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Tetradecanoylphorbol acetate and terbutaline stimulate surfactant secretion in alveolar type II cells without changing the membrane potential

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Alveolar type II cells were isolated from adult rat lungs after tissue dissociation with elastase. The effect of known secretagogues on transmembrane potential was examined in freshly isolated cells (day 0 cells) and in cells after one day of primary culture (day 1 cells). Freshly isolated type II cells were incubated with 3,3′-dipentyloxacarbocyanine (di-O-C_5(3)) or 3,3′-dipropylthiadicarbocyanine (di-S-C_3(5)), dyes whose intracellular fluorescence intensity is a direct function of the cellular transmembrane potential. Fluorescence was continuously recorded by fluorescence spectrophotometry. Type II cells rapidly incorporated the dyes, and the addition of gramicidin (1 μg/ml) depolarized the cells as indicated by a change in fluorescence. Neither 12-*O*-tetradecanoylphorbol 13-acetate (TPA) nor terbutaline plus 3-isobutyl-1-methylxanthine (IBMX), which stimulate surfactant secretion from isolated alveolar type II cells, changed the transmembrane potential. The lipophilic cation triphenylmethylphosphonium ($TPMP^+$) was used to quantitate the transmembrane potential of type II cells cultured for one day. Addition of TPA or terbutaline plus IBMX induced surfactant secretion but did not alter the transmembrane potential. To study further the relationship of secretion to the transmembrane potential, secretion was also determined in the presence of high extracellular potassium which depolarizes the cells and in the presence of choline in place of sodium. High potassium enhanced the basal secretion of phosphatidylcholine from 1.8% to 3.4% ($P < 0.01$, $n = 7$). Substitution of sodium chloride by choline chloride had no effect on basal secretion but enhanced TPA-induced secretion ($P < 0.01$). We conclude that high extracellular potassium induces membrane depolarization and stimulates surfactant secretion, but TPA or terbutaline plus IBMX stimulates secretion without detectable membrane depolarization and stimulation of secretion by TPA does not require extracellular sodium.

Introduction

Alveolar type II cells synthesize and secrete pulmonary surface active material Surface active

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Abbreviations TPA, 12-*O*-tetradecanoylphorbol 13-acetate, di-S-C_3(5), 3,3′-dipropylthiadicarbocyanine, di-O-C_5(3), 3,3′-dipentyloxacarbocyanine, $TPMP^+$, triphenylmethylphosphonium ion, TPB^-, tetraphenylboron, CCCP, carbonyl cyanide *m*-chlorophenylhydrazone, IBMX, 3-isobutyl-1-methylxanthine

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material decreases the work of breathing and provides for alveolar stability 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) [1], beta-adrenergic agonists, agents which elevate intracellular levels of cyclic AMP [2–4], and the calcium ionophore A23187 [5] all stimulate surfactant secretion by type II cells in vitro These agonists are thought to involve a separate series of secondary intracellular chemical messengers, but it is possible that they all involve a common series of events such as protein phosphorylation or changes in the transmembrane potential In many cells changes in the transmembrane potential occur after pharmacologic stimulation, and membrane depolarization is thought to be related to a variety of cellular functions including exocytosis (for a review, see Ref 6) In lung slices high potassium has been reported to stimulate surfactant secretion and this effect is suggested to be caused by membrane depolarization [7] The resting membrane potential of isolated alveolar type II cells has been measured [8,9] but the relationship of changes in the transmembrane potential to surfactant secretion is not known In this report we measured both changes in membrane potential and surfactant secretion in isolated alveolar type II cells to clarify the relationship of these two events In addition, we measured basal and stimulated secretion in the presence of potassium chloride which depolarizes the cells and in the presence of choline chloride in place of sodium chloride to eliminate entrance of extracellular sodium upon stimulation of secretion

Experimental Procedures

Animals and materials Pathogen-free Sprague-Dawley rats (weighing 200–250 g) were obtained from Bantam-Kingman, Inc (Freemont, CA) The sources of materials used in this work were as follows [^{3}H]triphenylmethylphosphonium ion ($TPMP^+$) (40 Ci/mmol), [*Me*-^{3}H]choline chloride (80 Ci/mmol), ^3H_2O (100 mCi/g), and [^{14}C]sucrose (671 mCi/mmol) from New England Nuclear (Boston, MA), elastase (porcine pancreas) from Cooper Biomedical (Malvern, PA), 12-*O*-tetradecanoylphorbol 13-acetate (TPA) from Consolidated Midland Corporation (Brewster, NY), terbutaline sulfate from Merrell Dow Pharmaceuticals Inc (Cincinnati, OH), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), gramicidin, 3-isobutyl-1-methylxanthine (IBMX), quin2, quin2-acetoxymethyl ester (quin2/AM), triphenylmethylphosphonium bromide ($TPMP^+$), tetraphenylboron (TPB^-), and valinomycin from Sigma (St Louis, MO), and 3,3′-dipropylthiadicarbocyanine iodide (di-S-C_3(5)) from Molecular Probes, Inc (Junction City, OR) The fluorescent probe, 3,3′-dipentyloxacarbocyanine (di-O-C_5(3)), was provided by Dr Seiichi Kitagawa (Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine)

Isolation of alveolar type II cells Alveolar type II cells were isolated from adult male Sprague-Dawley rats by tissue dissociation with elastase and partially purified on metrizamide density gradients [5] The cells were further purified by centrifugal elutriation as described previously [10] for experiments using freshly isolated cells (day 0 cells) or by adherence in primary culture for 22 h (day 1 cells) After elutriation the cell purity determined by modified Papanicolaou stain [11] was $91 \pm 1\%$ and the cell viability determined by Trypan blue exclusion was $95 \pm 3\%$ ($n = 9$) The cell purity after 22 h in culture was $96 \pm 2\%$ and viability was $99 \pm 1\%$ ($n = 10$)

Changes in transmembrane potential in freshly isolated alveolar type II cells The changes in transmembrane potential of freshly isolated cells was measured by using the fluorescent carbocyanine dyes, 3,3′-dipentyloxacarbocyanine (di-O-C_5(3)) or 3,3′-dipropylthiadicarbocyanine (di-S-C_3(5)) These dyes were chosen because with depolarization the fluorescence of di-S-C_3(5) is increased whereas the fluorescence of di-O-C_5(3) is decreased Di-S-C_3(5) has been used to measure resting membrane potential of freshly isolated rat alveolar type II cells by Castranova et al [8] The fluorescence was measured with a Perkin-Elmer model MPF66 fluorescence spectrophotometer equipped with thermostatted cuvette holder (37 °C) and stirrer The excitation and emission wavelengths were set at 622 nm (5 nm slit) and 665 nm (5 nm slit) for di-S-C_3(5) and 460 nm (4 nm slit) and 510 nm (5 nm slit) for di-O-C_5(3) Freshly isolated and elutriated cells were suspended in Hepes-buffered saline (145 mM NaCl, 5 mM KCl, 10 mM Hepes-K, 0 5 mM $CaCl_2$, 0 4

mM $MgCl_2$, and 5 5 mM glucose (pH 7 4) NaCl-Hepes buffer) For calibration of the dyes the potassium concentration of this buffer was changed by the equimolar substitution of KCl for NaCl The cells were added to a 3-ml cuvette containing 1 57 μM di-S-C_3(5) or 0 25 μM di-O-C_5(3) to obtain a final volume of 3 ml The final cell concentration was 1 10^6 cells/ml The dyes were dissolved in ethanol to form stock solutions of 1 88 mM di-S-C_3(5) and 0 3 mM di-O-C_5(3) The final concentration of ethanol in the cuvette was 0 08% The cells were allowed to equilibrate until the fluorescence level was steady (usually 10 min), and then various agents were added and the change in fluorescence was measured The cell viability did not change during the incubation with the dyes, and greater than 90% of the cells excluded Trypan blue after the measurements

Secretion of phosphatidylcholine The cells recovered from metrizamide density gradient were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 μg/ml gentamicin, 100 U/ml penicillin, 50 μg/ml streptomycin, and 1 μCi/ml [*Me*-^{3}H]choline chloride at a concentration of 1 10^6 cells/ml The cells were routinely plated at 2 10^6 cells/35 mm dish After incubation in an atmosphere of 10% CO_2/90% air for 22 h at 37°C, the dishes were washed with 10 ml of DMEM containing 1 mg/ml bovine serum albumin to remove non-adherent cells and radioactive materials Each dish was held at tilted position and was washed by flooding the top of the dish with 10 ml of the wash solution from a syringe and simultaneously aspirating from the bottom of the dish The monolayers were further washed with 10 ml of NaCl-Hepes buffer (contents, see above), choline chloride-Hepes buffer (containing 145 mM choline chloride instead of NaCl), or KCl-Hepes buffer (containing 150 mM KCl and no NaCl) in the same manner After washing 3 ml of the designated solution was left in the dishes The final wash buffer was then replaced with 1 6 ml of the fresh buffer as indicated After equilibration for 10 min the cells were incubated with various agents for 1 h at 37°C in the air atmosphere The medium was removed, centrifuged at 1000 rpm ($260 \times g_{max}$) for 10 min to remove any detached cells, and the lipids were extracted by the method of Bligh and Dyer [12] Routinely, the radioactivity in the total lipid fraction was determined [13] As determined previously, 98 4% of the radioactivity in the total lipid fraction under these conditions is phosphatidylcholine [13] The cells were harvested from dishes with a rubber policeman and treated in the same manner as the media The percentage release of phosphatidylcholine is calculated as follows % secretion = (total lipid cpm in medium/total lipid cpm in cells + total lipid cpm in medium) $\times$ 100 A portion of media was assayed for lactate dehydrogenase, a cytoplasmic enzyme, to quantitate cytotoxicity [14] The release of lactate dehydrogenase did not exceed 3% of total cellular activity through all the experiments in this study

Changes in transmembrane potential in day 1 cells Although the fluorescent dyes have an advantage in that a continuous measurement of the membrane potential is possible, with cells on coverslips we were not able to get stable tracings and reproducible changes with agents which depolarized the cells, e g high potassium and valinomycin Therefore, we used lipophilic cation $TPMP^+$ for adherent cells which were also used in parallel for studies of secretion This compound has been used to measure resting membrane potential of freshly isolated rabbit alveolar type II cells [9] The cells recovered from the metrizamide density gradient were suspended in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 μg/ml gentamicin, 100 U/ml penicillin, and 50 μg/ml streptomycin at the concentration of 5 10^5 cells/ml The cells were plated at 5 10^5 cells/well in 24-well culture dishes After incubation for 22 h, the monolayers were washed sequentially with DMEM and with NaCl-Hepes buffer Then 0 5 ml of NaCl-Hepes buffer or KCl-Hepes buffer containing various concentrations of [^{3}H]$TPMP^+$ and TPB^- was added and cells were equilibrated at 37°C for a various periods of time as indicated The agents or solutions to be tested were added to the dish and incubation was continued At designated times the buffer was aspirated and monolayers were rapidly washed twice with 0 5 ml of the buffer which was the same as used in previous incubation except non-radioactive $TPMP^+$ was added instead of [^{3}H]$TPMP^+$ Finally the cell-associated radioac-

tivity was released into 0 5 ml of 1% Triton X-100 and counted The portion of radioactivity which was not associated with the cells was determined by adding [^{14}C]sucrose to the medium Approximately 0 1% of total radioactivity of the added [^{14}C]sucrose remained after washing and this value was used to correct the [^{3}H]TPMP^{+} values Typically 4% of radioactivity of the added [^{3}H]TPMP^{+} was associated with the cells after equilibration

Intracellular water volume of alveolar type II cells In order to calculate the intracellular oncentrations of TPMP^{+} and quin2, we needed to measure the intracellular volume The intracellular water volume of alveolar type II cells cultured for one day was determined essentially as described by Gallo et al [9] by using $^{3}H_2O$ and [^{14}C]sucrose Type II cells cultured for one day in 100 mm dishes (1 10^6 cells/ml, 16 ml per dish) were released with trypsin, centrifuged, and suspended in NaCl-Hepes buffer at 10 10^6 cells/ml The number of cells recovered was (8 3 ± 0 8) 10^6 cells/dish, and the viability was 96 ± 3% ($n = 4$) The cells were incubated with $^{3}H_2O$ or [^{14}C]-sucrose in NaCl-Hepes buffer for 15 min or 3 min, respectively The final cell concentration was 2 10^6 cells/ml A 0 5-ml aliquot of the cell suspension was layered upon 0 3 ml of an oil cushion (6 parts dibutylphthalate to 1 part mineral oil, v/v) in a 1 5 ml microcentrifuge tube The tubes were spun for 90 s in a Fisher 235B microcentrifuge An aliquot of the supernatant was saved to determine radioactivity The bottom of the tube containing cell pellet was cut off with a razor blade and added to 0 5 ml of 0 1 M NaOH After incubation overnight the radioactivity was determined The total volume of the pellet was determined from the $^{3}H_2O$ content and the extracellular space from the [^{14}C]sucrose content The intracellular water space was taken as the difference between total and extracellular volumes and the value was 0 37 ± 0 04 μl/10^6 cells ($n = 4$, independent cell preparations) This value was used to calculate intracellular concentration of TPMP^{+} and quin2, and we assumed that the intracellular water space of the cells attached to the dishes is equal to that of trypsinized cells

Quin2 loading and measurement of $[Ca^{2+}]_i$ Quin2 loading and determination of $[Ca^{2+}]_i$ was performed as described by Moolenaar et al [15] with the following modifications [16]. The isolated alveolar type II cells were plated in 35-mm culture dishes containing three 13-mm diameter glass cover slips. After 22 h, non-adherent cells were washed away and the alveolar type II cell monolayers were loaded with quin2 by incubating them in DMEM containing 50 μM quin2/AM at 37°C Stock solution of quin2/AM was made in dimethylsulfoxide at the concentration of 50 mM Quin2/AM was directly added to medium from the stock solution, and the quin2/AM containing medium was briefly sonicated before addition After 30 min for loading the cells with quin2/AM, the medium was changed to DMEM without quin2 and incubation was continued for additional 30 min. Hydrolysis of the quin2/AM was determined to be virtually complete at this time since the emission spectrum shifted from that of quin2/AM which has a peak at 430 nm to that of the free acid which has a peak at 490 nm [17] After washing, the quin2 loaded monolayer was inserted into a thermostatted cuvette in a Perkin-Elmer model MPF-66 fluorescence spectrophotometer Cells were incubated in 3 0 ml of NaCl-Hepes buffer Fluorescence was continuously recorded at an excitation wavelength of 339 nm (5 nm slit) and an emission wavelength of 490 nm (20 nm slit) Values of $[Ca^{2+}]_i$ (in nM) were calculated from the observed fluorescence intensity (F) of intracellular quin2 and the intensity of Ca^{2+}-saturated dye (F_{max}) according to the following equation [15]

$$[Ca^{2+}]_i = 115((F - 0\ 16F_{max})/(F_{max} - F))$$

where 115 nM is the apparent K_d for Ca^{2+}-quin2 at cytoplasmic ionic conditions [17], and 0 16 F_{max} is the fluorescence intensity of the Ca^{2+}-free quin2 anion [18] F_{max} was determined by rapidly saturating intracellular quin2 with Ca^{2+} by permeabilizing the cells with 20 μM digitonin The autofluorescence of type II cell monolayers was determined and subtracted from F and F_{max} in the calculation In general, the fluorescence for quin2 loaded monolayers was 4–5 times the autofluorescence of type II cells on a coverslip A slow spontaneous decrease in fluorescent emission of quin2 loaded cells was observed (12 ± 4% ($n = 7$) of total Ca^{2+} dependent fluorescence emission

after 5 min) due to photobleaching of the probe, and the fluorescence tracing was corrected accordingly for the estimation of $[Ca^{2+}]_i$ The cellular quin2 concentration was estimated with a quin2-free acid standard and was 1 1 ± 0 4 mM ($n = 3$)

Other Radioactivity was measured by Beckman liquid scintillation counter model LS 1801 Statistical analysis was performed with analysis of variance and Tukey's test for multiple comparisons with the SAS statistical program

Results

The change in transmembrane potential of freshly isolated alveolar type II cells was measured by using the cationic fluorescent dyes, di-S-$C_3(5)$ or di-O-$C_5(3)$ When the cells were incubated with these compounds, they rapidly incorporated the dyes and reached a steady state level within 10 min An increase in fluorescence of di-S-$C_3(5)$ reflects an apparent depolarization and a decrease in fluorescence an apparent hyperpolarization, whereas an increase in fluorescence of di-O-$C_5(3)$ reflects an apparent hyperpolarization and a decrease in fluorescence a depolarization In order to verify that fluorescence of both dyes was a valid indication of changes in transmembrane potential in alveolar type II cells, we determined the applicability of the Goldman equation to this system, as shown for erythrocytes by Sims et al [19] Alveolar type II cells were equilibrated in Hepes-buffered salt solutions of identical concentrations of all components except Na^+ and K^+ which were varied so that the sum of their concentrations remained constant Then the change in fluorescence was measured when valinomycin (1 μM) was added to suspension in order to increase the membrane permeability to K^+ and to eliminate the K^+ gradient across the membrane The resulting change in the membrane potential was a function of the K^+ gradient before valinomycin was added and therefore of the external K^+ concentration As shown in Fig 1, except at very high concentrations (150 mM), the observed values follow the expected linear dependence of transmembrane potential on $\log[K^+]_{out}$ Therefore, the fluorescence changes observed for di-S-$C_3(5)$ or di-O-$C_5(3)$ can measure a change in transmembrane potential in alveolar type II cells

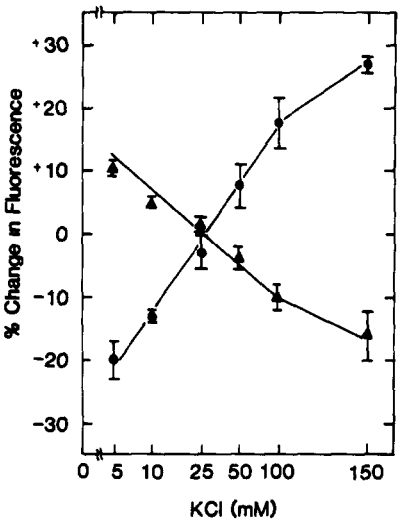

Fig 1 Effect of external potassium concentration on the relative change of the dye fluorescence upon addition of valinomycin Freshly isolated type II cells were incubated with 1 57 μM di-S-$C_3(5)$ or 0 25 μM di-O-$C_5(3)$ for 10 min at 37 ° C in different concentrations of external potassium, then valinomycin (1 μM) was added, and the change of fluorescence was recorded Each point represents the mean ± S E from three different experiments The symbols are ● for di-S-$C_3(5)$ and ▲ for di-O-$C_5(3)$

In the next set of experiments, the effect of stimulants of pulmonary surfactant secretion on the fluorescence of di-S-$C_3(5)$ or di-O-$C_5(3)$ was examined A concentration of TPA (50 ng/ml), which causes a marked secretory response, did not change the transmembrane potential whereas gramicidin (1 μg/ml) depolarized alveolar type II cells (Fig 2) In addition, terbutaline (100 μM) plus IBMX (50 μm) which also stimulates secretion had no effect on the fluorescence (data not shown) Castranova et al [8] reported that 10 μg/ml of TPA depolarized freshly isolated rat alveolar type II cells This concentration is extremely high for biologic effects in type II cells or in other mammalian cells In our laboratory, near maximal stimulation of surfactant secretion by TPA is observed at the concentration of 25 ng/ml Moreover, even when we added 10 μg/ml of TPA to freshly isolated type II cells loaded with di-S-$C_3(5)$, we could not find any change of fluorescence

We have used cells after one day in culture to study secretion [2–4,13] Therefore, we analyzed

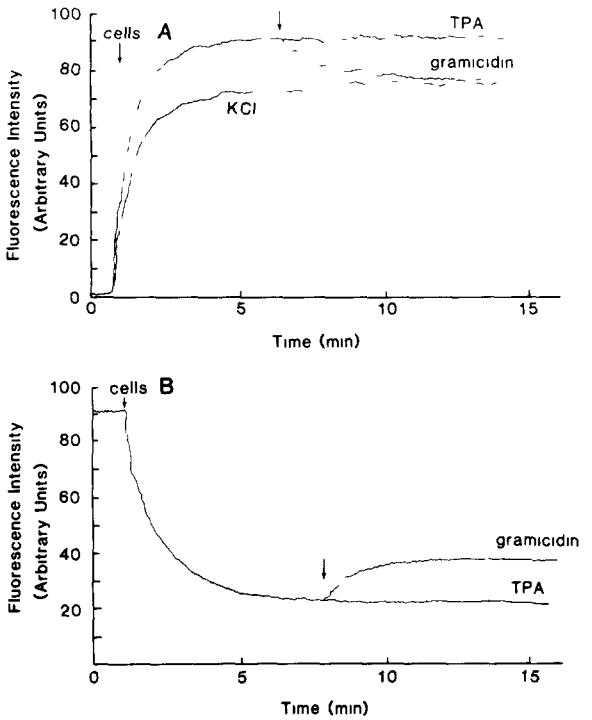

Fig 2 Transmembrane potential changes in freshly isolated alveolar type II cells Transmembrane potential changes were measured by the fluorescence changes of di-O-C_5(3) (A) or di-S-C_3(5) (B) as described under Experimental Procedures Gramicidin (1 μg/ml) or TPA (50 ng/ml) was added at the time indicated by the arrow The cellular fluorescence of di-O-C_5(3) was also measured in the presence of high potassium (150 mM) (A) The data represent three different experiments with similar findings The fluorescence of control cells remains stable at least 30 min after equilibration

the changes in transmembrane potential with day 1 cells in order to compare to the secretory response directly Neither we nor others have reported the secretory response of freshly isolated alveolar type II cells, because the cells are routinely incubated with a radioactive lipid precursor to radiolabel phosphatidylcholine We used the cationic lipophilic probe, $TPMP^+$, to measure transmembrane potential in day 1 cells $TPMP^+$ has been shown to be a permanent cation across cell membranes [19], and its equilibrium distribution has been demonstrated to be dependent on the transmembrane potential in a variety of systems including alveolar type II cells [9] Therefore, by estimating the transmembrane concentration gradient of $TPMP^+$, the changes in transmembrane potential can be calculated simply from the Nernst equation as described by Cheng et al without direct measurement of intracellular volume

$$E_m = 58 \log[TPMP^+]_{experimental}/[TPMP^+]_{control}$$

where $[TPMP^+]_{experimental}$ and $[TPMP^+]_{control}$ are the cell-associated amount of the hydrophobic cation in the experimental or resting condition, respectively The rate of uptake of $[^3H]TPMP^+$ by alveolar type II cells cultured for one day was determined When type II cells attached to the dish were incubated with 50 nM $[^3H]TPMP^+$, the cells gradually incorporated the probe, but equilibrium was not reached even after 5 h of incubation (Fig 3) Addition of TPB^- accelerated the uptake of $TPMP^+$ by type II cells (Fig 3) as reported in other systems including type II cells [9,21,22]. Upon addition of 2 μM of TPB^- the maximum accumulation of $TPMP^+$ was observed at 2 h and remained constant for at least addition 3 h (Fig 3) The distribution ratio of $TPMP^+$, $[TPMP^+]_{out}/[TPMP^+]_{cell\ associated}$ did not vary from the concentration of 10 nM to 400 nM of $TPMP^+$ in the presence of 2 μM TPB^- Therefore, the subsequent studies were performed with preincubation time of 2 h with 50 nM $[^3H]TPMP^+$ (2 μCi/ml) and 2 μM TPB^- This procedure did not affect basal secretion or secretion stimulated by TPA or terbutaline plus IBMX In addition, $TPMP^+$ and TPB^- did not cause cell damage, as assessed by trypan blue exclusion and lactate dehydrogenase release To determine the changes in transmembrane potential by using $[^3H]TPMP^+$ one must also measure the potential-independent uptake of this probe by type II cells To obtain this value we measured uptake in the presence of 150 mM KCl and 1 μM valinomycin, which should cause complete depolarization After equilibration with 50 nM $[^3H]TPMP^+$ and 2 μM TPB^- for 2 h, cells incorporated $2\,46 \pm 0\,24$ pmol/10^6 cells of $[^3H]TPMP^+$, and cells incubated with 150 mM KCl plus 1 μM valinomycin incorporated $0\,75 \pm 0\,07$ pmol/10^6 cells in 10 min at 37 °C (mean ± S E, $n = 9$ different experiments), which indicates some potential-independent accumulation of the probe The value of cell associated $TPMP^+$ described below has been corrected for this poten-

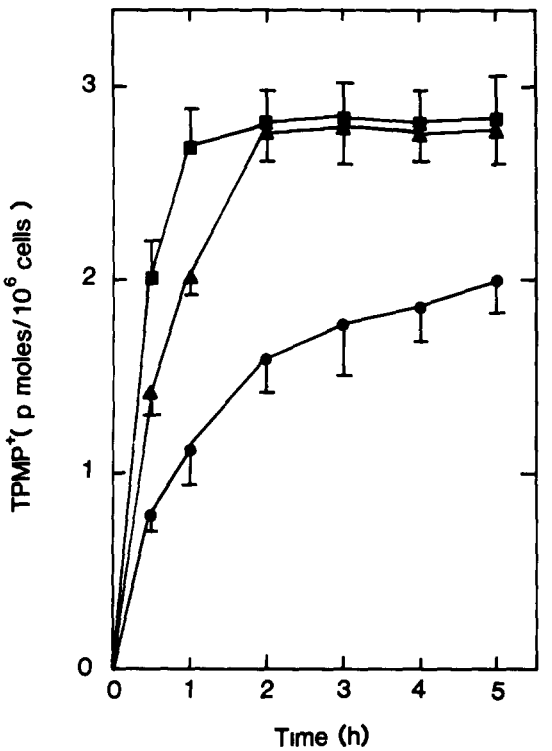

Fig 3 Uptake of $TPMP^+$ by alveolar type II cells in primary culture Alveolar type II cells cultured for 1 day were incubated with 50 nM $TPMP^+$ (2 μCi/ml) in the absence or presence of TPB^- at 37 °C Cell-associated $TPMP^+$ accumulation was determined as described under Experimental Procedures Each point represents the mean ± S E from four different experiments conducted in each duplicate The symbols are ● for $TPMP^+$ alone, ▲ with 2 μM TPB^- plus $TPMP^+$, and ■ with 5 μM TPB^- plus $TPMP^+$

tial-independent component which was determined in each experiment Potential-dependent accumulation of $TPMP^+$ reflects both the plasma and mitochondrial membrane potentials When type II cells were treated with the mitochondrial uncoupler CCCP (5 μM) for 10 min at 37 °C following the steady-state accumulation of $TPMP^+$, the corrected cell associated $TPMP^+$ accumulation decreased from 1 64 ± 0 15 pmol/10^6 cells of control value to 0 50 ± 0 05 pmol/10^6 cells (mean ± S E, $n = 8$ different experiments)

After the method was validated, the effect of depolarizing agents and the secretagogues, TPA or terbutaline plus IBMX, on the amount of cell-associated $TPMP^+$ was determined High potassium or gramicidin decreased the amount of cell-associated $TPMP^+$ (apparent depolarization, Table I) but TPA or terbutaline plus IBMX did not affect the value significantly after 1 min, 10 min, and 60 min incubation (Table I) or 30 min (not shown) The effects of these agents on the phosphatidylcholine secretion can be observed within 30 min [1,2,13] These results indicate that alveolar type II cells do not undergo membrane depolarization or hyperpolarization during the course of surfactant secretion induced by these agents

High potassium, which causes depolarization in a variety of cells, has been reported to cause phosphatidylcholine secretion in lung slices [7] Therefore, we examined the phosphatidylcholine secretion in media in which sodium chloride was

TABLE I

EFFECT OF HIGH POTASSIUM, GRAMICIDIN, TPA, AND TERBUTALINE PLUS IBMX ON THE ACCUMULATION OF $TPMP^+$

Alveolar type II cells cultured for 22 h were incubated with 50 nM [^{3}H]$TPMP^+$ (2 μCi/ml) and 2 μM TPB^- for 2 h, and then the test solution or compound was added The amount of cell-associated $TPMP^+$ was determined at various times as indicated The values have been corrected for potential-independent accumulation of the probe The data are expressed as mean ± S E from the number of experiments indicated Potassium chloride and gramicidin significantly decreased accumulation of $TPMP^+$ ($P < 0.01$)

Treatment	Accumulation (pmol $TPMP^+/10^6$ cells)		
Time	1 min ($n = 5$)	10 min ($n = 9$)	60 min ($n = 5$)
Control	1 65 ± 0 23	1 73 ± 0 16	1 66 ± 0 24
150 mM KCl		0 72 ± 0 23	
1 μM gramicidin		1 24 ± 0 12	
50 nM TPA	1 70 ± 0 17	1 71 ± 0 16	1 70 ± 0 19
100 μM terbutaline plus 50 μM IBMX	1 66 ± 0 20	1 70 ± 0 17	1 65 ± 0 20

totally substituted by equimolar concentration of potassium chloride or choline chloride As shown in Table II high potassium significantly increased basal secretion over control ($P < 0.01$) whereas substitution by choline did not significantly affect basal secretion Choline substitution potentiated TPA-induced secretion The data of Table I and II indicate that although transmembrane potential of alveolar type II cells does not change upon the addition of known secretagogues, high potassium can stimulate phosphatidylcholine secretion probably through membrane depolarization

Using the fluorescent calcium indicator, quin2, investigators have shown that high extracellular potassium elevates $[Ca^{2+}]_i$ through voltage-dependent calcium channels in an insulinoma cell line [23], brain synaptosomes [24], PC 12 cells [24], and the pituitary GH cell line [25] In general, elevation of $[Ca^{2+}]_i$ is thought to play a central role in cellular function such as secretion We have shown previously that an increase in $[Ca^{2+}]_i$ stimulates phosphatidylcholine secretion in type II cells [16] Therefore we examined the effect of high potassium on $[Ca^{2+}]_i$ in alveolar type II cell cultured on glass coverslips The resting level of $[Ca^{2+}]_i$ in alveolar type II cells was 143 ± 10 nM (mean $\pm$ S E., 12 monolayers in four different experiments) High potassium did not affect $[Ca^{2+}]_i$ in quin2-loaded alveolar type II cells in primary culture whereas the calcium ionophore, ionomycin, elevated $[Ca^{2+}]_i$ as shown in Fig 4 Therefore, high potassium-induced membrane depolarization does not apparently open voltage-dependent calcium channels in type II cells sufficient to change $[Ca^{2+}]_i$ The actual biochemical mechanism for stimulation by high extracellular potassium remains to be determined

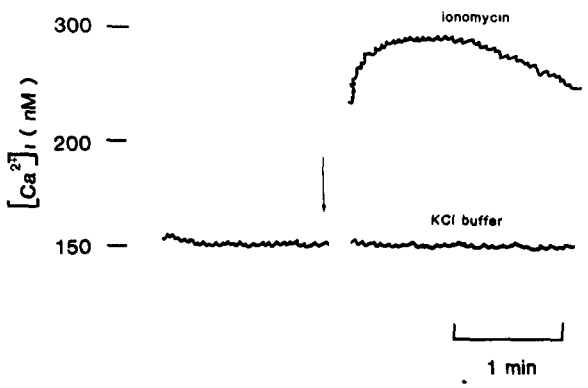

Fig 4 Effect of high potassium and ionomycin on $[Ca^{2+}]_i$ in alveolar type II cells in primary culture Alveolar type II cells which were cltured for one day on glass coverslips were loaded with quin2 (50 μM) and subjected to measurement of $[Ca^{2+}]_i$ as described under Experimental Procedures Monolayer was inserted into a cuvette containing NaCl-Hepes buffer and at the time indicated by an arrow ionomycin (0 4 μM) was added or the solution was totally replaced by KCl-Hepes buffer

TABLE II

SUBSTITUTION OF SODIUM CHLORIDE BY POTASSIUM CHLORIDE OR CHOLINE CHLORIDE ON PHOSPHATIDYLCHOLINE SECRETION

Freshly isolated alveolar type II cells were suspended in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1 μCi/ml of [*Me*-^{3}H]choline 2 ml of the suspension (1 10^6 cells/ml) were plated in 35 mm plastic dishes and incubated for 22 h in 10% CO_2/90% air atmosphere After washing the monolayers with DMEM and bovine serum albumin (1 mg/ml), the monolayers were washed again with 10 ml of NaCl-Hepes, KCl-Hepes, or choline Cl-Hepes buffer as indicated Incubation was continued for 1 h after addition of each agent at 37° C in air as the gas phase and % secretion was determined as described under experimental procedures The data are expressed as mean $\pm$ S E from seven different experiments each conducted in duplicate By analysis of variance with Tukey's test for multiple comparisons ($P < 0.01$), there was a significant effect of the dominant salt in the media on control (basal) secretion and on secretion stimulated by TPA but not on secretion stimulated by terbutaline plus IBMX Basal secretion is greater in KCl than in NaCl or choline chloride ($P < 0.01$), and TPA stimulated secretion is greater in choline chloride than in NaCl or KCl ($P < 0.01$)

Treatment	% Secretion, dominant salt in media		
	NaCl	KCl	Choline-Cl
Control	1 8±0 2	3 4±0 4	2 0±0 2
50 ng/ml TPA	13 0±1 7	11 0±1 6	16 2±2 1
100 μM terbutaline plus 50 μM IBMX	5 9±0 5	5 6±0 8	6 5±0 6

Discussion

Although various hormones and biologically active substances have been reported to change the transmembrane potential of their target tissues [6], our present results demonstrate that alveolar type II cells do not undergo membrane depolarization or hyperpolarization upon stimulation by agents that induce secretion of pulmonary surfac-

tant In order to measure transmembrane potential of freshly isolated cells we used the fluorescent carbocyanine dyes, di-S-$C_3(5)$ and di-O-$C_5(3)$ The advantage of using these dyes is that continuous recording is possible and relatively small change from the steady state level can be detected But in order to apply this method to a system one has to verify that fluorescence of the dye is a valid indication of changes in membrane potential in each system We confirmed the results of Castranova et al [8] that the fluorescence of di-S-$C_3(5)$ and di-O-$C_5(3)$ follows the Nernst equation as shown in Fig 1 and this method can be applied to measure changes in membrane potential in type II cells Although Castranova et al reported that TPA depolarized type II cells in our studies TPA or terbutaline plus IBMX, which stimulate surfactant secretion from alveolar type II cells, does not change the membrane potential (Fig 2) The reason for this discrepancy is not clear

In order to examine the changes in the membrane potential of type II cells in primary culture, which have been used to study phosphatidylcholine secretion, we initially attempted to use the carbocyanine dye id-O-$C_5(3)$ When alveolar type II cells cultured on glass coverslips were incubated with di-O-$C_5(3)$, the cells gradually incorporated the dye and reached steady state level at 2 h Although gramicidin (1 μg/ml) or high potassium depolarized these cells, addition of valinomycin always led to an increase in fluorescence (apparent hyperpolarization) independent of the concentration of extracellular potassium This phenomenon has been observed in granulocytes when the concentration of either cells or dye is too low [26] Hence, the concentrations of cells and dye were probably too low to use this dye to measure changes in the membrane potential in alveolar type II cells in primary culture Therefore, we used the radioactive cationic probe $TPMP^+$ which was used in freshly isolated rabbit alveolar type II cells by Gallo et al [9] Alveolar type II cells in primary culture gradually incorporated $TPMP^+$ and addition of TPB^- accelerates the incorporation as shown in Fig 3 The time required to reach at steady state level was 2 h in the presence of 2 μM TPB^-, and this is longer than that reported by Gallo et al for freshly isolated rabbit type II cells This may be because in primary culture only the apical surface of the cells is exposed to the probe A considerable amount of the probe partitioned in mitochondria in type II cells, because the absolute magnitude of mitochondria transmembrane potential is higher than plasma membrane potential [9] However, changes in the plasma membrane potential caused by high potassium or gramicidin were detectable as shown in Table I Therefore the $TPMP^+$ method can be applied to examine the effects of secretagogues on changes in the plasma membrane potential in type II cells in primary culture. Similarly, cells loaded with $TPMP^+$ and TPB^- can respond to secretagogues normally and do not show signs of cytotoxicity as determined by trypan blue exclusion or release of lactate dehydrogenase. Our results indicate that TPA or terbutaline, at levels that induce phosphatidylcholine secretion, does not change membrane potential (Table I)

High potassium has been reported to increase phosphatidylcholine secretion in lung slices, and this effect was not modified by atropine or propranolol [7] Our results also show that high potassium increases phosphatidylcholine secretion by isolated type II cells in vitro (Table II) Therefore, the effect of high potassium on lung slices could be a direct effect on type II cells Physiological mediators or events such as stretching the plasma membrane during hyperventilation may cause membrane depolarization in type II cells in vivo and, hence, may control surfactant secretion in the intact animal through this mechanism Although high potassium slightly increased phosphatidylcholine secretion, the depolarizing agent gramicidin (1 μg/ml) did not stimulate secretion However, gramicidin (1 μg/ml) inhibited TPA-induced secretion by 60% (single experiment in duplicate) The release of lactate dehydrogenase from gramicidin-treated cells did not exceed 3% Prolonged incubation with gramicidin (1 h for a secretion study), however, may disturb the cellular structure required for secretory response and mask the effect of membrane depolarization on secretion The stimulation of secretion by high potassium was not additive to that found for terbutaline plus IBMX or TPA This result suggests that there may be some negative regulation occurring between the depolarization pathway and the pathway stimulated by terbutaline or TPA In some

systems high potassium opens voltage-dependent calcium channels but in type II cells as shown in Fig 4, there was no change in intracellular calcium The mechanism of the stimulatory effect of high potassium on phosphatidylcholine secretion remains to be determined

Substitution of sodium by choline slightly enhanced TPA-induced phosphatidylcholine secretion (Table II) The mechanism of the modulation of the TPA-induced response by choline is unknown at present, but it is clear that a physiological concentration of extracellular sodium is not required for phosphatidylcholine secretion by isolated alveolar type II cells

In summary, we could not detect membrane depolarization due to secretagogues for type II cells in vitro Hence, our data do not support the hypothesis that a unifying event during stimulation of secretion is membrane depolarization We used both carbocyanine dyes which should detect early depolarization and the $TPMP^+$ method which should detect late or gradual depolarization We conclude that TPA, the class of single agonist which produces the greatest stimulatory effect on secretion in vitro, does not alter the membrane potential of type II cells at concentrations which stimulate secretion

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