Biochimica et Biophysica Acta 939 (1988) 335–342
Elsevier

BBA 73935

Activation of basolateral membrane K^+ permeability by bradykinin in MDCK cells

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(Received 14 September 1987)

Key words: Membrane permeability; Potassium ion channel; Bradykinin stimulation; Calcium ion dependence; Inositol trisphosphate

We study bradykinin-stimulated K^+ efflux in Madin-Darby canine kidney (MDCK) cells using ^{86}Rb as an isotopic tracer. Bradykinin brings about a rapid increase in the permeability of MDCK cells to K^+, the effect is dose-dependent with a plateau at 10^{-6} M. The effect seems to be mediated by Ca^{2+}-activated K^+ channels, localised at the basolateral aspect of the epithelium. Unlike α-receptors, which mediate a similar effect of adrenalin in these cells, bradykinin receptors seem to be present at both sides of the epithelium. Bradykinin increases the labelling of IP_3, and bradykinin-stimulated K^+ efflux persists even in cells which are bathed in Ca^{2+}-free medium, suggesting that the effects seen in the present work are probably due to Ca^{2+} release from intracellular stores. Some extracellular Ca^{2+} also might be involved in the bradykinin effect, consistent with the kinin-increasing membrane permeability to Ca^{2+}.

Introduction

The advantages of the use of established cell lines in studies of epithelial transport and its regulation are self-evident. One of the best characterised of these is the Madin-Darby canine kidney (MDCK) line. These cells were originally demonstrated to organise in vitro as a functional epithelium by Cereijido et al. [1] and Misfeldt et al. [2]. The extensive studies of Simmons and his co-workers (reviewed by Simmons et al. [3]) have revealed that MDCK cells can be a very good model of general Cl^- secretory epithelium. One of the important features of such epithelia is the presence of a basolateral Ca^{2+}-activated K^+ channel which is responsive to α-adrenergic stimulation and which is thought to mediate K^+ recycling across that membrane [4]. Recently, it has been shown that another agonist, bradykinin, can cause an increase in intracellular $[Ca^{2+}]$ in MDCK cells [5]. We have investigated whether such changes provoke an activation of K^+ channels similar to that of adrenalin. The results indicate that this is the case, but the origin of the Ca^{2+} mobilized by bradykinin effect seems to be different from that seen in adrenergic activation, as does the distribution of the receptors in the different membrane domains.

Abbreviations: MDCK, Madin-Darby canine kidney; IP_1, IP_2, IP_3, IP_4, inositol mono-, bis, tris and tetrakisphosphate, respectively. SEP-PAKs are prepacked anion-exchange cartridges.

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Materials and Methods

Materials

Inorganic salts were of Analar grade or equivalent. Hepes, sodium D-gluconate, adrenalin (bitartrate salt), bradykinin (triacetate salt), Ca^{2+} ionophore A23187, and quinine hydrochloride were all purchased from Sigma Chemical Co., Poole, U.K. ^{86}Rb as the Cl^- salt was obtained from Amersham International, U.K. ACCELL QMA anion-exchange SEP-PAKs were purchased from Waters Association, U.K.

Cell culture

Experiments were performed on MDCK cells at passages 68–77, grown in Dulbecco's modified Eagle's medium containing 10% foetal calf serum as described previously [6]. For K^+-efflux measurements in subconfluent monolayers [4] cells were seeded at a density of 300 cells/cm^2 in 35 mm-diameter wells of six-well culture trays (Nunc). Cells were fed every other day and used 5–6 days after seeding. Confluent monolayers on a permeable support were produced by seeding $2.0 \cdot 10^4$ cells/cm^2 on Millipore filters (30 mm Millicell-HA) used as inserts in six-well culture trays. Cells were used when confluent, as asessed on fixed and stained filters, 6–7 days after seeding. All cells used came as a kind gift from Dr. D.R. Tivey.

K^+-efflux measurements

It has been demonstrated that ^{86}Rb can be used in MDCK cells as a good tracer for potassium transport [7], $^{86}Rb^+$ has therefore been used routinely in the present work to measure K^+ efflux. Instantaneous rate constants for efflux (k in 10^2 min^{-1}) being measured according to the method of Brown and Simmons [4]. Briefly, cells were loaded with radioactivity by over-night incubation in the presence of 10 μCi/ml ^{86}Rb. Cells were briefly rinsed three times with ice-cold basic medium immediately before the experiment to remove extracellular ^{86}Rb (see below). Efflux was followed at 37 °C by replacing the bathing medium every 2 min and counting the radioactivity released from the cells. The modifiers were generally added after a 14 min control period and were kept in contact with the cells for 4 or 6 min. Cells were extracted with 0.5% Triton X-100 at the end of the experiment to assess the activity remaining in the cells. Because efflux rate will vary with the degree of confluency control, experiments were always run in cells grown in parallel. A similar approach was followed for cells grown to confluence on filters, with separate apical and basolateral samples being taken from either side of the filter. Radioactivity was measured in a liquid scintillation counter by the Cerenkov effect.

Composition of solutions

The basic medium (Hanks buffer) used throughout had the following composition (mM): NaCl 140, KCl 5, $CaCl_2$ 1.3, $MgCl_2$ 0.5, KH_2PO_4 0.44, K_2HPO_4 0.36, $NaHCO_3$ 4.2, glucose 5.5, Hepes 10; pH was adjusted to 7.2 with Tris. In experiments examining the effect of extracellular Ca^{2+}, $CaCl_2$ was omitted and 1 mM EGTA added. In solutions containing Ba^{2+}, all $NaHCO_3$ was replaced by equimolar concentrations of sodium D-gluconate.

Separation of inositol phosphates

This was performed in cells previously labelled with *myo*-[^{3}H]inositol. Cells grown on 35-mm-diameter wells of six-well trays were incubated in the presence of 10 μCi/ml *myo*[^{3}H]inositol for 48 h. The monolayers were washed with Hanks buffer and left for 15 s in the same buffer. Bradykinin (10^{-6} M) was then added, and incubation terminated after a timed interval by addition of 5% (v/v) perchloric acid. After storing for 5 min at 4 °C, the extract was neutralised by addition of 1.5 vol. of freshly prepared 1 : 1 freon/octylamine (v/v). The sample was centrifuged after vigorous vortexing and the upper phase recovered. After dilution with distilled water, inositol phosphates in samples were processed through ACCELL QMA anion-exchange SEP-PAK cartridges as described previously [8]. It was assumed that radioactivity eluting with ammonium formate/formic acid (pH 4.75) at 0.1, 0.2, 0.3 and 0.4 M in 5 mM disodium tetraborate corresponded to IP_1, IP_2, IP_3 and IP_4, respectively. Cartridges used were first checked with a [^{3}H]IP_3 standard to ensure that all loaded radioactivity eluted in the 0.3 M solution upon batch elution.

Ca^{2+}-electrode experiments

These were performed on cells suspended by

incubation with trypsin and EDTA and rendered permeable by incubation with saponin as described before [9]. Resuspended cells were incubated for 5 min at 37 °C in a medium containing 50 μg/ml saponin, washed and resuspended in a high K^+ low Na^+ buffer with no added Ca^{2+}. The solution in the electrode chamber had the following composition: 120 mM KCl, 6 mM $MgCl_2$, 1.2 mM KH_2PO_4, 5 mM potassium succinate, 5 mM Tris-ATP, 20 mM creatine phosphate, 0.5 mg · ml^{-1} bovine serum albumin, 50 μg · ml^{-1} creatine phosphokinase, and 25 mM Hepes (pH 7.2).

Results

Fig. 1 shows the fractional rate of ^{86}Rb efflux from subconfluent MDCK cells which had been previously loaded with the isotope. Addition of the Ca^{2+}-ionophore A23187 to the medium elicited a fast and transient 4-fold increase in K^+ efflux (from 2.9 ± 0.1 to 11.6 ± 0.5, mean ± S.D., $n = 3$). Addition of bradykinin at concentrations 10^{-8} to 10^{-6} M also provoked an increase in the rate of K^+ efflux which was dose-dependent. A K^+ channel activated by intracellular Ca^{2+} has been described in MDCK cells by Brown and Simmons [4]. This agent mediates the increased K^+ permeability provoked by the ionophore. To see whether the same channels are involved in the bradykinin-induced K^+ permeability, the effect of inhibitors on the bradykinin action was tested in the present work.

Fig. 2 shows the effect of quinine and Ba^{2+} on K^+ efflux from subconfluent monolayers of MDCK cells. The upper panel shows that both Ba^{2+} and quinine slightly depress the basal rate of

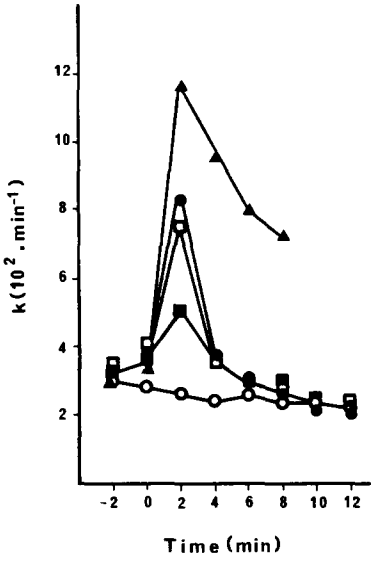

Fig. 1. Effect of bradykinin and Ca^{2+}-ionophore A23187 on the efflux of K^+ from subconfluent MDCK cells. The agents were added at time, 0 min. Symbol definition: ○ control with no addition; ■, □ and ● are 10^{-8}, 10^{-7} and 10^{-6} M bradykinin, respectively; ▲, $2 \cdot 10^{-5}$ M Ca^{2+}-ionophore A23187. Values shown are means of three independent experiments.

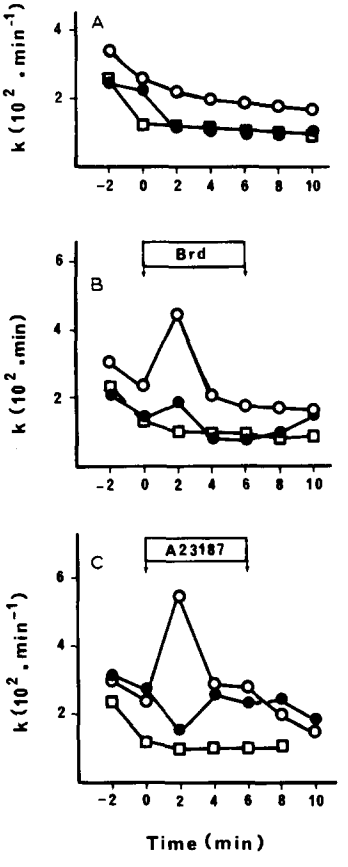

Fig. 2. Effect of channel blockers on bradykinin- and Ca^{2+}-ionophore-stimualted K^+-efflux from subconfluent MDCK cells. (A) shows control experiment and (B and C) show the effect of 10^{-6} M bradykinin and $2 \cdot 10^{-5}$ M A23187. ○ control solution; ●, solution containing 1 mM quinine; and □, solution containing 5 mM $BaCl_2$. Inhibitors were present from the beginning of the K^+-efflux measurement. Results are means of three measurements.

K^+ efflux. The bottom and middle panels show that the K^+-efflux stimulation elicited by bradykinin or by Ca^{2+}-ionophore is abolished by Ba^{2+} or quinine. These results suggest that the increase in K^+ permeability induced by bradykinin is due to the stimulation of Ca^{2+}-activated K^+ channels [4,7].

It has been demonstrated that Ca^{2+}-activated K^+ channels are located at the basolateral membrane of MDCK cells and presumably total K^+ efflux from subconfluent monolayers of cells should consist primarily of movement across basolateral membranes [4,7]. It is not clear however, that the channels activated by bradykinin are also present in the basolateral membrane nor which side of the epithelium possesses bradykinin

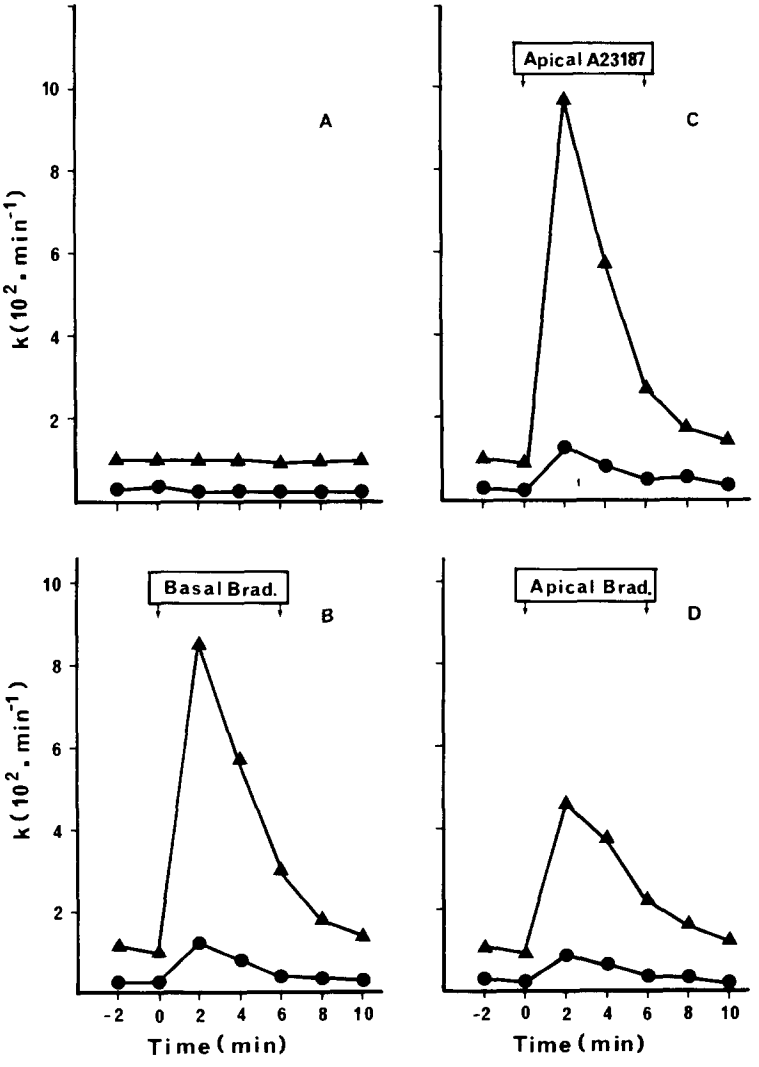

Fig. 3. Effects of A23187 and bradykinin on the efflux of K^+ from confluent monolayers of MDCK cells grown upon permeable support. ●, efflux across the apical membrane, and ▲ efflux across the basolateral membrane of the MDCK cells. At the times indicated, Ca^{2+}-ionophore ($2 \cdot 10^{-5}$ M) was added at the apical side of the monolayer (C). Bradykinin (10^{-6} M) was added either at the apical (D) or basal (B) side of the epithelium. Panel A shows data from experiments where no additions were made. Results are means of three separate experiments.

receptors mediating the effect. These two points have been investigated further using confluent monolayers grown upon permeable suppports.

Fig. 3A shows K^+ efflux from confluent monolayers of MDCK cells. Efflux across the basal side of the monolayer is seen to be 4-fold greater than that across the apical side. Addition of Ca^{2+}ionophore A23187 to the apical aspect of the epithelium elicited a 10-fold increase in the rate of K^+ efflux across the basal aspect of the monolayer. Efflux across the apical membrane was also increased, but the rate of efflux attained here was 8-times less than that across the basolateral side. Bradykinin increased K^+ efflux when added at either side of the epithelium. The effect seems greater, however, when added to the basolateral side. Under all circumstances the efflux was primarily through the basolateral side of the epithelium.

It has been previously demonstrated that bradykinin can induce an increase in intracellular free $[Ca^{2+}]$ from intracellular stores in MDCK cells [5]. If intracellular Ca^{2+} release was the only mechanism by which bradykinin increases K^+ permeability of MDCK cells, we would expect no effect of extracellular-Ca^{2+} removal on the augmented rate of K^+ efflux. Fig. 4 shows that the basal rate of efflux from a subconfluent monolayer of MDCK cells was slightly depressed when the bathing medium was made Ca^{2+} removal. Interpretation of this result is complicated by the depression in basal rate of K^+ efflux, but the contrast with adrenalin action, which is fully dependent on extracellular Ca^{2+} [4], is striking.

It is also shown in Fig. 4 that a second addition of bradykinin produces a much smaller effect even when a 12-min period had elapsed since the first exposure. This inactivation is more obvious in the absence of extracellular Ca^{2+}. The dependence of the inactivation on extracellular Ca^{2+} suggests that intracellular stores depleted by bradykinin action, have not been fully replenished before the second addition. That this might be the case is suggested by the results given in Table I showing Ca^{2+}-ionophore A23187 increases K^+ efflux after cells have been treated with bradykinin, but only if Ca^{2+} is present in the bathing medium. This is a consequence of first adding bradykinin as the ionophore is normally able to increase K^+ efflux in Ca^{2+} free solutions. Bradykinin is able to elicit an increased K^+ efflux after A23187 addition, provided that Ca^{2+} is present in the medium (Table I).

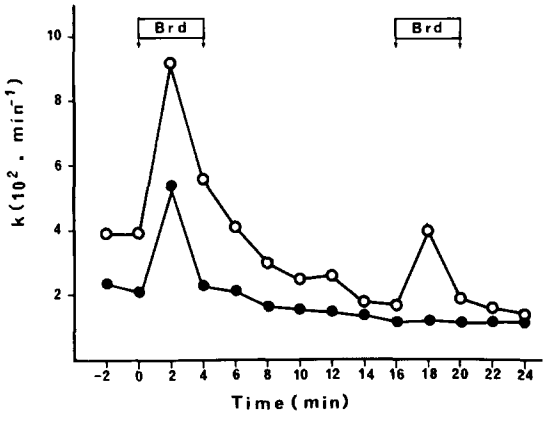

Fig. 4. Effect of Ca^{2+} removal on the bradykinin-stimulated K^+ efflux from MDCK cells. ○ results obtained under control conditions, and ●, data obtained in the absence of Ca^{2+} and presence of 1 mM EGTA. A 4 min exposure to bradykinin (10^{-6} M) was followed by a 12 min agonist-free incubation, after which a 10^{-6} M bradykinin was applied for a second time. Results are means of three separate measurements.

TABLE I

EFFECT OF BRADYKININ OR Ca^{2+}-IONOPHORE ON K^+ EFFLUX

Results are expressed as the ratio of fractional K^+ efflux during the first 2 min of agonist action (k_2) to K^+ efflux during the 2 min period before addition (k_0). The figures report the effect of successive additions (1st and 2nd addition, respectively) carried out on the same cells and separated by a period of 6 min without agonist or ionophore. Results are means ± S.D. of three separate experiments.

Experimental condition	k_2/k_0	
	1st addition	2nd addition
	10^{-6} bradykinin	$2.0 \cdot 10^{-5}$ M A23187
$+Ca^{2+}$	3.9 ± 0.4	3.5 ± 0.2
$-Ca^{2+}$ + 1 mM EGTA	2.4 ± 0.3	0.9 ± 0.1
	$2.0 \cdot 10^{-5}$ M A23187	10^{-6} M bradykinin
$+Ca^{2+}$	4.6 ± 0.15	2.0 ± 0.04
$-Ca^{2+}$ + 1 mM EGTA	2.2 ± 0.09	–

The underlying assumption made in the design of the above experiments is that the main effect of bradykinin is to increase intracellular Ca^{2+} by activation of phosphatidylinositol 4,5-bisphosphate hydrolysis and subsequent release of Ca^{2+} from intracellular stores [10]. The following two experiments show that in the particular strain of MDCK cells used here bradykinin stimulates polyphosphoinositol metabolism, and that IP_3 is able to evoke intracellular Ca^{2+} release.

Cells that had been previously labelled with [^{3}H]inositol were incubated in the presence of bradykinin and the production of water-soluble radioactive products was followed in the presence of LiCl. Table II shows that bradykinin evoked a detectable increase, possibly transient, of labelled IP_2 and IP_3 within 30 s of addition. A small increase was also noted in IP_1 and IP_4, but this was less marked for IP_4 than for the other inositol phosphates.

A continuous recording of free Ca^{2+} concentrations in a medium containing ATP and mimicking the intracellular composition is shown in Fig. 5. Addition of MDCK cells whose plasma membranes had been rendered highly permeable by incubation with saponin, led to a reduction in Ca^{2+} concentration to around $4.0 \cdot 10^{-7}$ M. This disappearance of Ca^{2+} from the medium, which is ATP-dependent and reversed by the Ca^{2+}-ionophore A23187, is similar to that observed in several other cell types [10–13], including transporting epithelia, and is believed to reflect sequestration into endoplasmic reticulum. Addition of 5 μM

TABLE II

EFFECT OF BRADYKININ ON THE CELLULAR CONTENT OF DIFFERENT INOSITOL PHOSPHATES IN MDCK CELLS

MDCK cells were labelled with *myo*-[^{3}H]inositol for 48 h and then washed before exposure to 10^{-6} M bradykinin for the times indicated. Different portions were prepared as described under 'Materials and Methods'. Data are expressed as means ± S.D. of three determinations, in counts per min (cpm). GPI; glycerophosphoinositol; IP_1, IP_2, IP_3, IP_4: inositol mono-, bis-, tris- and tetrakisphosphate, respectively. Except for GPI, all values are significantly different with respect to zero time ($P < 0.05$; two-tailed t-test of mean values).

Time (s)	0	20	30	60
GPI (cpm)	950 ± 24	940 ± 40	900 ± 24	969
IP_1 (cpm)	590 ± 36	820 ± 14	1130 ± 18	–
IP_2 (cpm)	210 ± 7	350 ± 12	500 ± 26	386 ± 4
IP_3 (cpm)	100 ± 7	181 ± 7	270 ± 23	203 ± 7
IP_4 (cpm)	77 ± 4	103 ± 5	122 ± 5	120 ± 12

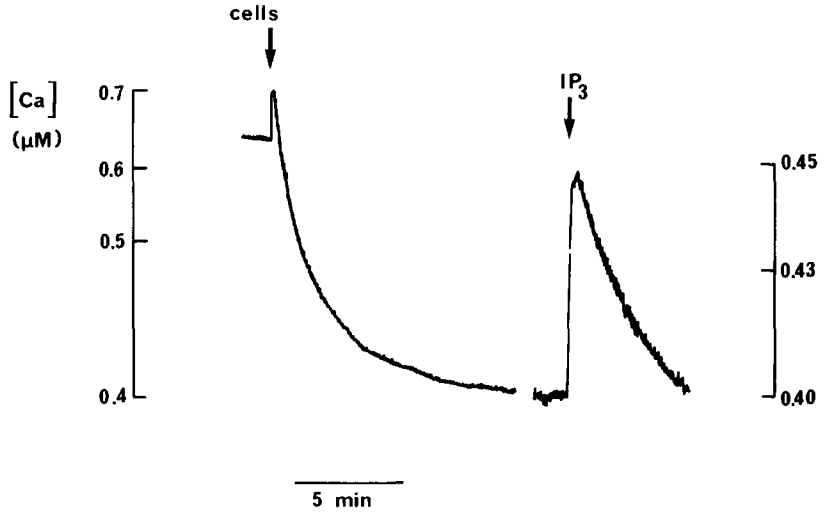

Fig. 5. Effect of IP_3 on the uptake of Ca^{2+} by permeabilised MDCK cells. Addition of MDCK cells previously incubated with 50 μg/ml saponin to the medium containing 5 mM ATP and mimicking the intracellular composition, is indicated by an arrow on the left of the figure. Addition of 5 μM IP_3 to the cells on the right. Notice a change in the gain of the recording at the gap. The trace shown is typical of four experiments.

inositol 1,4,5-trisphosphate induced a rapid release of Ca^{2+} followed by a slower reuptake to a level similar to that before addition.

Discussion

Confluent monolayers of MDCK cells respons to adrenergic stimulation by secreting Cl^- across the apical side of the epithelium [3]. As pointed out by Simmons et al. [14], this sort of response is more typical of the small intestine rather than the kidney, which is the tissue of origin of this line. A comparison of antigenic determinants, identified with monoclonal antibodies, also reveals more similarities of the MDCK cells with small-intestinal epithelium than with kidney tissue [3]. The membrane transport systems which are thought to be essential to Cl^- secretion are: a basolaterally located $Na/K/Cl_2$ cotransport accumulating Cl^- intracellularly, an apically located Cl^- conductance, and a basolaterally located K^+-permeability pathway allowing recycling of this cation [4,15,16].

We demonstrate here that bradykinin brings about an instantaneous increase in the permeability of MDCK cells to K^+. The effect is dose-dependent with a plateau being reached at about 10^{-7}–10^{-6} M. The effect seems to be mediated by Ca^{2+}-activated K^+ channels, as it can be blocked by Ba^{2+} and quinine, two agents which have been shown previously to inhibit these channels and which, in the present work, are shown to block the effects of Ca^{2+}-ionophore A23187. The cellular location of the effect also argues for a specific activation of Ca^{2+}-dependent K^+ channels [4,7], as experiments with confluent monolayers show the permeability pathway to be localised to the basal aspect of the epithelium. It is not possible to ascertain at present whether the small efflux observed across the apical aspect of the monolayer is genuine or due to leakage of basolateral K^+ across the cation-selective intercellular junctions.

It is interesting to note that, unlike α-adrenergic receptors which mediate a similar effect of adrenalin in these cells [4], bradykinin receptors seem to be present at both sides of the epithelium. A similar distribution has recently been observed for kinin receptors in a human colonic adenocarcinoma [17]. This is apparently at variance with what occurs in the small intestine where kinin is only effective from the basolateral side [18]. It must be stressed, however, that the mucosal surface of small intestine has a range of peptidases that could rapidly degrade any kinin presented to the apical aspect of the epithelium.

Pidikiti et al. [5] have already demonstrated an increase in intracellular $[Ca^{2+}]$ brought about by bradykinin in MDCK cells. The bradykinin effects seen in the present work are most probably brought about by a similar increase in Ca^{2+} mobilised from intracellular stores. This conclusion is based on the following pieces of evidence: bradykinin increases the production of IP_3 and other inositol phosphates in intact MDCK cells, suggesting receptor-mediated stimulation of polyphosphoinositide breakdown; IP_3 is shown to be able to release Ca^{2+} in MDCK cells permeabilised under conditions which preserve the intracellular organelles. The effect of bradykinin on K^+ efflux is initial present in cells that are bathed in Ca^{2+}-free solutions. Later inactivation of this effect seems to be due to depletion of intracellular Ca^{2+} stores. It is shown in Table I that the Ca^{2+}-ionophore is able to stimulate K^+ efflux in the absence of external Ca^{2+}. This can be interpreted on the basis of release of Ca^{2+} from intracellular stores. The fact that the ionophore is inactive if used after bradykinin stimulation in Ca^{2+}-free medium suggests that the peptide had depleted the intracellular stores.

We have observed that there is some difference between K^+ efflux in 1.3 mM Ca^{2+} medium and K^+ efflux in Ca^{2+}-free medium (Table I). Bradykinin-activated K^+ efflux is also greater in the presence of Ca^{2+} than in Ca^{2+}-free medium. This might imply that some extracellular Ca^{2+} is involved in the bradykinin effect. In this connection it is interesting to compare the effects of bradykinin on K^+ permeability of MDCK cells with that of adrenalin. Adrenergic stimulation occurring via α-adrenergic receptor activation seems completely dependent on extracellular Ca^{2+}. In fact our own recent experiments using Ni^{2+} as a blocker of plasma membrane Ca^{2+} channels indicate that while blocking completely adrenalin effects, Ni^{2+} has little effect on bradykinin-induced K^+ permeability [19]. This seems inconsistent with an α-adrenergic effect which is thought to occur via Ca^{2+} mobilisation [20]. Recently, it has been

postulated that one of the products of PIP_2 metabolism, namely IP_4, could serve as a second messenger gating plasma membrane Ca^{2+} channels [21]. If the adrenalin effect seen in MDCK cells is mediated by an α-adrenergic receptor, it might be of interest to explore whether it does indeed involve the production of inositol phosphates.

Acknowledgments

S.L.-B. was supported by a Fleming Award from the Spanish Ministerio de Educacion y Ciencia and the British Council. A.I. was supported by an Acciones Integradas grant of the British Council. K.A.W. gratefully acknowledges a fellowship from the Medical Research Council of Canada. We thank Drs. R.F. Irvine, M.W. Smith and D.R. Tivey for their comments on the manuscript.

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