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Effect of BHT 920 on calcium-activated K^+ channels in renal epitheloid MDCK cells

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In Madin Darby canine kidney (MDCK) cells, epinephrine has been shown to increase intracellular calcium, activate calcium-dependent K^+ channels and hyperpolarize the cell membrane. The present study has been performed to test for the possible involvement of α_2-adrenergic receptors. To this end, the effects of α_2-adrenoceptor agonist BHT 920 have been studied on cell membrane potential, ion channel activity and intracellular calcium: Similar to epinephrine, BHT 920 hyperpolarizes the cell membrane, increases intracellular calcium and activates inwardly rectifying K^+ channels (single channel slope conductances 30–80 pS) Half-maximal hyperpolarization is achieved at concentrations between 10 and 100 nmol/l. The hyperpolarizing effect of BHT 920 is abolished in the presence of α_2-adrenoceptor antagonist yohimbine (100 nmol/l) but not in the presence of α_1-adrenoceptor antagonist prazosin (100 nmol/l). At extracellular calcium activity below 100 nmol/l BHT 920 still leads to a transient hyperpolarization of the cell membrane but, in contrast to epinephrine, is unable to significantly increase intracellular calcium or significantly activate the calcium-sensitive K^+ channels. The observations indicate that stimulation of α_2-receptors participates in the epinephrine-induced increase of intracellular calcium, channel activation and hyperpolarization.

Introduction

Chloride secretion across monolayers of Madin Darby canine kidney (MDCK) cells [1,2] is stimulated by epinephrine [3,4] in part by an increase of the K^+ conductance of the basolateral cell membrane. In subconfluent MDCK cells, epinephrine has indeed been shown to hyperpolarize the cell membrane [5] by activation of potassium channels [6]. Epinephrine increases the intracellular calcium concentration by both recruitment of intracellular calcium and stimulation of calcium entry across the cell membrane [7]. The hyperpolarizing effect of epinephrine is abolished in the presence of the α-adrenoceptor antagonist, phentolamine [5]. Activation of β-adrenoceptors by isoproterenol leads to a slight depolarization of the cell membrane, an effect mimicked by cAMP and probably elicited by activation of chloride channels [8]. The present study has been performed to elucidate the effect of the putative α_2-adrenoceptor agonist BHT 920 [9,10]. The experiments reveal that BHT 920, like epinephrine, is able to increase intracellular calcium, to activate K^+ channels and to hyperpolarize the cell membrane.

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Methods

Cell culture

MDCK cells from the American Type Culture Collection [1,2] were used from passage 70 to 100. Serial cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin, equilibrated with 95% humidified air and 5% carbon dioxide at 37 °C. After growing to confluency, monolayers were dispersed by incubation in a calcium- and magnesium-free, trypsin-EDTA containing, balanced salts solution (pH 7.4) [11], 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 into a perfusion chamber. A chamber volume of 0.1 ml and a perfusion rate of 20 ml/min allowed rapid fluid exchange.

Solutions and chemicals

The control bath perfusate was composed of (in mmol/l): 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 with 5% carbon dioxide and 95% air (pH 7.4) and kept at 37 °C. In some experiments $CaCl_2$ was omitted and 1 mmol/l EGTA added, the calcium activity of less than 100 nmol/l was verified using a calcium selective electrode. Where indicated, epinephrine, prazosin, yohimbine (Sigma, Munich, F.R.G.) or BHT 920 (kindly supplied by Beiersdorf, Hamburg, F.R.G.) were added at the concentrations as specified.

Electrophysiological measurements

Measurements of the potential difference across the cell membrane (PD) were made using conventional microelectrodes (tip diameter < 0.5 μm, resistance 100–200 MΩ, tip potential < 5 mV), back-filled with 1 M KCl. The microelectrodes were made by pulling filament-containing borosilicate tubes (o.d. 1 mm, i.d. 0.5 mm, Hilgenberg, Malsfeld, F.R.G.) and were connected with a high-input-impedance electrometer (FD223, WPI, New Haven, CT). 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, Oberkochen, F.R.G.), using a piezostepper (PM 20 N, Frankenberger, Germering, F.R.G.), mounted on a Leitz micromanipulator (Leitz, Wetzlar, F.R.G.). Measurements were performed on a vibration-damped table. The potential differences were recorded on a chart recorder (BBC, Vienna, Austria).

Patch-clamp experiments

Patch-clamp experiments were carried out according to the method of Sakmann and Neher [12]. Single channel current events were measured by means of an L/M-EPC-7 amplifier (LIST-Electronics, Darmstadt, F.R.G.), stored on a VHS-video-tape recorder (ELIN-6101, Vienna, Austria) via pulse code modulation (SONY PCM-501ES). The experiments were performed under cell-attached configuration. Outward current from the cytoplasm to the pipette is given as positive. For analysis the current records were played back through an eight-pole Bessel filter (model 902 LPF, Frequency Devices, Haverhill MA, U.S.A.) set at 0.85 kHz and digitized into an Olivetti M28 computer at a sampling rate of 0.5 ms (2 kHz) using a 12-bit A/D-Converter (DASH16, Metrabyte Corporation, Taunton, MA, U.S.A.) and stored on a 40 Mbyte hard disk.

The open probability was calculated from amplitude histograms according to the equation:

$$P_0 = \left(\sum_{n=1}^{N} (n \cdot t_n) \right) / N \qquad (2)$$

where t_n are the fractions of the observed time interval when n channels are open, and N is the maximal number of channels observed under maximal stimulation.

The selectivity of the channels for potassium has been estimated from the shift of the reversal potential (V_0), when part of KCl was replaced by NaCl in the pipette, described previously in detail [13]. In short, the apparent potassium transference number (t_K) of the channels, i.e., the contribution of potassium to the channel conductance has been estimated from:

$$V_0 = t_K \cdot E_K + t_{Na} \cdot E_{Na} + t_{Cl} \cdot E_{Cl}$$

where E_K, E_{Na} and E_{Cl} are the equilibrium potentials and t_K, t_{Na} and t_{Cl} the apparent transference numbers for potassium, sodium and chloride, respectively. The change of V_0 (dV_0), E_K (dE_K) and E_{Na} (dE_{Na}) following partial replacement of pipette potassium with sodium allow calculation of t_K:

$$t_K = dV_0/dE_K - t_{Na} \cdot dE_{Na}/dE_K - t_{Cl} \cdot dE_{Cl}/dE_K$$

dE_{Cl} is virtually 0, dE_{Na} is negative. Thus, if the terms $t_{Na} \cdot dE_{Na}/dE_K$ and $t_{Cl} \cdot dE_{Cl}/E_K$ are neglected, and intracellular potassium concentration is assumed to remain constant, the above equation yields a minimum estimate of t_K.

The pipette was filled with a solution composed of 10 mmol/l Hepes-KOH (pH 7.4), 45 μmol/l Phenol Red, and either 145 mmol/l KCl or 50 mmol/l KCl plus 95 mmol/l NaCl. As reference electrode, a 150 mmol/l KCl-agar bridge was used throughout and placed at the solution exit of the chamber.

Fluorescence measurements

To load the cells with fura-2, subconfluent cell layers were incubated for 45 min with 2.5 μmol/l fura-2/AM (Molecular Probes, Junction City, U.S.A.). Measurements were made under an inverted phase-contrast microscope (IM-35, Zeiss, F.R.G.) equipped for epifluorescence and photometry (Hamamatsu, Herrsching, F.R.G.) [14]. Light from a xenon arc lamp (XBO75, Osram) was directed through a grey-filter (nominal transmission 3.16%, Oriel, Darmstadt,

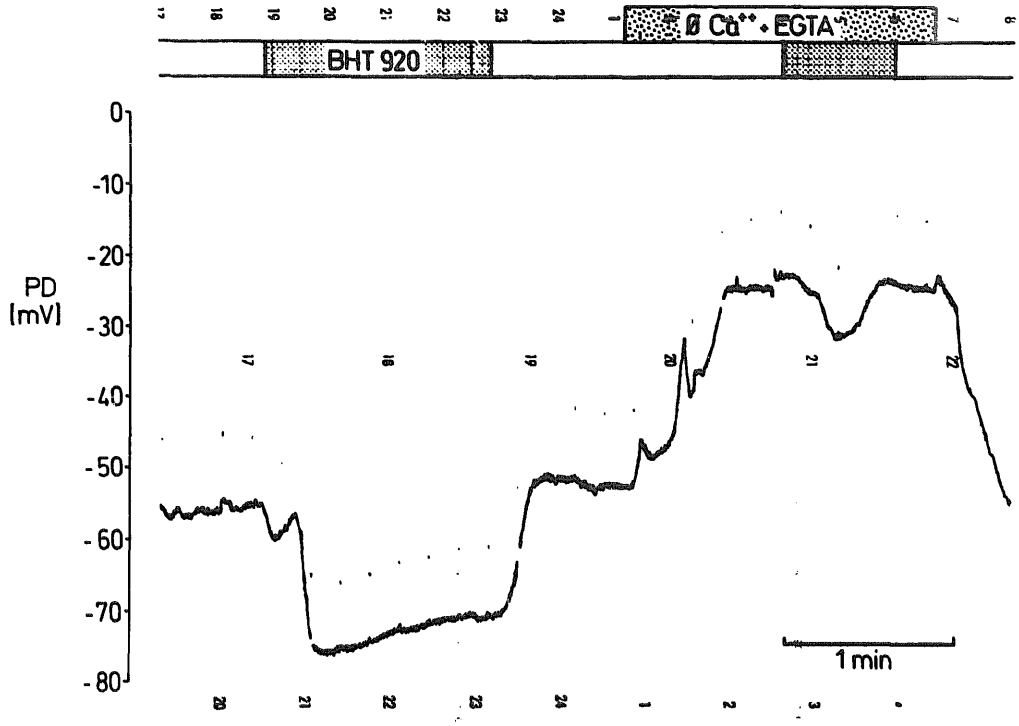

Fig. 1. Effect of BHT 920 (1 μmol/l) on the potential difference across the cell membrane (PD) in the presence and nominal absence (< 100 nmol/l) of extracellular calcium (original recording).

F.R.G.), a 340 nm interference filter (Halfwidth 10 nm, Oriel, Darmstadt, F.R.G.) and a diaphragm and was deflected by a dichroic mirror (FT425, Zeiss, F.R.G.) into the objective (Plan-Neofluar 63 × oil immersion, Zeiss, F.R.G.). The emitted fluorescence was directed through a 420 nm cut-off filter to a photomultiplier tube (R4829, Hamamatsu, Herrsching, F.R.G.). To reduce the region from which fluorescence was collected a pinhole was placed in the image plane of the phototubus (limitation to a circular area of 60 μm diameter). Intracellular calcium concentration in nmol/l ($[Ca^{2+}]_i$) was calculated from the observed fluorescence intensity of intracellular fura-2 (F), and the fluorescence intensity of calcium-saturated fura-2 (F_{max}) [15,16], according to:

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

Fluorescence values were corrected for cellular autofluorescence, 225 nmol/l is the apparent K_d for calcium-fura-2 at cytoplasmatic ionic conditions [17], and $0.33 \cdot F_{max}$ is the fluorescence intensity of the calcium free fura-2 [17]. For determination of F_{max} cells were exposed to 20 μmol/l digitonin (Sigma, Munich, F.R.G.) in control perfusate.

Statistical analysis

The data are given as arithmetic means ± S.E. of the mean. Statistical analysis was made by paired t-test, where applicable. Statistically significant differences were assumed at $P < 0.05$.

Results

Cell membrane potential

The potential difference across the cell membrane is −55.7 ± 1.7 mV ($n = 19$) in the absence of BHT 920. Addition of 1 μmol/l BHT 920 leads to a sustained

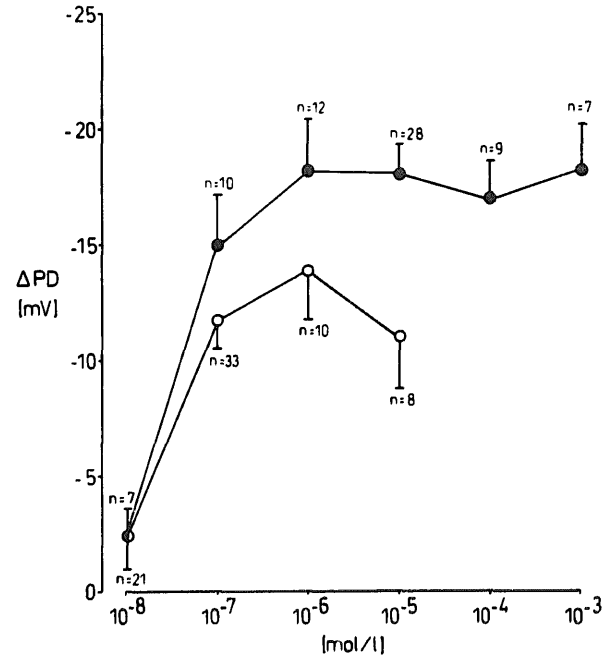

Fig. 2. Dose–response curve for BHT 920 (closed symbols) and for clonidine (open symbols). Hyperpolarization elicited by BHT 920 or clonidine as a function of the agonist concentration. Arithmetic means ± S.E., n = number of cells studied.

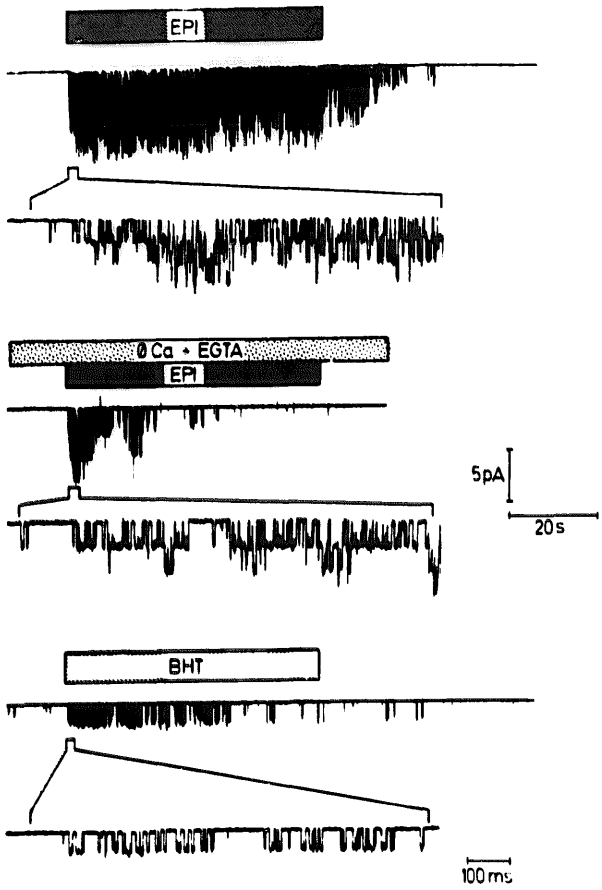

Fig. 3. Activation of ion channels in cell attached patches by epinephrine in the presence (upper panel) and nominal absence (< 100 nmol/l) of extracellular calcium (middle panel) as well as BHT 920 in the presence of extracellular calcium (lower panel). The experiments with epinephrine in the presence and absence of extracellular calcium have been performed subsequently in the same patch with 3 K^+ channels.

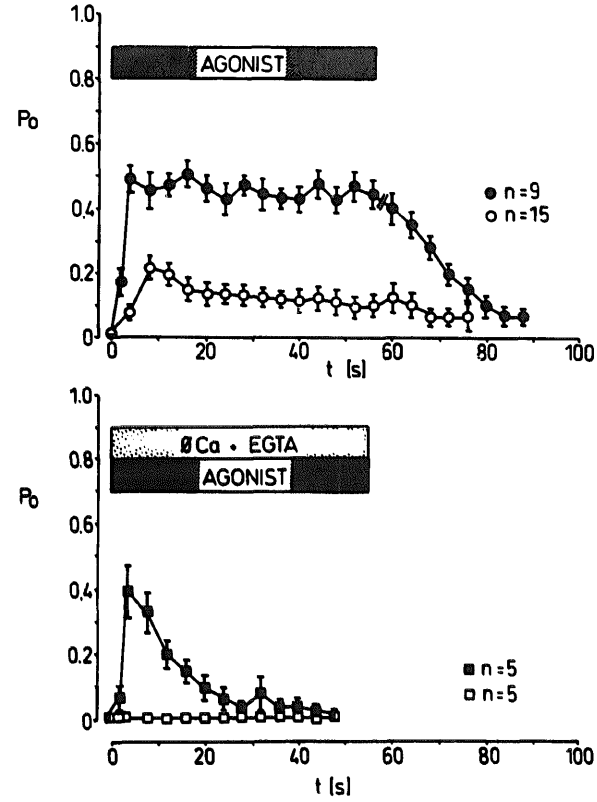

Fig. 4. Open probability of the epinephrine- (closed symbols) and BHT-920- (open symbols) activated ion channels in the presence (upper panel) and nominal absence (< 100 nmol/l) of extracellular calcium (lower panel). Arithmetic means ± S.E., n = number of patches studied.

hyperpolarization of the cell membrane (Fig. 1) by -18.3 ± 2.4 mV ($n = 12$). The dose-response curve (Fig. 2) indicates that the half-maximal effect is achieved at concentrations between 10 and 100 nmol/l.

A similar hyperpolarization as with BHT 920 is observed following application of clonidine, another α_2-adrenoceptor agonist (Fig. 2). The hyperpolarizing effect of BHT 920 is virtually abolished in the presence of 0.1 μmol/l α_2-adrenoceptor antagonist yohimbine (-0.9 ± 1.1 mV, $n = 10$), but is not significantly modified by 0.1 μmol/l α_1-adrenoceptor antagonist prazosin (-14.6 ± 1.7 mV, $n = 7$).

Reduction of extracellular calcium activity below 100 nmol/l depolarizes the cell membrane to -28.9 ± 3.2 mV ($n = 7$) and significantly blunts the hyperpolarizing effect of BHT 920 (-11.3 ± 3.1 mV, $n = 7$).

Ion channel activity

Both epinephrine (1 μmol/l) and BHT 920 (1 μmol/l) activate inwardly rectifying K^+ channels in cell attached patches (Figs. 3 and 4). The open probability for these channels is close to zero (0.02 ± 0.01, $n = 9$) prior to the addition of the agonists, but increases to 0.51 ± 0.04 ($n = 9$) following addition of epinephrine and to 0.21 ± 0.04 ($n = 15$) following addition of BHT 920. At an extracellular calcium activity reduced to less than 100 nmol/l, the activation of the ion channels by epinephrine is transient and that by

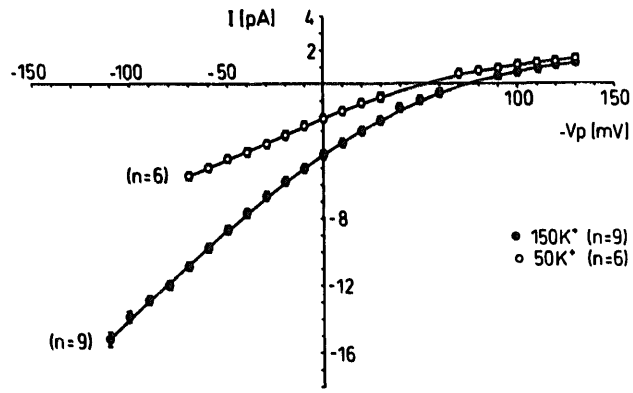

Fig. 5. Relation between single channel current (I) and the potential difference (V) between the pipette and the bath of the ion channels activated by epinephrine or BHT 920. Upper tracing at 50 mmol/l (open symbols), lower tracing at 150 mmol/l K^+ (closed symbols) in the pipette. The currents have been determined during the sustained hyperpolarization (−74 mV). Arithmetic means ± S.E., n = number of patches studied.

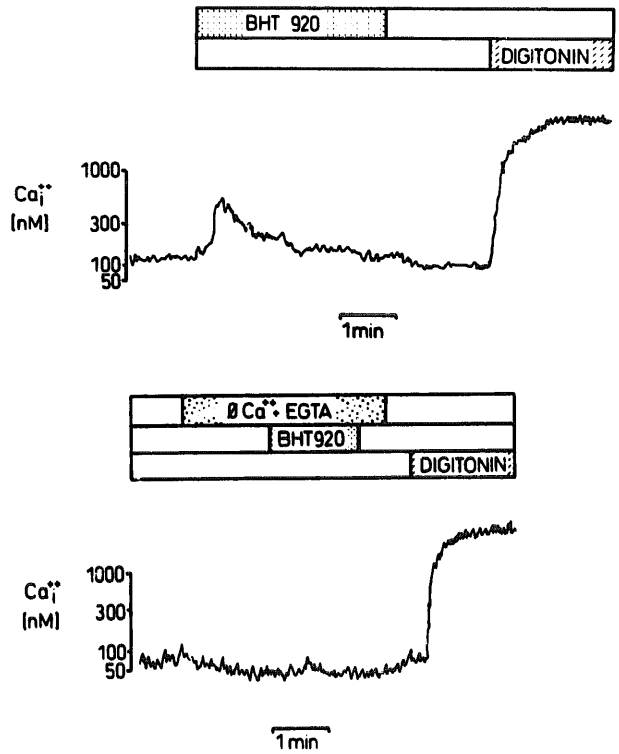

Fig. 6. Effect of BHT 920 (1 μmol/l) on intracellular calcium in the presence (upper panel) and nominal absence (< 100 nmol/l) of extracellular calcium (lower panel). Redrawn original tracings.

BHT 920 not detectable (Fig. 4). Fig. 5 gives the relation between single-channel current and potential difference between the pipette and the bath. The channels are inwardly rectifying with a single-channel slope conductance of 35 ± 1 pS at the reversal potential and of 79 ± 2 pS at zero potential between pipette and bath (150 K^+ in the pipette). The reversal potential is 74.1 ± 1.3 mV ($n = 9$, pipette-bath) at 150 mmol/l K^+ in the pipette, and 51.4 ± 1.8 mV ($n = 6$) at 50 mmol/l K^+ in the pipette. The shift of the reversal potential indicates that the channels are selective for potassium ($P_K/P_{Na} > 12$).

Subsequent addition of epinephrine and BHT 920 leads to the activation of the same channels in the same cell-attached patch. Thus, the two agonists indeed activate the same K^+ channels.

Intracellular calcium activity

BHT 920 increases intracellular calcium from 87 ± 11 nmol/l to 520 ± 133 nmol/l (Fig. 6). Reduction of extracellular calcium activity below 100 nmol/l leads to a decrease of intracellular calcium from 95 ± 2 nmol/l to 60 ± 6 nmol/l ($n = 8$). Subsequent application of BHT 920 (1 μmol/l) does not significantly increase intracellular calcium (66 ± 8 nmol/l, $n = 8$; Fig. 7).

Discussion

BHT 920, similar to clonidine, is known as α_2-adrenoceptor agonists [9,10]. The present study demonstrates that part of the effects of epinephrine [3–6,18] on MDCK cells are mimicked by BHT 920. Like epinephrine, the agonist increases intracellular calcium activity, activates calcium dependent K^+ channels and leads to a sustained hyperpolarization of the cell membrane. The effects of BHT 920 on the cell membrane potential are mimicked by clonidine, virtually abolished by α_2-adrenoceptor antagonist, yohimbine, but are not significantly modified by α_1-adrenoceptor antagonist, prazosin. Thus, α_2-receptors are likely to participate in the action of epinephrine on MDCK cells.

Under the conditions of this study (37 °C, bicarbonate present), we observed the activation of inwardly rectifying K^+ channels only, and occasionally the activation of a maxi K^+ channel [13,19,20,21].

As evident from Fig. 4, 1 μmol/l epinephrine increases the open probability of the calcium sensitive K^+ channels more than does 1 μmol/l BHT 920, even though BHT 920 exerts an almost maximal hyperpolarization at 1 μmol/l (Fig. 2). The difference may be due to additional activation of synergistic α_1-receptors by epinephrine. Unlike BHT 920, epinephrine leads to a significant increase of intracellular calcium activity in the absence of extracellular calcium [18]. Thus, the capacity of epinephrine to release intracellular calcium is apparently greater than that of BHT 920. Accordingly, BHT 920 is seemingly unable to activate the calcium-sensitive K^+ channels in the absence of extracellular calcium. On the other hand, BHT 920 still leads to a transient hyperpolarization of the cell membrane in the absence of extracellular calcium. This hyperpolarization could be due to a localized entry of calcium across the cell membrane escaping detection with the fura-2 fluorescence and activating K^+ channels not accessible to the patch-clamp pipette. Evidence for localized increases of intracellular calcium has been reported previously [22–24]. Alternatively, the hyperpolarization could be due to the activation of K^+ channels requiring a minimum level of, but not an increase in, intracellular calcium activity. In any case, the bulk hyperpolarization following application of BHT 920 results from activation of calcium sensitive K^+ channels. In conclusion, similar to epinephrine, BHT 920 hyperpolarizes the cell membrane at least in part by increase in intracellular calcium activity and subsequent activation of calcium-sensitive K^+ channels. Thus, α_2-adrenoceptors do participate in the effects of epinephrine on MDCK cells.

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References

1 Gstraunthaler, G. (1988) Renal Physiol. Biochem. 11, 1–42.
2 Madin, S.H. and Darby, N.B. (1958) As catalogued in: American Type Culture Collection Catalogue of strains. 2, 574–576.
3 Brown, C.D.A. and Simmons, N.L. (1981) Biochim. Biophys. Acta 649, 427–435.
4 Simmons, N.L., Brown, C.D.A. and Rugg, E.L. (1984) Fed. Proc. 43, 2225–2229.
5 Paulmichl, M., Defregger, M. and Lang, F. (1986) Pflügers Arch. 406, 367–371.
6 Kolb, H.A., Paulmichl, M. and Lang, F. (1987) Pflügers Arch. 408, 584–591.
7 Breuer, W.V., Mack, E. and Rothstein, A. (1988) Pflügers Arch. 411, 450–455.
8 Lang, F., Defregger, M. and Paulmichl, M. (1986) Pflügers Arch. 407, 158–162.
9 Kawasaki, H., Urabe, M. and Tasaki, K. (1989) Eur. J. Pharm. 164, 35–43.
10 Kobinger, W. and Pichler, L. (1980) Eur. J. Pharm. 65, 393–402.
11 Simmons, N.L. (1981) Biochim. Biophys. Acta 646, 243–250.
12 Sakmann, B. and Neher, E. (1983) Single channel recording Plenum, New York.
13 Friedrich, F., Weiss, H., Paulmichl, M. and Lang F. (1989) Am. J. Physiol. 256, C1016–C1021.
14 Almers, W. and Neher E. (1985) FEBS Lett. 192, 13–18.
15 Moolenaar, W.H., Tertoolen, L.G.J. and deLaat, S.W. (1984) J.Biol. Chem. 259, 8066–8069.
16 Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) J. Cell. Biol. 94, 325–334.
17 Grynkiewics, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
18 Pfeilschifter, J., Paulmichl, M., Wöll, E., Paulmichl, R. and Lang, F. (1991) Biochem. J. 274, 243–248.
19 Friedrich, F., Paulmichl, M., Kolb, H.A. and Lang F. (1988) J. Membr. Biol. 106, 149–155.
20 Lang, F., Friedrich, F., Paulmichl, M., Schoberberger, W., Jungwirth, A., Ritter, M., Steidl, M., Weiss, H., Wöll, E., Tschernko, E., Paulmichl, R. and Hallbrucker, C. (1990) Renal Physiol. Biochem. 13, 82–93.
21 Lang, F., Paulmichl, M., Pfeilschifter, J., Friedrich, F., Wöll, E., Waldegger S., Ritter M. and Tschernko E. (1991) Biochim. Biophys. Acta 1073, 600–608.
22 Foskett, J.K., Gunter-Smith, P.J., Melvin, J.E. and Turner, J. (1989) Proc. Natl. Acad. Sci. USA 86, 167–171.
23 Lang, F., Friedrich, F., Kahn, E., Wöll, E., Hammerer, M., Waldegger, S., Maly, K. and Grunicke H. (1991) J. Biol. Chem. 266, pp. 4938–4942.
24 Osypchuk, Y.V., Wakui, M., Yule D.I., Gallacher, D.V. and Petersen O.H. (1990) EMBO J. 9, 697–704.