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A FORSKOLIN AND VERAPAMIL SENSITIVE K^+ CURRENT IN HUMAN TRACHEAL CELLS

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A voltage-dependent K^+ current has been revealed in whole-cell recordings carried out on immortalized cells obtained from the human tracheal epithelium. At positive membrane potentials the current shows a time dependent inactivation which is accelerated by increasing the depolarizing step. Forskolin, a direct activator of adenylyl cyclase, and verapamil, a Ca^{2+} channel blocker, induce the K^+ current to inactivate more rapidly. Control experiments show that the action of these two compounds is not mediated by cyclic AMP and Ca^{2+}. The application of 1,9-dideoxyforskolin, an analogue which does not stimulate adenylate cyclase, inhibits the current in the same way as forskolin; on the contrary, the dibutyryl analogue of cyclic AMP is ineffective. Furthermore, eliminating extracellular Ca^{2+} does not affect K^+ current kinetics. Tetraethylammonium is an effective blocker of this current with an IC_{50} of 0.3 mM.

In recent years significant efforts have been devoted to development of epithelial cell lines for studies of specialized functions such as transepithelial ion transport. For this purpose, several cell lines have recently been obtained by transformation of human airway epithelium (1). These cells have been used to study Cl^- channels due to their relevance in cystic fibrosis (2). In particular, the line termed 9HTEo- has shown cAMP-dependent Cl^- channels (1) and volume-activated Cl^- currents (3) similar to those found in primary cultures of tracheal epithelial cells (4-6). In the present paper we further characterize the conductive properties of 9HTEo- cell membrane by decribing a new type of K^+ current which shows voltage dependent inactivation and inhibition by forskolin and verapamil. A similar current has not been previously described in epithelial cells.

METHODS

The 9HTEo- cell line is immortal and was established from the human tracheal epithelium as described by Gruenert et al. (1). Cells were cultured in 35 mm Petri dishes with a medium containing 45% Dulbecco's modified Eagle's medium, 45% Ham's F-12 medium, 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine. Experiments were carried out mainly on cells in the middle of confluent areas 1-7 days after cell plating.

Membrane currents were recorded with the whole-cell modification of the patch-clamp technique (7). The intracellular (pipette) solution was (in mM): 140 KCl, 0.18 $CaCl_2$, 1 $MgCl_2$, 2 EGTA, 1 Na_3ATP, 10 K-Hepes (pH=7.3; free Ca^{2+}=10 nM). Osmolality was adjusted to 290-300 mosmol/kg with mannitol.

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Abbreviations: ddFSK, 1,9-dideoxyforskolin; MPB-FSK, 7ß-desacetyl-7ß-[γ-(N-methyl-piperazino)-butyryl]-forskolin; TEA, tetraethylammonium.

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During whole-cell recordings the cell was superfused with a hypertonic Extracellular Standard Solution (ESS) which contained (in mM): 130 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 10 Na-Hepes (pH=7.3). This solution was made hypertonic (360 mosmol/Kg by addition of mannitol) in order to avoid spontaneous activation of volume-sensitive chloride currents (3). In ion selectivity studies 33-63 mM of K^+ or Rb^+ were applied by partially replacing NaCl and removing KCl in the ESS.

Membrane potentials are given taking the extracellular solution as ground. Positive or outward currents indicate cations flowing from the cytoplasmic to the extracellular side of the membrane. Data acquisition and analysis system have been described elsewhere (8). Experiments were performed at room temperature (22-24°C).

All chemicals were obtained from Sigma (Saint Louis, Missouri) with the exception of dd-FSK, MPB-FSK and verapamil which were purchased from Calbiochem (San Diego, California). Stock solutions were prepared by dissolving forskolin and ddFSK in DMSO at a concentration of 10 mM. 1% DMSO alone in the extracellular medium had no effect on K^+ currents. Verapamil and MPB-FSK were directly dissolved in the salt solution.

Data are presented as original recordings or as mean values ±SEM. Statistical significances were calculated using Student's *t* test.

RESULTS

As reported in Methods, whole-cell experiments have been carried out with hypertonic extracellular media in order to prevent activation of chloride currents (3). Under these conditions inward currents at negative membrane potentials remain low (40-90 pA at -80 mV). Instead, an outward transient K^+ current appears (72 out of 76 cells) as the membrane potential (V_m) is stepped to values > 0 (FIG.1A). The peak current at +80 mV had a mean value of 1.261±0.121 nA (n=36). Current activation is voltage-dependent: the rise time to peak is inversely related with the applied membrane potential. The inactivation process is also influenced by the applied membrane potential: in fact, after reaching the peak, the current decreases in a single exponential way as fast as the voltage pulse is depolarizing. For example, the inactivation time constant, which is obtained by fitting the current decay with an exponential function, is 131±9 and 51±3 ms at +20 and +80 mV respectively ($P<0.01$; n=16). At 0 mV the inactivation is very slow and requires seconds to reach the steady-state. Recovery from inactivation is obtained in 1-2 seconds by returning V_m to negative values (data not shown); therefore waiting time between voltage pulses was made longer (3-4 s) in order to avoid cumulative inactivation.

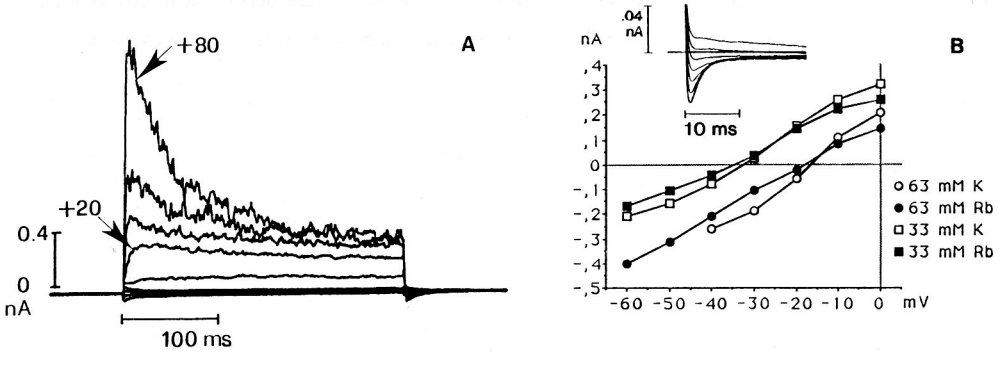

FIG.1- Whole-cell K^+ currents from a representative experiment. **(A)** Superimposed currents elicited by application of steps to membrane potentials in the range from -80 to +80 mV in increments of 20 mV. The holding potential is -50 mV. **(B)** K^+ and Rb^+ selectivity of the transient outward current. Tail currents (inset) were evoked by stepping the membrane potential to +80 mV for 10 ms and then returning to the various test potentials. The difference between the peak of the tail current and the current after 100 ms is plotted against the applied test potential at different extracellular concentrations (33-63 mM) of K^+ and Rb^+. Tail currents with 3 mM K^+ revert at -70 mV (not shown).

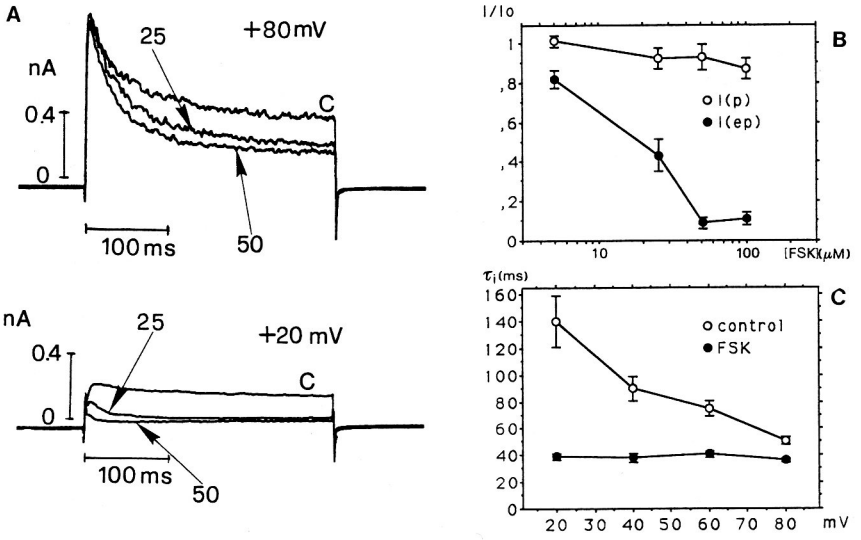

FIG.2- Effect of forskolin (FSK) on the transient K^+ current. **(A)** Samples of currents at two membrane potentials from a single experiment. Currents in control conditions (c) and with 25 or 50 μM forskolin (FSK) are indicated. **(B)** Effect of increasing concentrations of FSK on normalized peak I(p) and end pulse I(ep) currents. Data are shown as the ratio of the current with FSK to the current in its absence. Each point is the average of at least four experiments. FSK affects I(ep) more strongly than I(p). The difference is significant at the concentrations of 50 and 100 μM ($P<0.01$). **(C)** Voltage dependence of the inactivation time constant (τ_i) with and without 50 μM FSK. Data are mean values from six experiments. τ_i with forskolin is significantly smaller with respect to control ($P<0.01$).

The K^+ selectivity of the current has been assessed by measuring the reversal potential of tail currents at different extracellular K^+ and Rb^+ concentrations (FIG.1B). Tail currents reversed at -33.7±1.5 and -18±1.2 mV with 33 mM and 63 mM extracellular K^+ respectively (n=3). These values are slightly more positive than the expected Nernst potentials for K^+ (-37 and -21 mV). Reversal potentials with 33 mM and 63 mM extracellular Rb^+ are close to those obtained with K^+ (-36.1±1.0 and -21±2.8 mV; n=3). These data indicate that channels responsible for the transient outward current are not perfectly selective for K^+ over Na^+ ($P_K/P_{Na}=13.7$) and poorly discriminate between K^+ and Rb^+ ($P_K/P_{Rb}=1.1$).

The current sensitivity to various K^+ channel blockers in the external solution has been tested. TEA (n=21) is a strong blocker, with an apparent IC_{50} of 0.3 mM. Quinine is even more effective than TEA: at the concentration of 0.25 mM it inhibits K^+ currents by 67% (n=3). However, unlike TEA, quinine block was not totally reversible. Other blockers act at higher concentrations: Ba^{2+} (n=4) and 4-aminopyridine (n=4) reduce the current by 50% at 3 mM and 1 mM respectively. As for quinine, slow and incomplete recovery of current has been observed after washing out these two agents. Charibdotoxin at 10 nM (n=3) does not produce any effect.

We have found that the extracellular application of two not related compounds, the adenylyl cyclase activator forskolin (n=49) and the Ca^{2+} channel blocker verapamil (n=6), causes a strong and reversible inhibitory effect on K^+ currents. Both drugs act by increasing the apparent rate of inactivation. In fact, the currents measured at the end of the voltage pulse are more strongly affected than peak currents (FIG.2 and 3). This is particularly evident at the most positive potentials, i.e. +80 mV, where activation is fast. Instead, at lower potentials (0, +20 mV) the peak is also markedly reduced probably because the activation is slower and channels inactivate before the current has time to reach the maximal value. The graphics of FIG.2B and 3B

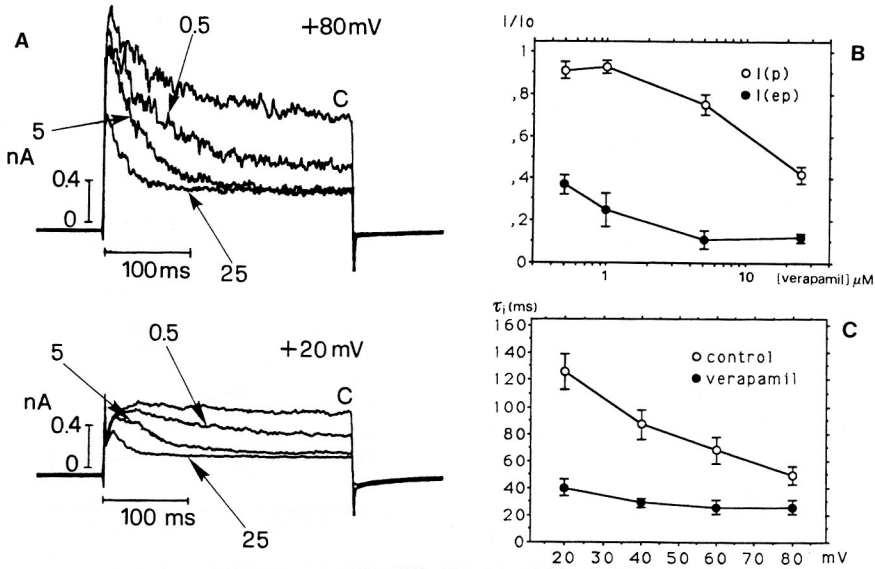

FIG.3- Effect of verapamil on the transient K^+ current. **(A)** Superimposed currents at two membrane potentials from a representative experiment. Increasing concentrations of verapamil (from 0.5 to 25 μM) cause progressive inhibition of K^+ current. **(B)** Normalized end pulse I(ep) and peak I(p) currents are plotted versus verapamil concentration. Each point is the average of four experiments. As forskolin, verapamil inhibits I(ep) more strongly than I(p) ($P<0.01$ at all concentrations). **(C)** Voltage dependence of the inactivation time constant (τ_i) with and without 5 μM verapamil in four experiments. τ_i with verapamil is significantly smaller with respect to control ($P<0.01$) at all membrane potentials but +80 mV.

show that verapamil is more potent than forskolin. For example, at 5 μM verapamil and forskolin reduce the end pulse current at+80 mV to 11±4% and 82±4% of the control respectively.

The effect of forskolin and verapamil on current inactivation is also shown by the behavior of the inactivation time constant (τ_i). In control conditions, τ_i is inversely related with the membrane potential whereas in the presence of these drugs (FIG.2C and 3C) it becomes significantly smaller and voltage independent.

Two forskolin analogues have been tested: MPB-FSK and ddFSK. These compounds also decrease K^+ current by increasing the inactivation rate (FIG.4). At 100 μM the hydrophylic MPB-FSK has the same effect of FSK (n=4): the peak at +80 mV is slightly reduced to 83±2% whereas the current at the end of the step is strongly blocked. At the same concentration ddFSK, which does not activate adenylyl cyclase (9), is more effective than forskolin and MPB-FSK in that the peak current is reduced by 56±1% (n=6). The ability of the dideoxi- analogue in blocking the K^+ current suggests that forskolin action is not mediated through the activation of adenylyl cyclase. This is confirmed by the fact that the external application of 1.0 mM dibutyryl-cAMP (n=3) does not change size and kinetics of the current (data not shown). Another evidence is that removal of ATP from the intracellular solution does not prevent forskolin from blocking the current.

A possible involvement of Ca^{2+} channels in the verapamil inhibition of K^+ currents has been investigated by replacing the ESS with another external medium containing 0 Ca^{2+} and 1 mM EGTA (data not shown). This intervention does not affect the current: this suggests that inhibition of K^+ currents by verapamil does not occurr through block of Ca^{2+} channels.

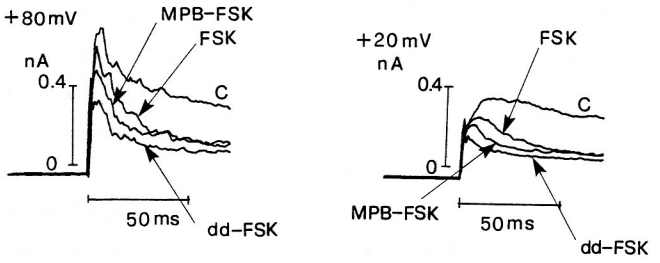

FIG.4- Comparison of the inhibitory effect of forskolin (FSK) with that of the analogues ddFSK and MPB-FSK at +80 and +20 mV. Drugs were applied at the concentration of 100 μM. Traces are from a representative experiment.

In 25 experiments, outside-out patches (7) have been obtained from the whole-cell configuration in order to reveal the channels responsible for the transient K^+ current. This search has been unsuccessful: K^+ channels with large conductance (>150-200 pS) have been observed but they do not show intrinsic inactivation or block by forskolin. This negative result might be due to a low density of forskolin-sensitive channels in the cell membrane. Alternatively, it is possible that these K^+ channels are located in the basolateral membrane where single channel recordings are not possible.

DISCUSSION

The present paper describes the biophysical and pharmacological characterization of a K^+ current in an immortalized epithelial cell line. Three main features characterize this current: a) the voltage-dependent inactivation which has not been observed in K^+ channels found in respiratory cells so far (5, 10, 11); b) the high sensitivity to TEA which makes the transient current described here different from the K^+ conductive pathways recently described in the tracheal epithelium (12); c) the block caused by forskolin and verapamil. K^+ currents inhibited by forskolin have been described in B and T lymphocytes (13, 14) and in pheochromocytoma (PC12) cells (15,16) but not in epithelial cells so far. Instead, the block of a K^+ channel by verapamil has been recently described in the thick ascending limb of Henle' s loop (17).

Our experiments are consistent with the findings for T lymphocytes and PC12 cells in that inhibition caused by forskolin was not mediated by the cAMP regulatory pathway (15, 16). Involvement of cAMP was reported in B lymphocytes (14).

A recent review (9) has underlined that forskolin affects the activity of various types of membrane proteins without mediation of cAMP. In addition to voltage-dependent K^+ channels, forskolin inhibits the glucose transporter (18) and the nicotinic acetylcholine receptor (19) independently of adenyl cyclase. In such cases the dideoxy derivative of forskolin is an useful probe for identifying the mechanisms which mediate forskolin effects. Since ddFSK does not bind to or activate adenylyl cyclase, its mode of action must be independent of cAMP. The inhibition caused by ddFSK in our experiments and the inactivity of dibutyrylcAMP, reinforce our conclusion that forskolin affects voltage-dependent K^+ currents of 9HTEo- cells in a cAMP-independent fashion. The effectiveness of the water-soluble MPB-FSK suggests that the hydrophobic properties of FSK are not important in its ability to inhibit K^+ currents.

Our observations show that forskolin and verapamil increase K^+ current inactivation and make it voltage-independent without the intervention of second messengers. A possible explanation of these effects is

that these drugs accelerate the intrinsic inactivation by allosterically affecting gating kinetics. Alternatively, it is possible that forskolin and verapamil act as blockers of the channel pore. In this case, to account for the observed effects, one should assume that these compounds enter the pore only when the channel is open and that kinetics of this process are slower than channel activation. A similar mechanism is reminiscent of the block of K^+ currents by quaternary ammonium ions in squid axon (20).

In conclusion, we have described a forskolin- and verapamil-sensitive K^+ current which is steadily expressed in the transformed human tracheal epithelial cell line, 9HTEo-. The biophysical and pharmacological characterization presented here will be useful in future experiments directed towards the understanding of the role played by this current in the native epithelium. The ability of forskolin, verapamil and their derivatives to affect physiological or pathological phenomena in airway epithelia could be a first indication of an involvement of these K^+ channels.

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