Cellular mechanisms of adrenaline-induced hyperpolarization in renal epitheloid MDCK cells

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The effects of adrenaline on the potential difference across the cell membrane, on formation of inositol phosphates and on intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) were analysed in cells without or with pretreatment with pertussis toxin or phorbol 12-myristate 13-acetate (PMA). In untreated cells, adrenaline leads to a sustained hyperpolarization, a stimulation of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ formation and a transient increase in $[\text{Ca}^{2+}]_i$ from 78 ± 7 to 555 ± 43 nM, followed by a plateau of 260 ± 23 μM . In the absence of extracellular Ca^{2+} the effect of adrenaline on both potential difference and $[\text{Ca}^{2+}]_i$ is transient. In cells pretreated with pertussis toxin, the effects of adrenaline on InsP_3 and $[\text{Ca}^{2+}]_i$ are still preserved, but the effect on potential difference is transient. In cells pretreated with PMA, the effect of adrenaline on InsP_3 formation is severely decreased and that on $[\text{Ca}^{2+}]_i$ abolished, whereas a transient hyperpolarizing effect is still present. This transient hyperpolarization is abolished by additional pretreatment with pertussis toxin. The observations suggest that adrenaline hyperpolarizes the cell membrane of MDCK cells by several distinct mechanisms. First, adrenaline stimulates the formation of InsP_3 and InsP_4 , which at least in part accounts for the release of intracellular Ca^{2+} and the entry of Ca^{2+} from the extracellular fluid. Stimulation of phospholipase C is not mediated by pertussis-toxin-sensitive G-proteins, but apparently is inhibited by activation of protein kinase C. Second, adrenaline hyperpolarizes the cell membrane by a mechanism independent from increase in $[\text{Ca}^{2+}]_i$ which is sensitive to pertussis toxin but is, at least in part, insensitive to PMA.

INTRODUCTION

In Madin–Darby canine kidney (MDCK) cells, adrenaline is known to hyperpolarize the cell membrane [1] by activation of K^+ channels [2]. The hyperpolarizing effect of adrenaline is abolished in the presence of the α -blocker phentolamine, and is thus thought to be mediated by α -receptors [1]. Activation of β -receptors by isoprenaline leads to a slight depolarization of the cell membrane, an effect mimicked by cyclic AMP and probably elicited by activation of chloride channels [3]. In the absence of extracellular Ca^{2+} the hyperpolarizing effect is transient and can be elicited only once, and is thus thought to be mediated by increased $[\text{Ca}^{2+}]_i$. It has indeed been shown that adrenaline in MDCK cells is able to increase $[\text{Ca}^{2+}]_i$ [4] and InsP_3 formation [5]. The present study has been performed to elucidate further the intracellular mechanisms accounting for the adrenaline-induced hyperpolarization. To this end, the effects of adrenaline on the potential difference across the cell membrane, on the formation of inositol phosphates and on $[\text{Ca}^{2+}]_i$ have been analysed in cells without or with pretreatment with pertussis toxin and/or phorbol ester.

MATERIALS AND METHODS

Cell culture

MDCK cells from the American Type Culture Collection [6,7] were used from passages 80 to 110. Serial cultures were maintained in Dulbecco's modified Eagle's medium with 10% (v/v) fetal-calf serum, 100 units of penicillin/ml and 100 μg of streptomycin/ml, equilibrated with humidified air/ CO_2 (19:1) at 37 °C. After growth to confluency, monolayers were dispersed by incubation in a Ca^{2+} - and Mg^{2+} -free trypsin–EDTA-containing

balanced salt solution (pH 7.4) [8], plated on sterile cover-glasses, and incubated again in the same medium as above for at least 48 h. For performance of the experiments, cover-glasses with incompletely confluent cell layers were mounted in a perfusion chamber. A chamber volume of 0.1 ml and a perfusion rate of 20 ml/min allowed rapid fluid exchange. Where indicated, the cells were exposed before the experiment for more than 2 h to 1 mg of islet-activating protein/l from the bacterium *Bordetella pertussis* (pertussis toxin; Sigma, Munich, Germany), which ADP-ribosylates certain G-proteins [9], or for 15–30 min to 100 nM-phorbol 12-myristate 13-acetate (PMA; Sigma), which is known to stimulate protein kinase C [10,11].

Solutions and chemicals

The control bath perfusate was composed of (in mM): 114 NaCl, 5.4 KCl, 0.8 MgCl_2 , 1.2 CaCl_2 , 0.8 Na_2HPO_4 , 0.2 NaH_2PO_4 , 20 NaHCO_3 , 5.5 glucose. The solution was equilibrated (pH 7.4) with air/ CO_2 (19:1) and kept at 37 °C. In some experiments CaCl_2 was omitted and 1 mM-EGTA added; the Ca^{2+} activity of less than 100 nM was verified with a Ca^{2+} -selective electrode. Where indicated, adrenaline (Sigma) was added at a concentration of 1 μM .

Inositol phosphate measurements

Sub-confluent MDCK cells in 35 mm-diameter dishes were labelled for 48 h with *myo*-[2- ^3H]inositol (20 $\mu\text{Ci}/\text{ml}$) in Dulbecco's medium free of inositol, containing 2% dialysed fetal-calf serum. After the prelabelling period the medium was removed, and the cells were rinsed several times to remove free [^3H]inositol and incubated for an additional 1 h in fresh medium. After this procedure the cells were incubated in 1 ml of Dulbecco's medium with or without adrenaline for the indicated time periods.

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concn.; PMA, phorbol 12-myristate 13-acetate.

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Thereafter, the reaction was terminated by rapid aspiration of the medium and addition of 1 ml of 15% (w/v) trichloroacetic acid. For extraction of inositol phosphate, the dishes were put on ice for 1 h. The trichloroacetic acid was removed with diethyl ether. The final extract was neutralized and applied to anion-exchange columns containing 1 ml of Dowex 1-X8 (100–200 mesh, formate form; Serva, Heidelberg, Germany). Free inositol and the inositol phosphates were eluted sequentially in accordance with Berridge [12] as described previously [13]. In some experiments inositol phosphates were separated by anion-exchange h.p.l.c. on a Partisil 10 SAX column (Whatman, Maidstone, Kent, U.K.) with an ammonium formate gradient (0–1.7 M), as described by Irvine *et al.* [14]. The peaks of inositol, $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ were identified with the elution profile of pure standards obtained from Amersham International (Amersham, Bucks., U.K.). The nature of $\text{Ins}(1,3,4)\text{P}_3$ was presumed on the basis of its co-elution with ATP [15].

Fluorescence measurements

To load the cells with fura-2 or BCECF [2',7'-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein], sub-confluent cell layers were incubated for 45 min with 2.5 μM -fura-2-AM or for 15 min with 3 μM -BCECF-AM (Molecular Probes, Eugene, OR, U.S.A.). Measurements were made with an inverted phase-contrast microscope (IM-35; Zeiss, Oberkochen, Germany) equipped for epifluorescence and photometry (Hamamatsu, Herrsching, Germany) [16]. Light from a xenon arc lamp (XB075, Osram) was directed through a grey filter (nominal transmission 3.16%; Oriel, Darmstadt, Germany), a 340 nm or 490 nm interference filter (half-width 10 nm; Oriel) and a diaphragm, and was deflected by a dichroic mirror (FT425; Zeiss) into the objective (Plan-Neofluar 63 $\times$ oil immersion; Zeiss). The emitted fluorescence was directed through a 420 or 530 nm-cut-off filter to a photomultiplier tube (R4829; Hamamatsu). To decrease the region from which fluorescence was collected, a pinhole was made in the image plane of the phototube (limitation to a circular area of 60 μm diameter). $[\text{Ca}^{2+}]_i$ (in nM) was calculated from the observed fluorescence intensity of intracellular fura-2 (F) and the fluorescence intensity of Ca^{2+} -saturated fura-2 (F_{max}) [17,18], according to:

$$[\text{Ca}^{2+}]_i = 225[(F - 0.33F_{\text{max}})/(F_{\text{max}} - F)] \quad (1)$$

Fluorescence values were corrected for cellular autofluorescence; 225 nm is the apparent K_d for Ca^{2+} -fura-2 under cytoplasmic ionic conditions [19], and 0.33 F_{max} is the fluorescence intensity of Ca^{2+} -free fura-2 [19]. For determination of F_{max} , cells were exposed to 20 μM -digitonin (Sigma) in control perfusate.

In one series, the excitation wavelength was alternated between 340 nm and 380 nm, and the ratio of the respective fluorescence values was utilized to quantify the $[\text{Ca}^{2+}]_i$ measurements [19]. Intracellular pH was determined by using the dye BCECF, calibrated with the high- K^+ /nigericin technique [20].

Electrophysiological measurements

Measurements of the potential difference across the cell membrane were made with conventional micro-electrodes (tip diameter < 0.5 μm , resistance 100–200 M Ω , tip potential < 5 mV), back-filled with 1 M-KCl. The micro-electrodes were made by pulling filament-containing borosilicate tubes (outer diam. 1 mm, inner diam. 0.5 mm; Hilgenberg, Malsfeld, Germany) and connected to a high-input impedance electrometer (FD223; WPI, New Haven, CT, U.S.A.). Measurements were referenced to a grounded Ag/AgCl electrode connected with the bath via a flowing 3 M-KCl/Agar bridge. Impalements were made under an inverted phase-contrast microscope (Invertoscop ID; Zeiss), by using a piezostepper (PM 20 N, Frankenberger,

Germering, Germany), mounted on a Leitz micro-manipulator (Leitz, Wetzlar, Germany). Measurements were performed on a vibration-damped table. The potential differences were recorded on a chart recorder (BBC, Vienna, Austria).

Statistical analysis

The data are given as arithmetic means $\pm$ S.E.M.. Statistical analysis was made by paired *t* test, where applicable. Statistically significant differences were assumed at $P < 0.05$.

RESULTS

Formation of inositol phosphates

As shown in Fig. 1, adrenaline (1 μM) leads to a transient increase in formation of $\text{Ins}(1,4,5)\text{P}_3$ and to a more sustained increase in $\text{Ins}(1,3,4,5)\text{P}_4$. Fig. 2 gives a dose-response curve of adrenaline-stimulated (InsP_3) formation. Table 1

Fig. 1. Effect of adrenaline (1 μM) on formation of $\text{Ins}(1,4,5)\text{P}_3$ (●), $\text{Ins}(1,3,4,5)\text{P}_4$ (▲) and $\text{Ins}(1,3,4)\text{P}_3$ (■) ($n = 5$).

Fig. 2. Dose-response curve of the adrenaline-induced stimulation of InsP_3 formation.

The increase in InsP_3 within 20 s (as % of control value) is plotted versus adrenaline concentration. Results are arithmetic means $\pm$ S.E.M. for five experiments.

Table 1. Effect of adrenaline (ADR, 1 μ M) on inositol metabolites (c.p.m./ μ g of cellular protein; $n = 5$)

Abbreviations: GPI, glycerophosphoinositol. ^asignificantly different from respective value before addition of adrenaline; ^bsignificantly different from respective value without pretreatment.

Pretreatment of cells	Treatment	GPI	InsP ₁	InsP ₂	InsP ₃
None	0	172 $\pm$ 11	216 $\pm$ 14	40.0 $\pm$ 4.0	21.0 $\pm$ 1.3
	ADR 20 s	204 $\pm$ 9	317 $\pm$ 14 ^a	53.0 $\pm$ 2.9 ^a	31.4 $\pm$ 1.3 ^a
	ADR 60 s	234 $\pm$ 3 ^a	291 $\pm$ 10 ^a	55.5 $\pm$ 3.2 ^a	21.8 $\pm$ 0.9
Pertussis toxin	0	187 $\pm$ 12	212 $\pm$ 14	38.9 $\pm$ 2.3	24.3 $\pm$ 2.6
	ADR 20 s	248 $\pm$ 7 ^{a,b}	194 $\pm$ 13 ^b	63.0 $\pm$ 3.9 ^{a,b}	34.3 $\pm$ 2.3 ^a
	ADR 60 s	210 $\pm$ 4	250 $\pm$ 14 ^{a,b}	50.4 $\pm$ 2.6 ^{a,b}	18.1 $\pm$ 1.6
PMA	0	198 $\pm$ 13	218 $\pm$ 13	43.3 $\pm$ 1.5	17.5 $\pm$ 1.0
	ADR 20 s	182 $\pm$ 3 ^b	130 $\pm$ 6 ^{a,b}	38.3 $\pm$ 1.1 ^b	21.8 $\pm$ 1.0 ^{a,b}
	ADR 60 s	195 $\pm$ 7 ^b	110 $\pm$ 7 ^{a,b}	43.7 $\pm$ 4.7	15.6 $\pm$ 1.7 ^b

Fig. 3. Effect of adrenaline (ADR, 1 μ M; ■) on intracellular Ca^{2+} activity (Ca_i) in cells without pretreatment (Co), in cells pretreated with pertussis toxin (PT) and in cells pretreated with PMA

(a) Effect of adrenaline at normal (1.2 mM) extracellular Ca^{2+} concentration; (b) effect of adrenaline at extracellular Ca^{2+} concentration decreased to less than 0.1 μ M ('0 Ca^{2+} ', ▨). Redrawn original recordings are shown.

summarizes the radioactivity of various inositol phosphates before and after application of adrenaline in untreated cells, in cells pretreated with pertussis toxin and in cells pretreated with PMA. In untreated cells, adrenaline leads to significant increases in InsP₁, InsP₂ and InsP₃. In cells pretreated with pertussis toxin, adrenaline in the first 20 s still leads to a significant increase in InsP₂ and InsP₃. Compared with cells without pretreatment, the effect on glycerophosphoinositol is even significantly enhanced. In cells pretreated with PMA, however, the effect of adrenaline on either inositol metabolite is significantly decreased. The effect of adrenaline on InsP₁ is even reversed in PMA-treated cells.

[Ca^{2+}]_i

According to fluorescence measurements (Fig. 3), [Ca^{2+}]_i is 86 $\pm$ 6 nM ($n = 15$) in the absence of adrenaline. Addition of adrenaline (1 μ M) to extracellular fluid leads to a significant increase in [Ca^{2+}]_i from 78 $\pm$ 7 to 555 $\pm$ 43 nM ($n = 10$), followed

by a partial decline to 260 $\pm$ 23 nM ($n = 10$). Decreasing extracellular Ca^{2+} activity to less than 0.1 μ M decreases [Ca^{2+}]_i significantly from 100 $\pm$ 15 to 50 $\pm$ 6 nM ($n = 5$). Subsequent application of adrenaline leads to a significant transient increase in [Ca^{2+}]_i to 348 $\pm$ 38 nM ($n = 5$).

In cells pretreated with pertussis toxin, [Ca^{2+}]_i is 76 $\pm$ 10 nM ($n = 14$) in the absence of adrenaline. In these cells, addition of adrenaline (1 μ M) to extracellular fluid leads to a significant increase in [Ca^{2+}]_i from 60 $\pm$ 9 to 366 $\pm$ 66 nM ($n = 7$), followed by a partial decline to 165 $\pm$ 26 nM ($n = 5$). Decreasing extracellular Ca^{2+} to less than 0.1 μ M decreases [Ca^{2+}]_i significantly, from 94 $\pm$ 16 to 43 $\pm$ 9 nM ($n = 7$). Subsequent application of adrenaline leads to a significant transient increase in [Ca^{2+}]_i to 251 $\pm$ 63 nM ($n = 7$).

In cells pretreated with PMA, [Ca^{2+}]_i is 86 $\pm$ 9 nM ($n = 15$) in the absence of adrenaline. Addition of adrenaline (1 μ M) to extracellular fluid has no significant effect on [Ca^{2+}]_i, both in the

Fig. 4. Dose-dependence of intracellular Ca^{2+} release

Mean values $\pm$ S.E.M. ($n = 6$) are shown of the transient increase in the fluorescence ratio R and in $[\text{Ca}^{2+}]_i$ after a gradual increase in adrenaline concentration to 1 nM, from 1 to 10 nM, from 10 to 100 nM and from 100 to 1000 nM.

presence (from 92 ± 13 to 118 ± 16 nM; $n = 9$) and in the nominal absence (from 43 ± 15 to 43 ± 16 nM; $n = 6$) of extracellular Ca^{2+} . In cells pretreated with the inactive 4 α -phorbol or 4 α -phorbol 12,13-didecanoate, adrenaline (1 μM) increases $[\text{Ca}^{2+}]_i$ to 801 ± 89 ($n = 10$) and 517 ± 40 ($n = 7$) nM respectively.

Fig. 5. Effect of adrenaline (ADR, 1 μM ; ■) on the potential difference across the cell membrane (PD) in cells without pretreatment (Co), in cells pretreated with pertussis toxin (PT) and in cells pretreated with PMA

(a) Effect of adrenaline at normal (1.2 mM) extracellular Ca^{2+} concentration; (b) effect of adrenaline at extracellular Ca^{2+} concentration decreased to less than 0.1 μM (0 Ca^{2+} , ■). Original recordings are shown.

To elucidate the dose-dependence of intracellular Ca^{2+} release, the adrenaline concentration was subsequently increased from 1 to 1000 nM in the continued absence of extracellular Ca^{2+} (Fig. 4). Addition of 1 nM-adrenaline, as well as increasing adrenaline from 1 to 10 nM and from 10 to 100 nM, each led to a significant transient increase in $[\text{Ca}^{2+}]_i$. Increasing the adrenaline concentration from 100 nM to 1 μM , however, did not significantly increase $[\text{Ca}^{2+}]_i$. Thus 100 nM-adrenaline leads to a full recruitment of adrenaline-sensitive intracellular Ca^{2+} .

Intracellular pH

This is 7.18 ± 0.02 ($n = 7$) in untreated cells and 7.20 ± 0.02 ($n = 7$) in cells pretreated with phorbol ester. Adrenaline does not significantly alter intracellular pH in untreated (7.17 ± 0.02 ; $n = 7$) or phorbol-ester-pretreated (7.19 ± 0.02 ; $n = 7$) cells.

Cell membrane potential

In the absence of adrenaline, the potential difference across the cell membrane averages -51.4 ± 1.1 mV ($n = 35$). As shown in Fig. 5, acute administration of adrenaline (1 μM) leads to a sustained, but fully reversible, hyperpolarization of the cell membrane, by -20.6 ± 0.8 mV ($n = 35$). Decreasing extracellular Ca^{2+} activity below 0.1 μM depolarizes the cell membrane significantly, to -23.8 ± 5.7 mV ($n = 6$). During continued decrease in extracellular Ca^{2+} , adrenaline leads to a significant transient hyperpolarization of the cell membrane by -23.8 ± 3.3 mV ($n = 6$).

In cells pretreated with pertussis toxin, the potential difference across the cell membrane averages -58.8 ± 1.4 mV ($n = 20$). In these cells acute administration of adrenaline (1 μM) leads to a transient hyperpolarization of the cell membrane by -10.0 ± 1.0 mV ($n = 19$). Decreasing extracellular Ca^{2+} activity below 0.1 μM depolarizes the cell membrane significantly to -24.3 ± 3.7 mV ($n = 15$). During continued decrease in extracellular Ca^{2+} , adrenaline still leads to a significant transient

Fig. 6. Potential difference across the cell membrane (PD) of phorbol-ester-pretreated cells as a function of extracellular K^+ concentration in both the absence (●) and the presence (○) of 1 μM -adrenaline

Results are arithmetic means $\pm$ S.E.M. for n experiments.

Fig. 7. Dose-dependence of adrenaline-induced hyperpolarization in the absence of extracellular Ca^{2+}

Mean values $\pm$ S.E.M. ($n = 9$) are shown of the transient hyperpolarizations following a gradual increase in adrenaline concentration to 1 nM, from 1 to 10 nM, from 10 to 100 nM and from 100 to 1000 nM.

hyperpolarization of the cell membrane by -7.0 ± 2.0 mV ($n = 15$).

In cells pretreated with PMA, the potential difference across the cell membrane averages -44.8 ± 0.8 mV ($n = 28$). In these cells acute administration of adrenaline (1 μM) still leads to a hyperpolarization of the cell membrane by -21.4 ± 1.3 mV ($n = 28$), which is in some cells sustained ($n = 8$), but in others only transient ($n = 20$). This hyperpolarization is paralleled by an increase of K^+ selectivity (Fig. 6). The hyperpolarization cannot be blocked by 0.1 mM-ouabain (-21.4 ± 1.7 mV; $n = 13$). Decreasing extracellular Ca^{2+} activity below 0.1 μM depolarizes the cell membrane significantly to -19.0 ± 3.6 mV ($n = 8$). During continued decrease in extracellular Ca^{2+} , adrenaline still leads to a significant, transient, hyperpolarization of the cell membrane by -9.9 ± 3.8 mV ($n = 7$). If the cells are pretreated with both PMA and pertussis toxin, adrenaline has no significant effect on the potential difference across the cell membrane (-1.2 ± 1.7 mV; $n = 13$). To elucidate the dose-dependence of adrenaline-induced hyperpolarization in the absence of extracellular Ca^{2+} , the adrenaline concentration was subsequently increased from 1 to 1000 nM in the continued absence of extracellular Ca^{2+} (Fig. 7). Adding 1 nM-adrenaline, or increasing adrenaline concentration from 1 to 10 nM, did not significantly alter cell membrane potential. However, increasing the adrenaline concentration from 10 to 100 nM or from 100 to 1000 nM each led to a significant transient hyperpolarization.

DISCUSSION

The present study confirms the conclusions from electrophysiological studies [1] that adrenaline is able to increase intracellular Ca^{2+} activity in MDCK cells by recruitment of Ca^{2+} from both intracellular stores and extracellular fluid. The mobilization of Ca^{2+} from intracellular stores is evidenced by the transient increase in $[\text{Ca}^{2+}]_i$ at extracellular Ca^{2+} activities of less than 0.1 μM ('nominal absence of extracellular Ca^{2+} '). The recruitment of extracellular Ca^{2+} accounts for the sustained portion of increased intracellular Ca^{2+} activity, which is observed in the presence, but not in the nominal absence, of extracellular Ca^{2+} . In fact, an adrenaline-stimulated Ca^{2+} channel has been

identified in muscle fibres [21] and hippocampal neurons [22]. Adrenaline has been shown to increase $[\text{Ca}^{2+}]_i$ via α_1 -receptors in several other tissues (for review see [23]).

The release of intracellular Ca^{2+} by α_1 -agonists is most likely the result of stimulated $\text{Ins}(1,4,5)\text{P}_3$ formation [5]. An increased inositol phosphate turnover has been reported in several other tissues after the addition of α_1 -agonists (for review see [23]).

The activation of phospholipase C does apparently not require pertussis-toxin-sensitive G-proteins. On the other hand, pretreatment with pertussis toxin virtually abolishes the sustained portion of adrenaline-induced hyperpolarization. Thus pretreatment with pertussis toxin dissociates two distinct mechanisms by which adrenaline hyperpolarizes the cell membrane. In previous studies, both pertussis-toxin-sensitive and -insensitive effects of adrenaline have been reported: adrenaline binding and stimulation of phosphorylase in hepatocytes [24], as well as adrenaline binding and stimulation of phosphoinositide hydrolysis and prostaglandin E_2 formation in MDCK cells [25], were found to be insensitive to pretreatment with pertussis toxin. On the other hand, noradrenaline-induced inhibition of Ca^{2+} -channels in dorsal root ganglion neurons [26], adrenaline-induced inhibition of adenylate cyclase [27–31], inhibition of insulin secretion [32,33] and stimulation of phosphoinositide turnover [34] have been reported to be blocked by pertussis toxin.

Pretreatment of the cells with phorbol ester blocks the effect of adrenaline on formation of $\text{Ins}(1,4,5)\text{P}_3$. Thus the adrenaline-induced stimulation of phospholipase C is apparently inhibited by activation of protein kinase C. Similar observations have been made in other tissues [35–39].

Despite the inhibition of $\text{Ins}(1,4,5)\text{P}_3$ formation, and despite the complete abolition of the effect of adrenaline on the intracellular Ca^{2+} activity, adrenaline still elicits a hyperpolarization of the cell membrane in cells pretreated with phorbol ester.

Furthermore, the dose-response curves for adrenaline-induced increase in $[\text{Ca}^{2+}]_i$ and hyperpolarization in the absence of extracellular Ca^{2+} are clearly dissociated: in the absence of extracellular Ca^{2+} , 1 nM- and 10 nM-adrenaline lead to a significant increase in $[\text{Ca}^{2+}]_i$ without significant hyperpolarization. Only the marked increase in $[\text{Ca}^{2+}]_i$ after addition of 100 nM-adrenaline activates the K^+ channels sufficiently to produce a significant hyperpolarization. Increasing the adrenaline concentration from 100 nM, on the other hand, does not lead to a further appreciable release of intracellular Ca^{2+} , but leads to a marked additional hyperpolarization. This hyperpolarization is apparently not mediated by increase of intracellular Ca^{2+} . Obviously, very localized alterations of $[\text{Ca}^{2+}]_i$ in close vicinity to the K^+ channels may escape detection by the fura-2 fluorescence measurements. Such very localized release of intracellular Ca^{2+} has been claimed to occur in the parotid acinar cells under muscarinic stimulation [40]. Clearly, if such localized release of intracellular Ca^{2+} does occur, its regulation is distinct from the regulation of bulk Ca^{2+} release from intracellular stores. The seemingly Ca^{2+} -independent hyperpolarization is not the result of activated Na^+/K^+ -ATPase, which is known to enhance the K^+ conductance in other tissues [41]. Furthermore, the pH measurements rule out a possible role of intracellular alkalization, which enhances the K^+ conductance in MDCK cells [42] and has been observed upon stimulation of α_2 receptors in neuroblastoma $\times$ glioma cells [43,44]. However, additional pretreatment with pertussis toxin virtually abolishes this hyperpolarization. This observation points to G-protein-mediated activation of K^+ channels.

In conclusion, adrenaline hyperpolarizes the cell membrane of MDCK cells by at least three distinct intracellular mechanisms. First, adrenaline stimulates release of intracellular Ca^{2+} , probably by formation of $\text{Ins}(1,4,5)\text{P}_3$, a mechanism not mediated by

pertussis-toxin-sensitive G-proteins, but apparently inhibited by activation of protein kinase C. Secondly, adrenaline stimulates entry of Ca^{2+} from extracellular fluid, a mechanism again not mediated by pertussis-toxin-sensitive G-proteins, but apparently inhibited by activation of protein kinase C. Thirdly, adrenaline hyperpolarizes the cell membrane by a mechanism independent of increase in $[\text{Ca}^{2+}]_i$ by a mechanism sensitive to pertussis toxin.

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REFERENCES

- Paulmichl, M., Defregger, M. & Lang, F. (1986) *Pflügers Arch.* **406**, 367–371
- Kolb, H. A., Paulmichl, M. & Lang, F. (1987) *Pflügers Arch.* **408**, 584–591
- Lang, F., Defregger, M. & Paulmichl, M. (1986) *Pflügers Arch.* **407**, 158–162
- Breuer, W. V., Mack, E. & Rothstein, A. (1988) *Pflügers Arch.* **411**, 450–455
- Slivka, S. R. & Insel, P. A. (1987) *J. Biol. Chem.* **262**, 4200–4207
- Gstraunthaler, G. (1988) *Renal Physiol. Biochem.* **11**, 1–42
- Madin, S. H. & Darby, N. B. (1958) *American Type Culture Collection Catalogue of Strains* **2**, 574–576
- Simmons, N. L. (1981) *Biochim. Biophys. Acta* **646**, 243–250
- Katada, T. & Ui, M. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3129–3133
- Kikkawa, U. & Nishizuka, Y. (1986) *Annu. Rev. Cell. Biol.* **2**, 149–178
- Nishizuka, Y. (1988) *Nature (London)* **334**, 661–665
- Berridge, M. J. (1983) *Biochem. J.* **212**, 849–858
- Pfeilschifter, J., Kurtz, A. & Bauer, C. (1986) *Biochem. J.* **234**, 125–130
- Irvine, R. F., Letcher, A. J., Heslop, J. P. & Berridge, M. J. (1986) *Nature (London)* **320**, 631–634
- Irvine, R. F., Ånggård, E. E., Letcher, A. J. & Downes, C. P. (1985) *Biochem. J.* **229**, 505–511
- Almers, W. & Neher, E. (1985) *FEBS Lett.* **192**, 13–18
- Moolenaar, W. H., Tertoolen, L. G. J. & de Laat, S. W. (1984) *J. Biol. Chem.* **259**, 8066–8069
- Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) *J. Cell Biol.* **94**, 325–334
- Gryniewicz, G., Poenie, M. & Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Ganz, M. B., Boyarsky, G., Sterzel, R. B. & Boron, W. F. (1989) *Nature (London)* **337**, 648–651
- Arreola, J., Calvo, J., Garcia, M. C. & Sanchez, J. A. (1987) *J. Physiol. (London)* **393**, 307–330
- Gray, R. & Johnston, D. (1987) *Nature (London)* **327**, 620–622
- Exton, J. H. (1986) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **20**, 211–262
- Lynch, C. J., Prpic, V., Blackmore, P. F. & Exton, J. H. (1986) *Mol. Pharmacol.* **29**, 196–203
- Terman, B. I., Slivka, S. R., Hughes, R. J. & Insel, P. A. (1987) *Mol. Pharmacol.* **31**, 12–20
- Holz, G. G., IV, Rane, S. G. & Dunlap, K. (1986) *Nature (London)* **319**, 670–672
- Jeffries, W. B., Fallet, R., Gong, G., Van Dreal, P. & Pettinger, W. A. (1988) *Am. J. Physiol.* **255**, F1107–F1115
- Murphy, T. J. & Bylund, D. B. (1988) *J. Pharmacol. Exp. Ther.* **244**, 571–578
- Nomura, Y., Kawata, K., Kitamura, Y. & Watanabe, H. (1987) *Eur. J. Pharmacol.* **134**, 123–129
- Rapiejko, P. J., Northup, J. K., Evans, T., Brown, J. E. & Malbon, C. C. (1986) *Biochem. J.* **240**, 35–40
- Takaichi, K. & Kurokawa, K. (1988) *J. Clin. Invest.* **82**, 1437–1444
- Metz, S. A. (1988) *Diabetes* **37**, 65–73
- Ullrich, S. & Wollheim, C. B. (1988) *J. Biol. Chem.* **263**, 8615–8620
- Moreno, F. J., Mills, I., Garcia-Sainz, J. A. & Fain, J. N. (1983) *J. Biol. Chem.* **258**, 10938–10943
- Corvera, S., Schwarz, K. R., Graham, R. M. & Garcia-Sainz, J. A. (1986) *J. Biol. Chem.* **261**, 520–526
- Earp, H. S., Hepler, J. R., Petch, L. A., Miller, A., Berry, A. R., Harris, J., Raymond, V. W., McCune, B. K., Lee, L. W. & Grisham, J. W. (1988) *J. Biol. Chem.* **263**, 13868–13874
- Hepler, J. R., Earp, H. S. & Harden, T. K. (1988) *J. Biol. Chem.* **263**, 7610–7619
- Owen, N. E. (1986) *J. Cell Biol.* **103**, 2053–2060
- Rosenbaum, J. S., Azhar, S. & Hoffman, B. B. (1987) *Biochem. Pharmacol.* **36**, 4335–4340
- Foskett, J. K., Gunter-Smith, P. J., Melvin, J. E. & Turner, R. J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 167–171
- Lang, F., Messner, G. & Rehwald, W. (1986) *Am. J. Physiol.* **250**, F953–F962
- Paulmichl, M., Gstraunthaler, G. & Lang, F. (1985) *Pflügers Arch.* **405**, 102–107
- Baron, B. M. & Limbird, L. E. (1988) *Biochim. Biophys. Acta* **971**, 103–111
- Isom, L. L., Cragoe, E. J., Jr. & Limbird, L. E. (1987) *J. Biol. Chem.* **262**, 6750–6757

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