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Calcium Mobilization and Exocytosis After One Mechanical Stretch of Lung Epithelial Cells

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Deep inflation of the lung stimulates surfactant secretion by unknown mechanisms. The hypothesis that mechanical distension directly stimulates type II cells to secrete surfactant was tested by stretching type II cells cultured on silastic membranes. The intracellular Ca^{2+} concentration was measured in single cells, before and after stretching. A single stretch of alveolar type II cells caused a transient (less than 60 seconds) increase in cytosolic Ca^{2+} followed by a sustained (15 to 30 minutes) stimulation of surfactant secretion. Both Ca^{2+} mobilization and exocytosis exhibited dose-dependence to the magnitude of the stretch-stimulus. Thus, mechanical factors can trigger complex cellular events in nonneuron, nonmuscle cells and may be involved in regulating normal lung functions.

EVIDENCE FROM EXPERIMENTS WITH whole animals or isolated lungs suggests that deep inflation of the lung augments secretion of pulmonary surfactant, the phospholipid-rich, chemically heterogeneous material that lowers surface tension at the air-liquid interface within the lungs (*1*). The mechanism by which an increase in lung volume apparently stimulates surfactant secretion is unknown. Because alveolar surface area may increase by as much as 80% during inflation to total lung capacity (*2*), one hypothesis is that mechanical distension of the alveolus causes surfactant to be secreted by alveolar type II epithelial cells. (Surfactant components are synthesized by the type II cell, stored in intracellular organelles, and secreted by exocytosis.) We found that a single stretch of alveolar type II cells cultured on elastic membranes caused a transient increase in cytosolic Ca^{2+}, after which surfactant secretion persisted for 30 min. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured in single cells before and after stretching. Calcium was mobilized from intracellular rather than extracellular stores. Thus, mechanical factors can trigger complex cellular events in a nonneuron, nonmuscle cell and may be of general importance in regulating cellular functions.

Rat alveolar type II cells were cultured for 20 to 24 hours on one of two types of circular elastic silicone membrane dishes (*3*). In experiments designed to study surfactant secretion, cells were incubated during this period with [^{3}H]choline (1 μCi/ml) to label phosphatidylcholine (PC), the major component of surfactant (*4*). Release of lactic acid dehydrogenase (LDH) into the media was measured as one indicator of cellular damage (*5*).

Membranes were stretched by applying hydrostatic pressure beneath the membranes (*6*). To imitate deep inflation of the lung, we distended membranes once and then returned them to the relaxed position. Secretion from cells on stretched or unstretched membranes was then measured. Changes in membrane surface area for each type of membrane were determined by measuring the height of membrane displacement and calculating the increase in surface area from the formula for the area of a sphere (*7*). We compared the increase of measured two-dimensional cellular surface area (CSA) (*8*) to the increase in calculated membrane surface area (MSA) by photographing living cells on membranes in stretched and relaxed states and digitizing cellular surface area (Fig. 1). For cells cultured on both types of membranes, stretch-induced increases in secretion relative to unstretched controls correlated well with increases in CSA rather than with increases in MSA.

We measured secretion of [^{3}H]phosphatidylcholine ([^{3}H]PC) over 1 hour from cells on silastic sheeting and Sylgard membranes (Table 1). One hour after a single stretch that increased mean CSA by 16% (silastic sheeting membranes) or 17% (Sylgard membranes), [^{3}H]PC secretion by cells on both types of membranes (Table 1 and Fig. 2) was significantly increased when compared to control membranes studied concurrently in the same device.

There was a direct correlation between the magnitude of stretch (as measured by an

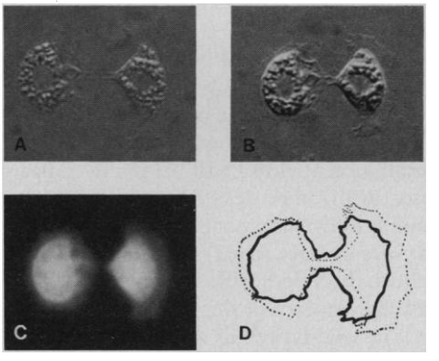

Fig. 1. Living rat alveolar type II cells cultured for 22 hours on Sylgard membranes. Photographs were taken at equal magnification on a Leitz Orthoplan microscope with differential interference contrast optics (×40 lens). Cells were loaded with fluorescein diacetate (50 μM for 1 hour) and the membrane was held and stretched in a modified Sykes-Moore chamber (Bellco Glass) with a no. 1 cover slip bottom. (**A**) Two connecting cells shown in the stretched state. The calculated increase in MSA was 52%. The measured increase in two-dimensional CSA was 23% for the cell on the left and 25% for the cell on the right compared to the relaxed state (**B**). (**C**) Staining with the vital dye fluorescein acetate indicates viability of the two cells. (**D**) Overlaid outlines of the two cells, showing the difference in shape and area. Cells were covered with a thin layer of medium (MEM with 25 mM Hepes buffer with Krebs salts).

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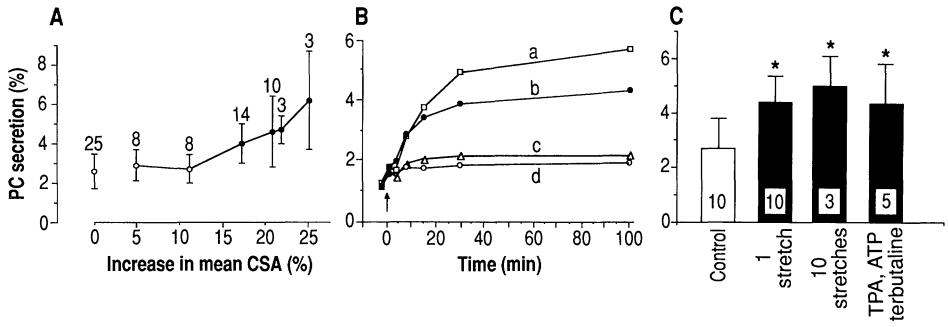

Fig. 2. Secretory response of alveolar type II cells to mechanical stimuli. (**A**) Dose response of secretion of [^{3}H]PC (mean ± SD) to increases in mean CSA. Numbers on top of error bars (mean ± SD) indicate the number of experiments performed with different cell isolations at each mean increase in CSA. ○, Points that are not statistically significantly different from unstretched controls; ●, points that are different from controls [analysis of variance followed by a Newman-Keuls multiple range test ($P < 0.05$)]. A stretch of 25% increase in mean CSA stimulated secretion more than one of 17% ($P < 0.05$). (**B**) Time course of [^{3}H]PC secretion by type II cells cultured on Sylgard membranes. [^{3}H]PC secretion was measured simultaneously on six membranes in the same stretching device. Data shown are from one of four experiments performed with different isolations of type II cells. Aliquots (120 μl) of media (of a total of 1.32 ml of medium per membrane) were removed at the indicated time points. At zero time (arrow), membranes were stretched (increasing mean CSA by 21%) or control solutions or chemical secretagogues (10^{-6} M ATP + 10^{-8} M TPA + 10^{-5} M terbutaline) were added. At subsequent time points, additional aliquots of media were removed. Line a, unstretched + TPA, ATP, terbutaline; line b, stretched; line c, stretched + SP-A; line d, unstretched controls. (**C**) Unstimulated, single stretch–, multiple stretch–, and agonist-stimulated secretion of [^{3}H]PC on Sylgard membranes during 1 hour. A single stretch was performed as described in the text. Ten stretches were performed at the beginning of the experiment, and the membranes were then allowed to relax for the rest of the experimental period. Membranes were stretched so that the mean CSA increased by 17%. Membranes treated with agonists (10^{-4} M ATP, 10^{-8} M TPA, and terbutaline 10^{-5} M) were not stretched. By Dunnett's test, all treatment groups were different from unstretched controls ($P < 0.05$).

increase in mean CSA) and the magnitude of [^{3}H]PC secretion (Fig. 2A). A threshold distension exists below which secretion is not augmented. Because membranes often ruptured when distension was greater than that indicated in Fig. 2A, we could not determine whether secretion reaches a plateau with respect to the magnitude of stretch. The kinetics of stretch-induced secretion (Fig. 2B) indicated that secretion did not occur en bloc after a stretch, but continued to be augmented for at least 30 min. However, the rate of secretion after a single stretch decreased over the 30-min period. By 100 min after a stretch, the stimulatory effect had virtually ceased. We compared the magnitude of secretion stimulated by a single stretch to that stimulated by multiple stretches or known chemical secretagogues for type II cells. Ten consecutive stretches stimulated secretion only 1.1 times more than a single stretch of the same magnitude (Fig. 2C). Continuous exposure to 10^{-8} M 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) resulted in a secretory response that was somewhat greater than that elicited by a single stretch (16% increase in CSA) but was not statistically significantly different from it (Table 1). On Sylgard membranes, stretch-induced secretion (17% increase in CSA) was comparable to secretion stimulated by continuous exposure to three secretagogues [10^{-8} M TPA + 10^{-4} M adenosine triphosphate (ATP) + 10^{-5} M terbutaline] (Fig. 2, B and C).

It appears unlikely that stretch-induced secretion in our system is an artifact of cellular damage or detachment of cells during the stretch. Neither vital dye exclusion nor total radioactivity was lower in cells on stretched membranes (*9*). LDH release from cells on stretched membranes was not different from cells on unstretched control membranes (Table 1). Occasionally LDH release exceeding 1% was observed from cells on unstretched and stretched membranes; results from those membranes were discarded (*10*). Further evidence supporting the concept that secretion of [^{3}H]PC is not due merely to damage of cell membranes is that we could inhibit secretion by surfactant protein A (SP-A) (Table 1 and Fig. 2B) or cold temperature (Table 1). SP-A inhibits agonist-induced PC secretion in type II cells (*11*); the mechanism of inhibition is unknown. Increased secretion is also unlikely to be caused by the motion of medium covering cells during the stretch because cells on plastic dishes incubated for 3 hours on a rocking platform with 27 cycles per minute did not show differences in either baseline or TPA-induced secretion compared to cells on stationary dishes (*12*).

Metabolites of arachidonic acid are released after distension of the lung (*13*). The stimulatory effect of lung inflation on secretion is inhibited by indomethacin in some experiments (*14*), but not in others (*15*). We tested whether the addition of a cyclooxygenase inhibitor (indomethacin) or a lipoxygenase inhibitor (BW 755 C) blocked stretch-induced secretion in type II cell cultures. Neither indomethacin nor compound BW 755 C blocked the secretory response to stretch (Table 1).

Both cyclic adenosine monophosphate (cAMP) (*16*) and $[Ca^{2+}]_i$ (*17*) have been implicated as cellular second messengers in surfactant secretion. A single stretch did not

Table 1. Factors affecting secretion. Stretch-induced (16% increase in CSA) and TPA-induced secretion of [^{3}H]PC during 1 hour from type II cells cultured on silastic sheeting (mean ± SD, n = number of cell isolations). Human proteinosis SP-A (1.25 μg/ml) (a gift of S. Hawgood) was added to appropriate wells 3 min before stretch. Results were analyzed by an analysis of variance and a Newman-Keuls test. Influence of low temperature and inhibitors of the cycloxygenase and lipoxygenase on stretch-induced secretion of type II cells cultured on Sylgard membranes (mean ± SD, n = number of cell isolations). Higher and lower concentrations of indomethacin and BW 655 C than shown in the table were also tested. Higher concentrations resulted in LDH release. All concentrations were ineffective in blocking stretch-induced secretion. Compounds were added to experimental membranes 30 min before stretch. Results were analyzed as described above.

Treatment	[^{3}H]PC (% of total)		n	LDH (% of total)	
	Unstretched	Stretched		Unstretched	Stretched
	Cells cultured on silastic membranes				
None	1.7 ± 0.9	2.9 ± 0.9*	6	0.2	0.2
TPA (10^{-8} M)	3.2 ± 1.1*		6	0.1	
SP-A		1.3 ± 0.7	6		0.3
	Cells cultured on Sylgard membranes				
None	2.6 ± 0.9	4.6 ± 1.8*	10		
4°C	2.4 ± 0.5	2.4 ± 0.6	3		
Indomethacin (10^{-5} M)	2.4 ± 1.0	4.4 ± 1.3*	3		
BW 755C (10^{-5} M)	2.2 ± 0.5	3.8 ± 0.8*	4		

*Value is different from unstretched control ($P < 0.05$).

cause an increase in cellular cAMP measured 5 and 30 min later, whereas terbutaline, a β-adrenergic agonist, stimulated cAMP at 5 min (Fig. 3A). We next measured $[Ca^{2+}]_i$ in single cells, monitoring the same cell before and after stretching (Fig. 3, B to D). For technical reasons, the earliest time points we could measure after stretch and relaxation occurred 8 to 10 s after relaxation was complete. Because $[Ca^{2+}]_i$ was greatest at this time and then rapidly fell, it is likely that the maximal $[Ca^{2+}]_i$ induced by stretch were greater than those we were able to measure. When experiments were performed in medium containing Ca^{2+} and Mg^{2+}, $[Ca^{2+}]_i$ increased 3.5 times after stretch and relaxation (baseline $[Ca^{2+}]_i$, 0.107 ± 0.039 μM; after stretch $[Ca^{2+}]_i$, 0.369 ± 0.099 μM; $n = 9$ cells, six different cell isolations) (Fig. 3B). Intracellular Ca^{2+} concentrations then declined to baseline at variable rates (within 60 s). (Return to basal $[Ca^{2+}]_i$ + 50% was reached at 46 ± 42 s after measurement was restarted.)

To determine whether the increases in $[Ca^{2+}]_i$ observed after stretch and relaxation were from intracellular or extracellular sources, we also measured $[Ca^{2+}]_i$ in cells stretched in Ca^{2+}- and Mg^{2+}-free medium containing 0.2 mM EGTA. Although $[Ca^{2+}]_i$ was somewhat lower in this medium, the response was similar, with $[Ca^{2+}]_i$ increasing 3.5 times after stretch and relaxation (baseline $[Ca^{2+}]_i$, 0.073 ± 0.034 μM; after stretch $[Ca^{2+}]_i$, 0.216 ± 0.083 μM; $n = 7$ cells from five different cell isolations) (Fig. 3C). Thus, stretch produces a rise in $[Ca^{2+}]_i$ mediated by release of Ca^{2+} from intracellular stores rather than by allowing Ca^{2+} to enter from extracellular stores, such as may occur through stretch-dependent Ca^{2+} channels in the plasma membrane (*18*).

We also examined the dose-dependence of $[Ca^{2+}]_i$ on the magnitude of the stretch stimulus (Fig. 3D). In the cell shown, an increase in $[Ca^{2+}]_i$ first appeared after a stretch resulting in an 11% increase of CSA. The subsequent larger stretch to an ~17% increase of CSA produced a larger increase in $[Ca^{2+}]_i$. Thus, the threshold for increase in $[Ca^{2+}]_i$ appears to be close to the threshold of secretion with respect to the magnitude of stretch. When examining single cells, we have noticed that the stretch threshold varies somewhat among cells, due to variability among cells in the CSA/MSA ratio (and therefore in the extent to which they are stretched) or in the cellular response to stretch stimuli.

Addition of rat SP-A to the medium before stretching did not affect the increase in $[Ca^{2+}]_i$ (*19*), suggesting that the inhibitory effect of SP-A on secretion occurs late in exocytosis. This is supported by the fact that SP-A inhibits secretion stimulated by chemical agonists that act by stimulating different cellular second messengers.

We conclude from these results that secretion of PC in alveolar type II cells can be directly stimulated by a single brief mechanical distension. Mechanical stimuli can trigger complex cellular events in nonneuron, nonmuscle cells. The effect of a single stretch stimulus is prolonged, stimulating secretion for 15 to 30 min.

The lung is a distensible organ that undergoes phasic changes in volume. Periodic deep breaths such as sighs and yawns occur in most mammalian species and are thought to be important in maintaining lung compliance, presumably by stimulating surfactant secretion. The time course observed here, in which 75% of the maximal secretory response occurred within 15 min of the stretch, supports the concept that mechanical forces acting directly on cells may have an important role in lung physiology.

The mechanism of stretch-induced secretion appears to involve transient increases in $[Ca^{2+}]_i$ from intracellular sources similar to those observed after addition of chemical secretagogues or hyposmotic swelling (*20*) in a wide variety of cell systems. We postulate that there is a tension- or distortion-sensitive signaling mechanism located in the cell membrane or attached to cytoskeletal elements and that this mechanism is able to release Ca^{2+} from intracellular sources by an unknown mechanism. The model system described here affords an opportunity to study the mechanisms by which mechanical forces may be coupled to cellular events.

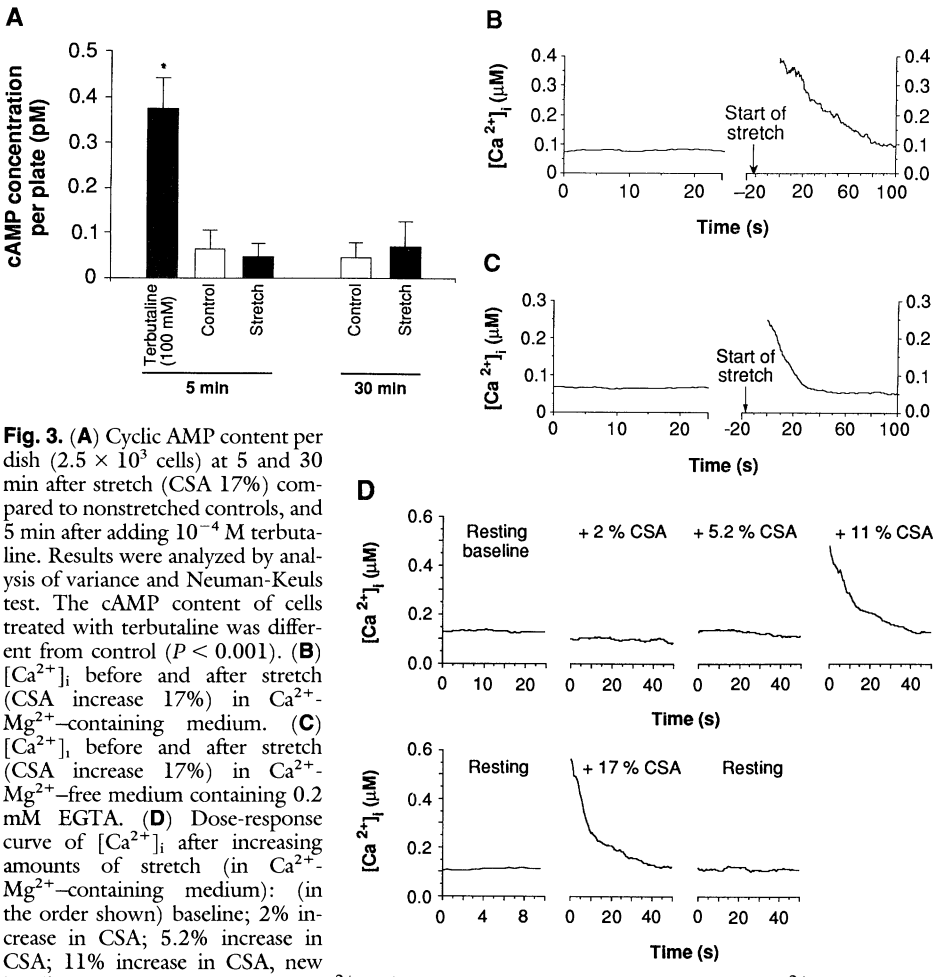

Fig. 3. (**A**) Cyclic AMP content per dish (2.5×10^3 cells) at 5 and 30 min after stretch (CSA 17%) compared to nonstretched controls, and 5 min after adding 10^{-4} M terbutaline. Results were analyzed by analysis of variance and Neuman-Keuls test. The cAMP content of cells treated with terbutaline was different from control ($P < 0.001$). (**B**) $[Ca^{2+}]_i$ before and after stretch (CSA increase 17%) in Ca^{2+}-Mg^{2+}–containing medium. (**C**) $[Ca^{2+}]_i$ before and after stretch (CSA increase 17%) in Ca^{2+}-Mg^{2+}–free medium containing 0.2 mM EGTA. (**D**) Dose-response curve of $[Ca^{2+}]_i$ after increasing amounts of stretch (in Ca^{2+}-Mg^{2+}–containing medium): (in the order shown) baseline; 2% increase in CSA; 5.2% increase in CSA; 11% increase in CSA, new baseline; 17% increase in CSA; $[Ca^{2+}]_i$ after completion of the experiment. The $[Ca^{2+}]_i$ and cAMP were measured as in (*21*).

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3. Surgical silastic sheeting 0.005 inch (0.127 mm) thick (Dow Corning) was cut to appropriate size, and a silicone ring (3.2 cm outer diameter, 2.3 cm inner diameter, 0.25 cm thick; gift of Baxter Health Care Co.) was glued to each membrane with house-

hold silicone glue and allowed to dry for at least 24 hours. The resulting "membrane dish" had a surface of 4.15 cm^2. Membrane dishes of the same size were also prepared by casting 0.5 ml of a mixture of ten parts Sylgard 184 silicone elastomer and one part Sylgard 184 curing agent (Dow Corning) into 35-mm plastic culture dishes and allowing them to polymerize at 65°C overnight. When the membranes were still sticky, but firm, the silicone ring was allowed to polymerize into the membrane to avoid the use of glue. Sylgard-type membranes were 0.23 mm thick and optically clear. Both types of membranes were sterilized and coated overnight with bovine plasma fibronectin (200 μg/ml).

4. Type II cells were isolated from pathogen-free adult male Sprague-Dawley rats (Bantin-Kingman) [L. G. Dobbs, R. F. Gonzalez, M. C. Williams, *Am. Rev. Respir. Dis.* **134**, 141 (1986)]. We cultured 2.5×10^5 cells/ml on each membrane in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (University of California at San Francisco Cell Culture Facility), 2 mM glutamine, penicillin (100 U/ml), and gentamicin (50 μg/ml). [^{3}H]Choline was obtained from Amersham Corporation. Secretion of [^{3}H]PC was measured as previously described [L. G. Dobbs, R. F. Gonzalez, L. A. Marinari, E. J. Mescher, S. Hawgood, *Biochim. Biophys. Acta* **877**, 305 (1986)]. Medium and cells were harvested and centrifuged, and lipids were extracted [J. Folch, M. Lees, G. H. S. Stanley, *J. Biol. Chem.* **226**, 497 (1957)]. Radioactivity was measured in a liquid scintillation counter (Beckman Instruments LS 7500).
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6. The stretching devices we constructed provide space for two groups of three membranes per device. Each group of three membranes can be stretched independently. Thus three experimental membranes (stretched) were compared to three control membranes (unstretched) under equivalent conditions. Membranes were held in place by an acrylic top plate (2 cm high) with round borings forming the wells. Pressure was applied manually with a syringe and transduced to the membranes by fluid; the amount of stretch was controlled by the volume of fluid added.
7. Height was measured from the middle of the distended elastic membrane. Volume added was plotted against the calculated increase in membrane surface area. The volume:MSA relationship was reproducible and linear over the range used. It was nearly identical for both types of membranes.
8. Cellular surface area is defined here as the area that is occupied by the cell in two dimensions as viewed through a microscope and not as a true three-dimensional measurement of cellular surface. Cellular surface area was measured by digitizing cell area on photographs (Calcomp 6000). Although there was cell-to-cell variation in CSA/MSA ratio, this ratio was higher (0.75 ± 0.36; mean ± SD, $n = 20$) for silastic sheeting than it was for the Sylgard membranes (0.40 ± 0.11, $n = 6$), possibly because of the smoother Sylgard surface. We do not know the reason for the difference between MSA and CSA. Cell attachment sites to fibronectin may be broken when cells are stretched. Alternatively, the cells or the matrix may slide on the membrane, resulting in less distension of the cells compared to the membrane.
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10. Data from 23 of 300 stretched and 13 of 210 unstretched membranes were discarded because LDH release was greater than 1%; LDH released from cells on these discarded membranes averaged 2.2 ± 1.6% for stretched and 3.2 ± 3.4% for unstretched membranes.
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21. Cyclic AMP content was determined with a radioimmunoassay (Rainen, Du Pont) after extraction of the cells at 4°C with 0.1 M HCl and rapid freezing. Results were similar to previously published observations [E. J. Mescher, L. G. Dobbs, R. J. Mason, *Exp. Lung Res.* **5**, 173 (1983)]. [Ca^{2+}]$_i$ was measured with an ACAS 470 laser cytometer (Meridian Instruments Inc.). Cells cultured on Sylgard membranes were incubated for 1 hour with medium containing 2 μM Indo-1-AM (Molecular Probes Inc.) and transferred to a stretching chamber (37°C) adapted to the ACAS stage. To measure rapid changes in [Ca^{2+}]$_i$, we used linescans (120 points; step size 0.25 μm) to measure [Ca^{2+}]$_i$ in a single cell over time (additional image scans correlated well with the [Ca^{2+}]$_i$ values obtained from linescans). The ratio of fluorescence at 485 nm and 405 nm was calculated for each point. A calibration curve relating [Ca^{2+}]$_i$ to fluorescence was constructed according to the method described [M. H. Wade and S. A. McQuiston, *ACAS 470 Training Manual* (Meridian Instruments Inc., Okemos, MI, 1988), p. 37]. [Ca^{2+}]$_i$ was measured for various lengths of time before stretching. Measurement of [Ca^{2+}]$_i$ during stretch was not feasible because the focal plane changed when the membrane was distended. Stretch was carried out by adding warmed fluid to the chamber with a syringe and followed by immediate relaxation. The same cell was quickly identified and centered, and measurement of fluorescence was restarted as quickly as possible. Stretch and relaxation were usually completed in 12 s. The time required to recenter the cell and restart the instrument was more variable; time from start of stretch to restart of measurement was 21.7 ± 8.9 s for experiments in Ca^{2+}-Mg^{2+}–containing medium and 19.4 ± 4.9 s for experiments in Ca^{2+}-Mg^{2+}–free medium. The background determined from a cell-free area of the membrane was subtracted and [Ca^{2+}]$_i$ from linescans was averaged.
22. Supported in part by grants from the NIH (HL-24075 and HL-34356) and Deutsche Forschungsgemeinschaft. We thank J. Nellenbogen and D. Johnson for expert technical assistance, J. R. Wright for help in preparing rat SP-A, and J. A. Clements, M. C. Williams, J. Goerke, and M. J. Fulwyler for helpful discussions and suggestions. We also thank Burroughs-Wellcome Inc. for their gift of BW 755C.

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Recognition by Human $V_\gamma 9/V_\delta 2$ T Cells of a GroEL Homolog on Daudi Burkitt's Lymphoma Cells

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All human γδ T cells coexpressing the products of the variable (V) region T cell receptor (TCR) gene segments $V_\gamma 9$ and $V_\delta 2$ recognize antigens from some mycobacterial extracts and Daudi cells. Exogenous and endogenous ligands on the cell surface, homologous to the groEL heat shock family, induced reactivities that resembled superantigen responses in this major subset of human peripheral blood γδ T cells. Stimulation of human $V_\gamma 9/V_\delta 2$ T cells is not restricted by human leukocyte antigens (HLA), including nonpolymorphic β_2-microglobulin (β_2M)–associated class Ib molecules. These data may be important for understanding the role of γδ T cells in autoimmunity and in responses to microorganisms and tumors.

A SUBSET OF T CELLS EXPRESS THE γδ T cell receptor (TCR) (*1*) and have limited variable (V) region repertoire (*2*). Antigens that stimulate γδ T cells are only now being identified (*3*), and it is not clear whether they need to be processed and presented as is the case for αβ T cells (*4*). The nonclassical major histocompatibility complex (MHC) class Ib molecules also have limited polymorphism and have been proposed as antigen-presenting molecules for γδ T cells (*5*). Both murine (*6, 7*) and human (*8*) γδ T cells can respond to mycobacterial extracts. In the mouse, the 65-kD mycobacterial heat shock protein (hsp65) and peptides from it can stimulate γδ T cells (*6*). Hsp65 is homologous to the groEL protein of *Escherichia coli* and similar stress proteins in prokaryotic and eukaryotic cells (*9, 10*). Examination of over 2000 human γδ T cell clones revealed that all of those expressing both $V_\gamma 9$ and $V_\delta 2$ (*11, 12*) are particularly efficient killers of the Daudi Burkitt's lymphoma cell line (*13*). In this study, we examined the proliferative responses of human γδ T cells to endogenous and exogenous antigens. The proliferative response of human $V_\gamma 9/V_\delta 2$ T cells to Daudi cells and bacterial extracts could be inhibited by antiserum to the mammalian groEL related heat shock proteins (hsp58 or hsp60) (*14*). This serum immunoprecipitated a molecule of corresponding molecular size from the surface of Daudi cells. We