage inhibition of the response to ATP versus the TTX concentration curve is somewhat unusual and is discussed later, but greater than 50% inhibition is achieved even at a concentration of TTX as low as 10 nM. The inhibition curves had almost identical form whether the combined data, with variable control responses, or limited data, with constant control responses were used.

In a final set of experiments identical in format to those of Fig. 3 we used atropine (0.1 μM) instead of TTX to examine for its effect on responses to ATP (300 μM). Five paired experiments were performed and the charge transfer occurring in 15 min following ATP was 0.35 ± 0.05 μeq (n = 10) in controls and 0.34 ± 0.04 μeq (n = 10) in atropine-treated tissues. We can conclude that effects at muscarinic cholinoceptors are unlikely to be involved in the responses to ATP.

Lack of Involvement of Eicosanoids

The rat colon is capable of generating large quantities of eicosanoids, and some secretagogues act par-

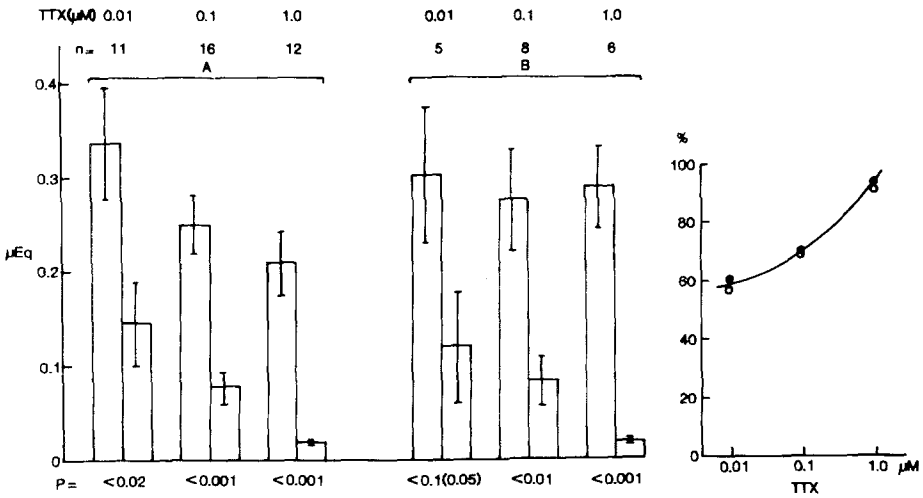

Fig. 4. Effect of TTX on the responses to ATP. A series of experiments identical to those shown in Fig. 3 were carred out using different concentrations of TTX. The composite data are given here. Throughout the ATP concentration used was 300 μM applied to the basolateral side of preparations of 0.6 cm^2. Responses were terminated after 10 min and the areas under the curve integrated to give the measurements in μeq. On the left (*A*) are the results using paired preparations, while in the center are the results from single preparations (*B*), in which the tissues were not exposed to TTX until after the control responses had been obtained. (This corresponds to responses labeled *1* and *2* in Fig. 3.) Mean values ±SE are given. The number of observations, their significance using an unpaired *t* test, and the TTX concentration are given. The single value in parentheses was obtained using a paired *t* test. On the right is shown the percentage inhibition of the response to ATP versus the TTX concentration obtained from the data in the center (●) or the left-hand side (○) of the figure

tially through the generation of these (Cuthbert, Halushka, Margolius & Spayne, 1984); it is possible that ATP might act in this way, too. We have shown previously that indomethacin (5 μM) effectively prevents eicosanoid formation in the colon by inhibition of fatty acid cyclooxygenase (Cuthbert et al., 1984). Five experiments with paired preparations were made as follows. ATP (300 μM) was added to one preparation of each pair in the absence of indomethacin while the second received ATP only after incubation with indomethacin (5 μM) for 10 min. After washing ATP away from the first preparation the response was repeated in the presence of indomethacin. Net charge transfer during 10 min was 0.281 ± 0.054 μeq in the absence and 0.284 ± 0.047 μeq in the presence of indomethacin, these values being the means ±SE for five paired tissues. On rechallenging the control preparations after removal of ATP and incubation with indomethacin, the value was 0.263 ± 0.056 μeq. It can be concluded that eicosanoid formation plays no part in the response to ATP.

Effects of Phosphodiesterase Inhibitors on Responses to ATP

As will be discussed, adenosine derivatives have been shown to affect cyclic nucleotide metabolism (Grasl & Turnheim, 1984), and it might be expected that the actions of ATP will be potentiated by inhibitors of phosphodiesterase. On the other hand, theophylline blocks the effects of purines in some systems, so the effect this agent may have on ATP responses is unclear. Consequently, we examined the effects of two phosphodiesterase inhibitors, theophylline and isobutylmethylxanthine (IBMX), on responses to ATP. The experimental design again involved paired preparations. In one of each pair ATP was added before, and in the other after, preincubation for 15 min with theophylline or IBMX. After washing away the drugs a cross-over test was employed. In all instances the ATP concentration was 300 μM, a concentration producing a submaximal effect, allowing detection both of potentiation or inhibition of the effect. The results of these experiments are given in Table 1. The mean reponse to ATP was increased in the presence of both theophylline and IBMX, but this increase was significant only with theophylline.

Lack of Antagonism of ATP by α,β-Methylene ATP

In a number of systems the effects of purines are antagonized by α,β-methylene ATP, apparently by desensitization of the receptors. Consequently, α,β-methylene ATP itself shows initial agonist effects while subsequent addition of ATP causes no

effect even after the methylene compound is removed (Kasakov & Burnstock, 1982). We were unable to show antagonism with α,β-methylene ATP on the colon epithelium with a number of experimental designs. In the example illustrated in Fig. 5 the most favorable conditions for demonstrating antagonism were employed by using only a low concentration of ATP (50 μM), even lower than the concentration of α,β-methylene ATP (100 μM). With ATP, 50 μM, the response was not well maintained during 10 min, but a response similar in form could be obtained in the presence of α,β-methylene ATP. The latter itself caused a minor, but sustained, increase in SCC. Although there was tachyphylaxis to the effects of ATP upon repeated administration, this was no less severe in the absence of α,β-methylene ATP than in its presence.

Effects of Inhibitors on ATP-Stimulated SCC

From the direction of the SCC responses to ATP it is probable that electrogenic secretion of anions, electrogenic cation absorption, or a mixture of the two is responsible. Before embarking on measurements of ion flux we examined the effects of blocking agents with defined actions on the responses to ATP. We chose to use high concentrations of piretanide as a blocker of NaCl cotransport, acetazolamide as a carbonic anhydrase inhibitor, and diphenylamine-2-carboxylate (DPC) as a blocker of epithelial chloride channels (Greger & Schlatter, 1985). Typical results are shown in Fig. 6. The pair of preparations illustrated in Fig. 6*a* were made from adjacent sections of colon. The response to ATP was reasonably similar in form and size in both preparations. Transport was inhibited by addition of piretanide or DPC to the basolateral bathing solution. The response to piretanide was faster in onset than that to DPC, which characteristically showed temporary pauses during the fall in SCC. The residual SCC appeared to show a further small decrement in SCC when acetazolamide was added to the piretanide-treated preparation. Two other paired preparations are illustrated in Fig. 6*b*. On this occasion the preparations were chosen from colon segments 4–5 cm apart. In this instance the responses to ATP were rather discrepant, the most caudal piece giving the smaller response. These responses emphasize the need to choose pieces as close as possible to each other for measurements of ion flux. In these two preparations acetazolamide was without effect on SCC added before the other blocking agents. DPC added to the apical side of one preparation and the basolateral side of the other produced inhibition of SCC; the residual SCC was sensitive to piretanide. As discussed later, the combined evidence using blocking drugs suggests that ATP causes electrogenic chloride secretion in the colon, with perhaps a minor involvement of bicarbonate.

Table 1. Effects of theophylline (1 mM) and IBMX (0.1 mM) on the responses to ATP (300 μM)

ATP	Plus inhibitor	n	P
0.280 ± 0.23	0.367 ± 0.034 (theophylline)	10	<0.05
0.309 ± 0.023	0.362 ± 0.049 (IBMX)	10	NS

Values are given in μeq and represent the charge transfer occurring during 10 min following addition of ATP.

Ion Fluxes Associated with SCC Changes Caused by ATP

To discover which ion movements are affected by ATP we have measured ion fluxes of Na^+

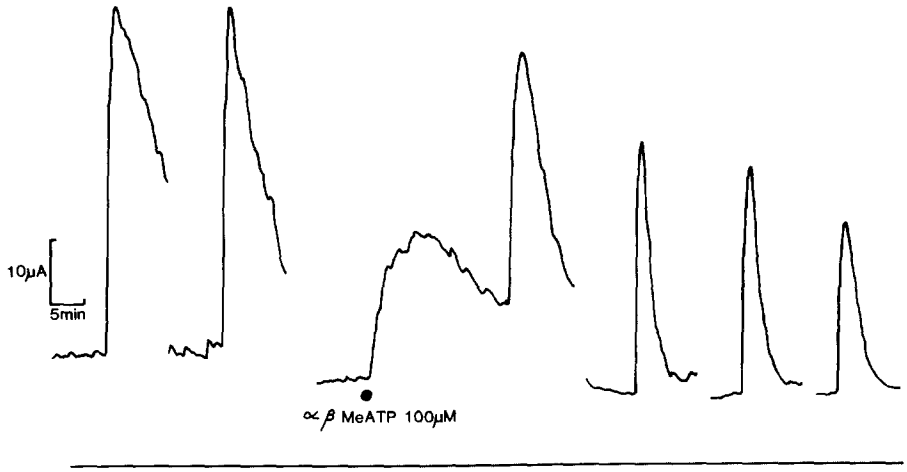

Fig. 5. SCC responses in a single preparation (0.6 cm^2) to repeated (six times) addition of ATP (50 μM) to the basolateral bathing fluid. The horizontal line indicates zero SCC. The ATP was washed away 10 min after each application. Before the third application of ATP the tissue was exposed on the basolateral side to α,β-methylene ATP (100 μM) for 20 min after which ATP was added in the presence of the analogue

and Cl . and the changes in these in response to ATP.

The protocol for the flux experiments is illustrated in Fig. 7. After paired tissues had been allowed to equilibrate with the isotope (either ^{22}Na or ^{36}Cl), added to the apical (*M*) side of one preparation and the basolateral (*S*) side of the other, samples were taken from the *trans* side at 20-min intervals. In all, five samples were taken so that fluxes could be calculated for the periods *P*1 to *P*4. Addition of ATP (1 mM) during *P*3 caused a rapid increase in SCC, which was maintained throughout *P*3. At the beginning of *P*4, DPC (150 μM) was added to the apical bathing solution causing a partial reversal of the ATP effect. The areas *A* and *B* (Fig. 7) were marked on the SCC traces and integrated to yield the changes in electrogenic transport caused by ATP and the inhibitor.

The data relating to Cl^- and Na^+ fluxes are given in Tables 2 and 3. ATP increased the $S \rightarrow M$ flux of chloride but reduced the flux in the opposite direction. As a result of these changes net chloride absorption in *P*2 was converted to net chloride secretion in *P*3. Addition of DPC during *P*4 caused the fluxes (and SCC) to return towards the control values, but the flux values were not significantly different from those in *P*3. Sodium fluxes were also affected by ATP addition, there being a significant reduction in $M \rightarrow S$ and net fluxes. Again the inhibitor tended to restore the values towards controls,

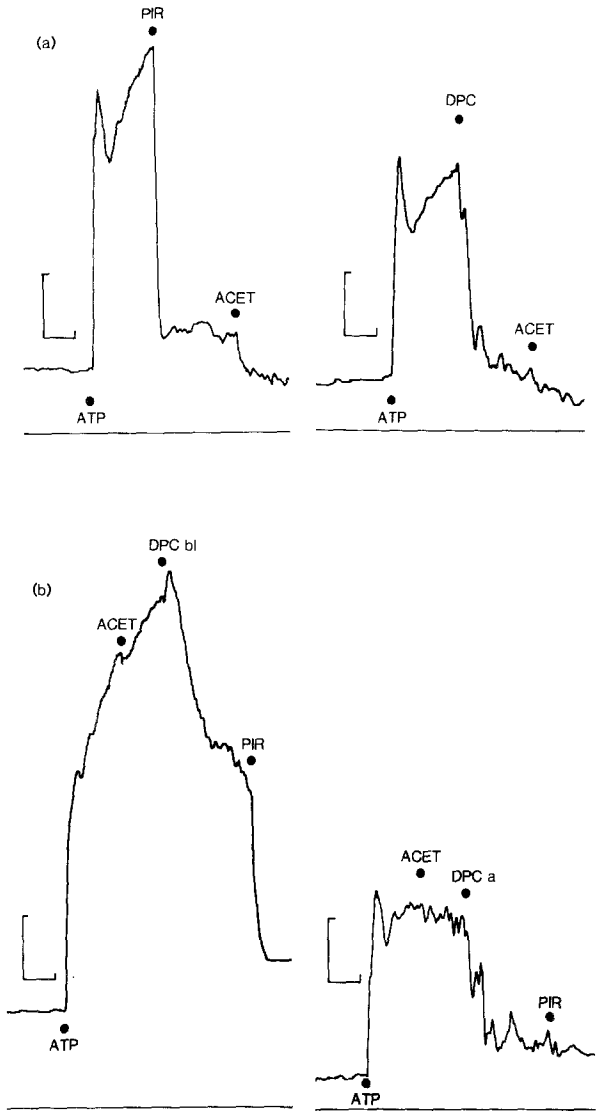

Fig. 6. Effect of inhibitors on the responses to ATP. (*a*) Paired preparations, each 0.6 cm^2, were exposed to ATP (0.5 mM) on the basolateral side. Piretanide (*PIR*, 1 mM) or DPC (150 μM) were added on the same side after the response to ATP had reached a plateau. Acetazolamide (*ACET*, 214 μM) was added to both sides of the tissue after the response to the inhibitors had reached steady state. (*b*) Paired preparations, each 0.6 cm^2, taken from pieces of colon 4–5 cm apart. After stimulation with ATP (0.5 mM) acetazolamide (*ACET*, 214 μM) was added to the solutions bathing both sides of the tissue. DPC (150 μM) was added to the basolateral (*DPC bl*) bathing solution of one preparation and the apical (*DPC a*) bathing solution of the other. Finally, piretanide (*PIR* 1 mM) was added to the basolateral side of both preparations. In all preparations horizontal lines indicate zero SCC. In each trace calibrations are 20 μA and 5 min

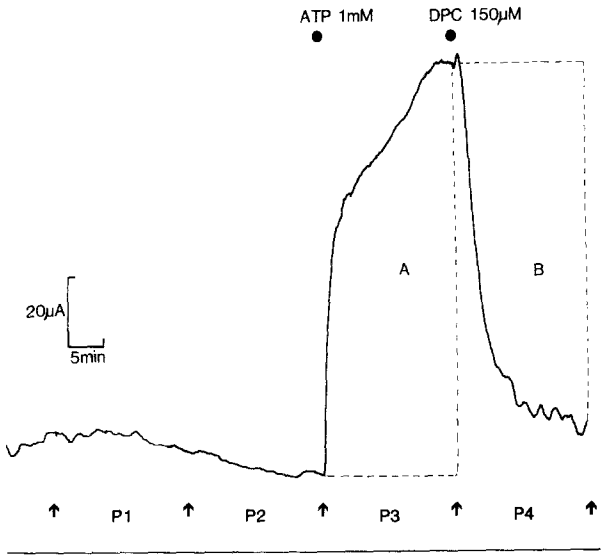

Fig. 7. Figure illustrating the protocol for flux experiments. SCC for a single preparation, 0.6 cm^2. Samples (1 ml) were taken from the side opposite to which the radioactive species was added at the five times indicated by the arrows, dividing the experiment into four periods *P*1 to *P*4, each of 20 min. Small samples (100 μl) were taken from the *cis* side at the mid points of *P*2 and *P*3. ATP (1 mM) was present during *P*3 and *P*4 on the basolateral side. DPC (150 μM) was present on the apical side during *P*4. The horizontal line indicates zero SCC. Areas *A* and *B* were integrated to give ΔSCC values. These represent, respectively, the increase in electrogenic transport caused by ATP (*P*3) and the reduction in electrogenic transport caused by DPC (*P*4)

Table 2. Chloride fluxes in colonic epithelium[a]

	Flux (μeq/0.6 cm^2/20 min)			
	*P*1	*P*2	*P*3	*P*4
$S \rightarrow M$	2.24 ± 0.41	2.12 ± 0.49	3.11 ± 0.48[b]	2.78 ± 0.33
$M \rightarrow S$	3.32 ± 0.62	3.61 ± 0.62	2.53 ± 0.66[c]	2.68 ± 0.51
Net	1.09 ± 0.58	1.49 ± 0.56	−0.57 ± 0.54[d]	−0.09 ± 0.45
ΔSCC	—	—	0.88 ± 0.12	0.52 ± 0.08[d]

[a] Effects of ATP.

Each value is the mean ±SE for seven measurements.

Seven paired preparations were used, one of each pair was used to measure $S \rightarrow M$ flux and the other for the $M \rightarrow S$ flux. Periods *P*1 and *P*2 were control periods during which basal SCC was recorded. ATP (1 mM) was present in the basolateral bathing solution during periods *P*3 and *P*4. Diphenylamine-2-carboxylate (150 μM) was present in the apical solution during period *P*4. Net fluxes were calculated from the two unidirectional fluxes and were considered positive if there was net chloride absorption. ΔSCC values represent either the increase in SCC caused by ATP, 1 mM (*P*3), or the reduction in SCC caused by DPC, 150 μM (*P*4). The ΔSCC values for a pair of preparations was averaged and the seven averages used to calculate the values given above. The values in *P*2, *P*3 and *P*4 were compared with the immediately preceding values using a paired Student *t* test. The values in *P*3 were significantly different from those in *P*2 ([b]$P < 0.01$; [c]$P < 0.05$; and [d]$P < 0.005$).

Table 3. Sodium fluxes in colonic epithelia[a]

	Flux (μeq/0.6 cm^2/20 min)			
	*P*1	*P*2	*P*3	*P*4
$S \rightarrow M$	1.14 ± 0.23	1.03 ± 0.26	1.37 ± 0.12	1.46 ± 0.31
$M \rightarrow S$	2.75 ± 0.28	2.56 ± 0.32	1.51 ± 0.28[b]	2.13 ± 0.37
Net	1.61 ± 0.23	1.53 ± 0.40	0.14 ± 0.26[b]	0.66 ± 0.37
ΔSCC	—	—	0.89 ± 0.11	0.32 ± 0.13[c]

[a] Effects of ATP.

Each value is the mean ±SE for 6 experiments. The protocols were the same as for chloride fluxes. Periods *P*1 and *P*2 were control periods; ATP (1 mM) was added to the basolateral bathing solution during *P*3 and diphenylamine-2-carboxylate (150 μM) was added to apical solution during *P*4. Net fluxes were calculated from the two unidirectional fluxes and were all positive, i.e., net absorption. ΔSCC for the paired preparations were averaged and the six averages used to calculate the values in the table. ΔSCC values represent either increases (ATP) or decreases (diphenylamine-2-carboxylate) in SCC. The values in *P*2, *P*3 amd *P*4 were compared with the immediately preceding values using a paired Student *t* test. Values marked with superscripts are significantly different from the corresponding values in *P*2 or *P*3 ([b]$P < 0.05$, [c]$P < 0.05$ one-tailed).

but values in *P*4 were not significant from those in *P*3.

Considering fluxes of both ions the net influx of Na^+ and of Cl^- are virtually identical during *P*2, and it is probable that electroneutral NaCl absorption is the explanation. For example, a net sodium influx of 1.5 μeq/0.6 cm^2/20 min would require a basal SCC of 120 μA, while resting currents in flux experiments were around 10–20 μA.

During the action of ATP net sodium absorption falls to only 0.14 μeq/0.6 cm^2/20 min, presumably representing a reduction in NaCl absorption. As there is a net chloride secretion during the action of ATP, electrogenic chloride secretion will have a value of 0.71 μeq/0.6 cm^2/20 min (i.e., 0.57 + 0.14). This value is not unlike the integrated value of the SCC response to ATP, which was identical in both sets of experiments (0.885 μeq/0.6 cm^2/20 min^{-1}), there being a 20% shortfall of net chloride flux.

Discussion

Effects of adenine nucleotides and nucleosides on epithelial transport have been reported previously. Kohn et al. (1970) reported ATP increased transepithelial potential in rat ileum and colon, but a greater sensitivity to apical application was reported. Here the effects of ATP have been shown to be exerted predominantly, if not exclusively, on the basolateral side of the epithelium. The small responses to ATP added to the apical solution could be due to the penetration from the opposite side. A basolateral effect of ATP was found in rabbit colon (Grasl & Turnheim, 1984), but this agent and adenosine were equipotent in increasing SCC, quite different from the pattern reported by us. SCC is also increased in the frog cornea by adenosine (Spinowitz & Zadunaisky, 1979), an agent that also increases NaCl secretion in rectal gland tubules of the dogfish (Greger, Schlatter, Wang & Forrest, 1984).

Before coming to the major new finding, namely the probable involvement of neural elements of the lamina propria in the response to ATP, we will consider the nature of the ATP receptors and the ions involved in the SCC responses. Classification of purinoceptors still poses considerable problems in the absence of a specific, high affinity antagonist. Based on the relative agonist potencies of ATP, ADP, AMP and adenosine, the receptors might be considered to be the P_2-type. Against this suggestion is the failure of α,β-methylene ATP to desensitize the receptors to ATP. In other systems α,β-methylene ATP, in micromolar concentrations, completely desensitizes tissues to exogenous ATP and to the effects of noncholinergic, nonadrenergic nerve stimulation (Kasakov & Burnstock, 1982; Meldrum & Burnstock, 1983; Sneddon & Burnstock, 1984). Two classes of P_1 receptors have been proposed which either activate or inhibit adenylate

cyclase (Londos, Cooper & Wolff, 1980). Adenosine effects at these receptors are blocked by methylxanthines, but in turn these latter are phosphodiesterase inhibitors, which potentiate effects mediated via cyclic AMP. We found that both theophylline and IBMX increased the response to ATP, suggesting the transport effect was mediated by cAMP, but without any evidence for antagonism at surface receptors. Grasl and Turnheim (1984) in rabbit colon were able to demonstrate both inhibition of the SCC effect by theophylline and accumulation of cAMP in response to adenosine. In rabbit ileum inhibition of SCC effects of purines was not seen with theophylline or IBMX, but was apparent with 8-phenyltheophylline (Dobbins, Lawrenson & Forrest, 1984). It is clear therefore that adenosine antagonists can give a variety of results because of the mixed actions of these compounds. While our results are consistent with the ATP effect being mediated through cyclic nucleotides, as indeed was suggested first by Kohn et al. (1970), we feel that the type of the purinoceptors involved must await more selective antagonists, especially since ATP also raises cAMP content in ileum (Dobbins et al., 1984), suggesting that ATP might activate adenosine receptors. It is clear, however, that the effects of ATP in the rat colon do not involve eicosanoid formation, a process which has been implicated in other responses (Burnstock, Cocks, Paddle & Staszewska-Barczak, 1975).

Turning to the ion currents stimulated by ATP, the picture is much clearer. The inhibition of the ATP effect by the loop diuretic piretanide, applied basolaterally, is a good indication that the SCC response is due to electrogenic chloride secretion. Piretanide inhibits NaCl cotransport (Zeuthen, Ramos & Ellory, 1978) and so prevents chloride entry through the basal pole of the cell, according to the model of electrogenic chloride secretion (Frizzell, Field & Schultz, 1979). The new inhibitor, diphenylamine-2-carboxylate, DPC, is considered to be a chloride channel blocker (Greger & Schlatter, 1985), preventing chloride exit through the apical membrane of the secreting cells. We have found that this agent does not exhibit the sidedness shown by piretanide (Cuthbert, 1984), presumably because its lipid solubility allows it to gain entry from either side of the tissue. There is little evidence that bicarbonate is actively secreted in this tissue as acetazolamide has no effect on ATP-stimulated SCC (Fig. 6*b*) except in one special condition. Acetazolamide does cause a minor reduction in SCC after piretanide but not after DPC (Fig. 6*a*). In rabbit colon rheogenic bicarbonate secretion was apparent only in the absence of chloride (Grasl & Turnheim, 1984), and it was suggested that HCO_3^- and Cl^- exit through the same apical channels. Therefore, in the presence of piretanide a residual anion secretion, sensitive to acetazolamide, might be expected. It would not be seen, however, if the apical channels were blocked by DPC and if both anions used the same channel. Our data are not inconsistent with these suggestions.

Net absorption of sodium is reduced by ATP, while net chloride absorption is converted to a net secretion. From the directions of the fluxes sodium absorption cannot be responsible for the electrical response to ATP. Allowing for neutral NaCl absorption, net chloride secretion caused by ATP equals the difference between net cation absorption and net anion secretion, i.e., 0.71 μeq/0.6 cm^2/20 min. This value is close to the average SCC response (0.88 μeq/0.6 cm^2/20 min) and not significantly different from it. Thus the major ion carrier in response to ATP must be chloride but the possibility that other ions may contribute in a minor way is not excluded. DPC caused a significant reduction in SCC in both sets of flux measurements; net movements of chloride and sodium during *P*4 moved toward the control values, although the values of flux in *P*4 were not significantly different from those in *P*3.

The most surprising finding in this study is the ability of TTX to block the effects of ATP upon SCC, provoking the idea that ATP acts indirectly via intraneuronal elements in the epithelium. This is not so for all secretagogues since we found TTX had no effect on the chloride secretory response to forskolin (data not given), a result which is also true for kinins (Manning et al. 1982). However, for a large number of secretagogues the locus of action has been assumed to be on the basolateral aspects of the epithelial cells, which is the conclusion made in recent works with ATP and adenosine (Dobbins et al., 1984; Grasl & Turnheim, 1984). Further, this latter view is supported by the finding that monolayers of pure MDCK cells respond to ATP by secreting chloride (Simmons, 1979), but the peak responses here are less than 10 μA cm^{-2}, only a fraction of the response we have found. We cannot preclude a small direct component in our responses, but TTX inhibition greater than 90% was obtained.

Our suggestion of an indirect action via neural elements depends on the specificity of TXX action. This agent is considered specific for voltage-sensitive sodium channels for which it has a high affinity of around 10^9 M^{-1} (Colquhoun & Ritchie, 1972). Extrapolation of our results indicates that TTX is extremely potent at inhibiting ATP, with an apparent EC_{50} of around 10^{-9} M. The shape of our concentration response curve for TTX is unlike that which would be expected from simple mass action kinet-

ics. However, a system in which there is an interaction of ATP with a nerve cell body in the enteric plexus, maybe several synapses away from the release of the final effector neurotransmitter, is unlikely to behave in linear fashion, but rather as an amplifying cascade. A simple relation, therefore, between TTX concentration and effect is not to be expected.

There is much evidence to suggest that nonadrenergic, noncholinergic nerves may liberate ATP as a transmitter or cotransmitter (Burnstock, 1972). Whether such purinergic nerves are involved in the control of intestinal secretion is unknown. Our results add nothing to the debate about purinergic systems but would be consistent with the possibility that they are present in the intestinal mucosa.

We have not tried to identify the neurotransmitter released by ATP in this study, particularly as inhibitors for likely candidates such as substance P and VIP are not presently available. Further, we know that the colon is almost unresponsive to histamine but very sensitive to 5-hydroxytryptamine, yet commonly available antagonists to the latter are rather ineffective. Our data with atropine seem to rule out a major role for acetylcholine acting at muscarinic receptors. This is in agreement with the results of Hubel (1984), who showed that secretory responses in the ileum mucosa produced by transmural electrical stimulation were sensitive to TTX but not atropine.

In summary, we find that ATP causes chloride secretion in the colon by an indirect mechanism, most likely involving neurones in the intramural plexus. It will be necessary to examine other secretagogues to discover if they too have indirect actions. Finally it will be important to identify the final effector neurotransmitter which either causes secretion or releases the epithelium from an inhibitory influence.

This work was supported by NIH grant HL 17705-10.

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Received 5 February 1985; revised 8 April 1985