C51

Human Keratinocytes Release ATP and Possess Three Mechanisms for Nucleotide Interconversion at the Cell Surface

H.E. Burrell, B.J. Foster, K.A. Buckley, J.M. Quayle, A.W. Simpson and J.A. Gallagher

Human Anatomy and Cell Biology, University of Liverpool, Liverpool, UK

Extracellular nucleotides activate the P2 family of receptors, of which there are two subgroups. P2X receptors are ligand-gated ion channels while P2Y receptors are G protein-coupled. Activation of P2 receptors is important in autocrine and paracrine regulation in many tissues. In the epidermis, the $P2Y_2$ receptor is expressed in the proliferative basal layer (Dixon *et al.*, 1999), the $P2X_5$ receptor is present in the differentiated layers, and the $P2X_7$ receptor is present in the apoptotic squamous layer (Grieg *et al.*, 2003). In this study, we have investigated the source of extracellular nucleotides in a human keratinocyte cell line (HaCaT). We have used a combination of luciferin-luciferase luminometry, pharmacological inhibitors and confocal microscopy to show that HaCaT cells release ATP into the culture medium, and that there are three mechanisms for nucleotide interconversion, resulting in cell surface ATP-generation. HaCaT cells were seeded at a density of 1 x 10^5 cells/well in 12 well plates and grown to confluence overnight and serum-starved for 1 hour prior to treatment. Values from controls (addition of treatments to medium alone) were subtracted from those in the presence of cells. Mean basal release of ATP was ≈100 nM/10^6 cells/hour and was significantly higher than that of SaOS-2 cells (an osteoblast cell line; $p<0.0001$, students' t-test, n=8). Addition of 10 μM ADP, to culture medium resulted in a 6-fold elevation of the ATP concentration. This effect occurs immediately and interestingly, ATP-generation and degradation appear to be balanced since the concentration of ATP remained stable for 90 minutes after addition (n=4). Addition of 10 μM GTP or UTP each resulted in a 3-fold increase in the ATP concentration, whilst 10 μM 2MeSADP, UDP or ATP had no effect (n=4). ADP to ATP conversion was inhibited by 10-300 μM diadenosine pentaphosphate, 12-48 μM oligomycin or 10 mM UDP, suggesting the involvement of cell surface adenylate kinase, F_1F_0 ATP synthase and nucleoside diphosphokinase (NDPK) respectively (n=4). These findings were supported by immunohistochemical localisation. Simultaneous addition of 10 μM ADP and 20 μM GTP elevated ATP above that for each nucleotide alone indicating that GTP is acting as a phosphate donor in the reaction $N_1TP + N_2DP \leftrightarrow N_1DP + N_2TP$ (n=4). However, the activity of NDPK, F_1F_0 ATP synthase or the forward reaction of adenylate kinase does not fully account for the ATP content of the culture medium. We postulate that this is due to the reverse reaction of adenylate kinase (AMP + GTP ↔ ADP + GDP). We have previously shown that HaCaT cells express multiple P2 receptors (Burrell *et al.*, 2003). In this study we now identify the potential sources of ATP required to activate these receptors and thus provide better understanding of the role of nucleotides in normal epidermal homeostasis and wound healing.

Burrell *et al.* (2003). *J. Invest. Dermatol.* **120**, 440-447.

Dixon *et al.* (1999). *Br. J. Pharmacol.* **127**, 1680-1686.

Grieg *et al.* (2003). *J. Invest. Dermatol.* **120**, 1007-1115.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C52

Mechanical compression activates chondrocyte calcium signalling in a cycle-dependent manner involving the release of ATP

M.M. Knight, B. Pingguan, D. Lee and D. Bader

Medical Engineering Division, Dept of Engineering, Queen Mary, University of London, London, UK

The process of mechanotransduction is essential for the health and homeostasis of the articular cartilage. However, the underlying signalling pathways are poorly understood. This study examines the influence of cyclic mechanical compression on chondrocyte Ca^{2+} signalling and the involvement of mechanosensitive release of cellular ATP.

The study used a well-characterised model consisting of isolated bovine (from an abattoir) articular chondrocytes cultured in agarose constructs [1]. After 24h, constructs were incubated in lluo-4 AM (Molecular Probes) and mounted in a custom made compression rig [1] on a confocal microscope (Perkin Elmer). Cyclic compression at 1Hz between 0% and 10% strain was applied for 1, 10, 100 or 300 cycles. This was followed by a 5min period in which images of fluo-4-labelled cells, approximately 20 cells per field of view, were captured every 4s using a ×20 objective. Separate constructs were held at 10% static compression and subjected to an identical 5min imaging period in the compressed state. Control constructs remained unstrained. In further studies constructs were bathed in apyrase (10 units/ml) during 1Hz cyclic compression for 10 cycles and throughout the subsequent 5min imaging period. Unstrained controls were also bathed in apyrase. Student's t tests ($p<0.05$) were used to compare the percentage of cells showing Ca^{2+} transients in samples of individual constructs (n=8-15).

In unstrained agarose constructs, 50% of cells exhibited spontaneous Ca^{2+} transients over the 5min period. Static 10% compression produced no change in Ca^{2+} signalling. Previous studies have shown increased signalling at 20% compression [1] indicating that the effect may be strain magnitude dependent. Cyclic compression for 1, 10 or 100 cycles was followed by a statistically significant increase in the percentage of cells exhibiting Ca^{2+} transients (Fig. 1). However, 10 or 100 cycles did not induce additional responses compared to 1 cycle, suggesting a redundancy of signal transduction. The involvement of a paracrine/autocrine mechanism was confirmed by the finding that the ATP scavenger, apyrase, abolished the up regulation of Ca^{2+} signalling produced by cyclic compression. After 300 cycles, Ca^{2+} signalling was not significantly different to that in unstrained controls, possibly associated with ATP receptor fatigue. In conclusion, cyclic compression activates Ca^{2+} signalling via mechanosensitive release of ATP as part of a chondrocyte mechanotransduction pathway.

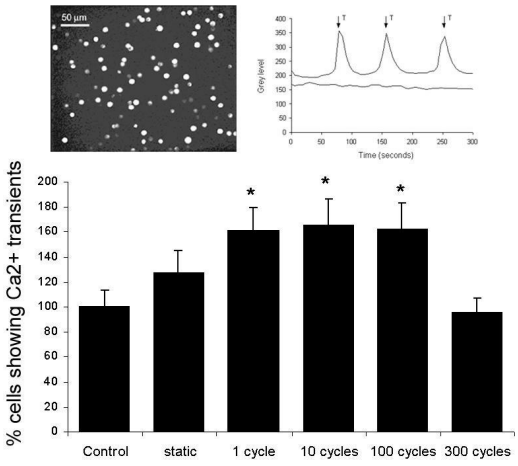

Figure 1. Percentage of cells exhibiting calcium transients in 5min period during static compression or following cyclic compression for 1, 10, 100 or 300 cycles. Values represent mean ± S.E.M. normalised to unstrained control (*p<0.05). Inset shows image of fluo-4-labelled cells and represenative Ca^{2+} transients for a single cell.

Roberts SR, Knight MM, Lee DA & Bader DL (2001). J Appl Physiol 90, 1385-1391.

M.M.K. is funded as an EPSRC Advanced Research Fellow. B.P. is funded by the Malaysian Government.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C53

A calcium-dependent pathway in swelling-induced ATP release from airway epithelial cells

S. Okada, A.M. Paradiso, E.R. Lazarowski and R.C. Boucher

Cystic Fibrosis Center, the Univ of North Carolina, Chapel Hill, NC, USA

In airway epithelia, extracellular ATP regulates mucociliary clearance by stimulating cell surface $P2Y_2$ receptors. However, the mechanisms of ATP release and the magnitude of endogenous ATP accumulation in the thin layer of liquid covering airway surfaces are poorly understood. We monitored in real-time apical ATP concentrations adjacent to the cell surface of airway epithelial cells cultured in an air-liquid interface system. Firefly luciferase fused to the IgG-binding domain of *Staphylococcus aureus* protein A (SPA-luc) was bound to the apical surface of polarized airway epithelial Calu-3 cells via an antibody against an endogenous cell surface glycoprotein, i.e. MUC1. Basal ATP levels monitored by cell-attached luciferase were similar to those measured in bulk mucosal medium (depth 2.6 mm) (7.6±0.7 nM and 7.3±0.6 nM by cell-attached and bulk measurement, respectively, n=6). A robust ATP release was observed following a 33% hypotonic challenge by cell-attached luciferase, which was 20 times higher than the peak ATP level measured in bulk mucosal medium following a hypotonic challenge (645.4±63.5 nM and 34.1±5.6 nM by cell-attached and bulk measurement, respectively, n=6). Utilizing luciferase dissolved in a thin film covering the apical cell surface (height 12 µm), ATP levels were similar to those detected by cell-surface attached SPA-luc both in basal and stimulated conditions (7.1±0.6 nM and 614.1±56.2 nM in basal and stimulated conditions, respectively, n=6).

Utilizing these methods, Calu-3 cells were tested to address whether cell-swelling induced ATP release from airway epithelia is dependent on changes in intracellular calcium concentration ($[Ca^{2+}]_i$). Although Calu-3 cells lack functional purinergic receptors, a 33% hypotonic challenge triggered an $[Ca^{2+}]_i$ elevation in cells bathed bilaterally in nominally calcium free buffer (fura-2 fluorescence Δ340/380 = 0.509±0.114, n=10). BAPTA treatment of these cells abolished hypotonicity-induced $[Ca^{2+}]_i$ elevations (fura-2 fluorescence Δ340/380 = 0.026±0.016, n=10) and significantly reduced ATP release (223.1±20.3 nM and 607.8±43.1 nM in BAPTA and vehicle treated, respectively, n=6, P<0.05). Bafilomycin A_1 treatment significantly reduced hypotonicity-induced ATP release (341.7±33.6 nM and 615.8±55.3 nM in bafilomycin A_1 and vehicle treated, respectively, n=6, P<0.05). These observations suggest the involvement of a calcium-dependent pathway, possibly via exocytosis, in swelling-induced ATP release. However, elevation of $[Ca^{2+}]_i$ without cellular swelling, i.e. by thapsigargin or ionomycin, resulted in an increase in ATP release to 2- to 5-fold higher than basal levels (27.2±4.8, 21.7±4.1 and 6.9±0.6 nM in thapsigargin, ionomycin and vehicle treated, respectively, n=6). Thus, Ca^{2+}_i partially regulated swelling-induced ATP release, but $[Ca^{2+}]_i$ elevation itself without cellular swelling was not sufficient to trigger maximal ATP release in Calu-3 cells.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C54

Real-time measurement of ATP release from nerve-mediated contractions in guinea pig detrusor smooth muscle

C. McCarthy[1], C. Wu[1], D. Newgreen[2] and C.H. Fry[1]

[1]Institute of Urology, University College London, London, UK and [2]Discovery Biology, Pfizer Global Research & Development, Sandwich, UK

Two neurotransmitters, ACh and ATP, initiate nerve-mediated contractions in detrusor smooth muscle from guinea pig. In human detrusor from stable bladders ACh alone is the excitatory transmitter. However with human detrusor from unstable bladders ATP emerges as an additional transmitter and there is speculation that this may contribute to the pathology (Bayliss et al 1999). We are interested in the reasons why ATP has this action in human detrusor and one hypothesis is that nerve-mediated ATP release is enhanced. To this end we have evaluated the use of an amperometric ATP sensor in guinea pig detrusor smooth muscle.

Detrusor strips were obtained from humanely killed guinea-pigs. Preparations were superfused at 37°C in a modified (glycerol 2mM) $HCO_3^-/C0_2$ Tyrodes solution (pH 7.3). An ATP sensitive sensor was calibrated in 10µM ATP solution and then placed on the muscle strip parallel to a null sensor. Contractions were elicited either by electrical field stimulation (EFS) or by agonists (carbachol 0.3µM or KCl 120mM).

Raising the frequency of EFS in the range 2-12 Hz generated increasing sensor responses (n=4). These responses were completely abolished by the neurotoxin TTX (1 μM). To quantify ATP release, the signals were integrated over five seconds, following the end of the stimulation. At 2 Hz and 12 Hz ATP release was over the range 0.14-0.54 $\mu mol.l^{-1}.s^{-1}.mg$ wet weight $^{-1}$ and 0.21-7.87 $\mu mol.l^{-1}.s^{-1}.mg$ wet $weight^{-1}$ respectively. A fraction of the ATP release with EFS may result from muscle contraction itself. This was assessed by contracting the preparation with carbachol and KCl, to elicit contractures of similar magnitude to that generated by 2-4 Hz EFS. Signals were generated by the ATP sensor that, except in one case, represented 10-30% of the signal produced by EFS. When the sensor was away from the muscle, EFS and KCl produced no response, although carbachol generated a small signal.

We have demonstrated that it is possible to record ATP release from detrusor preparations and that a fraction of this can be attributed to a nerve mediated response. This method offers a sensitive and dynamic technique to investigate the role of ATP in regulating detrusor contractions.

Bayliss M et al. (1999). J Urol 162, 1833-1839.

We thank the BBSRC for financial support and Prof N Dale for the generous gift of the ATP sensors.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

SA21

Introductory overview of ATP release

G. Burnstock

Autonomic Neuroscience Centre, University College London, London, UK

Until recently, it was usually assumed that the only source of extracellular ATP acting on purinoceptors was damaged or dying cells, but it is now recognised that ATP release from healthy cells is a physiological mechanism. There is an active debate, however, about the precise transport mechanism(s) involved. There is compelling evidence for exocytotic vesicular release of ATP from nerves, but for ATP release from non-neuronal cells, various transport mechanisms have been proposed, including ATP binding cassette (ABC) transporters, connexin or pannexin hemichannels or possibly plasmalemmal voltage-dependent anion channels (pl-VDAC), as well as vesicular release.

During purinergic mechanosensory transduction associated with pain and physiological reflexes, the ATP that acts on $P2X_3$ and $P2X_{2/3}$ receptors on sensory nerve endings is released by mechanical distortion from urothelial cells during distension of bladder and ureter and from mucosal epithelial cells during distension of the colorectum. ATP is released from vascular endothelial cells by shear stress or hypoxia and from odontoblasts, chromaffin cells, immune cells, Merkel cells, epithelial cells in the lung, cancer cells, endocrine cells, keratinocytes in the skin, glomus cells in the carotid body, astrocytes, platelets and red blood cells. Perhaps surprisingly, evidence has been presented that the release of ATP from urothelial cells in the ureter (as well as from endothelial cells) is largely vesicular, since monensin and brefeldin A, which interfere with vesicular formation and trafficking, inhibited distension-evoked ATP release, but not gadolinium, a stretch-activated channel inhibitor, or glibenclamide, an inhibitor of two members of the ABC protein family. A novel mechanism for transport of ATP has been proposed recently that involves activation of the membrane-bound protein cystic fibrosis transmembrane regulator (CFTR) by actin freed from binding by defects of the spectrin cytoskeleton produced by membrane deformation.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

SA22

Calcium-dependent ATP release from epithelial cells

S. Tatur[1] and R. Grygorczyk[2]

[1]Centre de recherche, Centre hospitalier de l'Universite de Montreal, Montreal, QC, Canada and [2]Medicine, University of Montreal, Montreal, QC, Canada

Mechanical perturbations, including hypotonic cell swelling, evoke rapid ATP release from a variety of cell types. The mechanism(s) of such release from epithelial cells and the regulatory pathways remain obscure. Two prevailing models propose conductive release, via ATP-permeable channels, or exocytosis of ATP-filled vesicles (Lazarowski et al. 2003). In a previous study, we showed that hypotonicity-evoked ATP release from human lung epithelial A549, airway epithelial 16HBE14o$^-$ cells and 3T3 fibroblasts does not involve gadolinium-sensitive channels or volume-sensitive, 5-nitro-2-(3-phenylpropylamino) benzoic acid-inhibitable anion channels. It was, however, tightly dependent on intracellular Ca^{2+} elevation and abolished in BAPTA-loaded cells, implicating vesicular exocytosis (Boudreault & Grygorczyk 2002, Boudreault & Grygorczyk 2004). In the present study, we explored, in more detail, modulation of ATP secretion via the intracellular Ca^{2+} and protein kinase C (PKC) signaling pathways in A549 cells.

ATP release from confluent A549 cell monolayers was investigated using a custom-made flow-through chamber (Boudreault & Grygorczyk 2004). ATP content in the samples was measured by lucifersase-luciferin assay, and ATP calibration curves were corrected for any inhibitory effect of the tested drugs on luciferase reaction.

Under control conditions, 50% hypotonic shock triggered transient ATP release that peaked at around 1 min 45 s, and declined to baseline during the next 15 min. The peak rate of ATP secretion was 401 ± 49 pmol min^{-1} $(10^6$ cells$)^{-1}$, while cumulative ATP release during 15 min was $1{,}142 \pm 151$ pmol $(10^6$ cells$)^{-1}$ (n = 5). Removal of extracellular Ca^{2+} had no effect on ATP secretion, indicating that it depends entirely on Ca^{2+} release from intracellular stores. Thapsigargin (1 μM, 1 h), a sarco-endoplasmic reticulum Ca^{2+}-ATPase inhibitor, caused 57% ± 15% reduction of the peak rate of ATP release (n = 4, 23% ± 3% decrease of total ATP released). Caffeine (10 mM), which depletes intracellular Ca^{2+} stores by activating ryanodine-sensitive Ca^{2+} release channels, diminished ATP release by 34% ± 9% (n = 4). Heparin (1 mg/ml, 30 min), known to interfere with the IP3 receptor channel, reduced the peak of ATP release by 18% ± 6% (n = 4). Precise evaluation of the heparin effect was, however, hampered by its strong interference with the luciferase reaction. The kinetics of ATP release were strongly affected by treatment with PMA (1 μM, 15 min), a PKC activator. The peak rate was reduced by 71% ± 4% (n = 4), but release did not decline to baseline as rapidly as in control, untreated cells, and remained significantly elevated for more than 10-15 min. As a result, total ATP released during the 15-min period was slightly enhanced to 128% ± 19% compared to control cells. Brefeldin A (10 μM, 2.5 h), which blocks protein secretion by disrupting the Golgi complex and vesicle trafficking, reduced the peak rate of ATP release by 53% ± 24% (n = 3), suggesting that protein transport vesicles contribute to ATP release. Together with previously-found complete inhibition of ATP release by low temperature (Grygorczyk & Boudreault 2004), our data strongly support the concept that Ca^{2+}-dependent vesicular exocytosis is a major mechanism of cell swelling-induced ATP secretion from A549 epithelial cells. This secretion is modulated by PKC and Ca^{2+} release from intracellular stores, involving at least two types of Ca^{2+} release channels. It does not, however, require direct extrcellular Ca^{2+} entry.

Lazarowski ER, Boucher RC & Harden TK (2003). Mol Pharmacol 64, 785-795.

Boudreault F & Grygorczyk R (2002). Am J Physiol Cell Physiol 282, C219-C226.

Boudreault F & Grygorczyk R (2004). J Physiol 561, 499-513.

Supported by the Canadian Institute of Health Research and the Canadian Cystic Fibrosis Foundation.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

SA23

Regulated release of nucleotides and UDP-sugars from the secretory pathway in epithelial cells

E.R. Lazarowski, C. van Heusden and S. Kreda

Medicine, University of North Carolina, Chapel Hill, NC, USA

Extracellular nucleotides regulate several components of the mucociliary clearance (MCC) process that removes noxious materials from the lung. Adenine and uridine nucleotides promote ion transport, coordinate cilia beating, and stimulate mucin secretion. While it is well established that extracellular ATP and other nucleotides promote MCC activities by activating cell surface purinergic receptors, it is largely unknown how nucleotides reach the extracellular space. Circumstantial evidence exists for a plasma membrane channel that conducts cytosolic nucleotides and for the involvement of a secretory pathway(s), but unambiguous proof of conductive or vesicular nucleotide release is lacking. Using radiolabeling and fluorescence HPLC-based approaches, adenosine, ATP/UTP, UDP, and UDP-glcuose, the naturally occurring agonists for the A2b, P2Y2, P2Y6, and P2Y14 receptors, respectively, were quantified in airway surface liquid with nanomolar sensitivity. Unlike short-lived ATP and UTP, UDP-glucose released into ASL was hydrolyzed only slowly. Nucleotides and UDP-sugars were released selectively to the mucosal surface of polarized Calu-3 cell monolayers. Since adenine nucleotides, UDP, and UDP-sugars participate in phosphorylation, sulfatation, and glycosylation reactions in the lumen of the Golgi apparatus, we hypothesize that apical surface expression of glycoproteins provides a mechanism for the vesicular release of nucleotides from airway epithelia. Electron-microscopy of Calu-3 cells revealed prominent sub-apical electro-lucent granules that resemble mucin-packed core-dense granules of goblet cells. In addition, confocal microscopy indicated MUC1 and MUC5AC immunoreactivity in the apical region of Calu-3 cells. Stimulation of mucin secretion with ionomycin resulted in enhanced release of nucleotides and UDP-sugars from Calu-3 cells as well as from A459, SPOC1 and HT-29 epithelial cells. Calu-3 cells labeled with FM 1-43, a water-soluble probe that becomes fluorescent upon binding to lipids, displayed robust ionomycin-promoted vesicle-plasma membrane recycling. These results suggest that nucleotides and nucleotide-sugars in the lumen of the secretory pathway are released to the extracellular environment via regulated exocytosis. In chronic lung diseases characterized by mucin-obstructed airways, UDP-sugar release from goblet cells may provide paracrine signaling to P2Y14 receptors on inflammatory cells

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

SA24

Exploring the Functional Role of Hemichannels on ATP Release

L. Bahima[1], M. Martin-Satue[1], X. Grandes[1], M. Elias[1], J. Blasi[1], L. Barrio[2] and C. Solsona[1]

[1]Pathology and Experimental Therapeutics, IDIBELL-University of Barcelona, Hospitalet de Llobregat, Barcelona, Spain and [2]Unit of Experimental Neurology, "Ramon y Cajal" Hospital, Madrid, Madrid, Spain

Gap junctions communicate and coordinate the activity of neighbouring cells. They have been found in various cells types such as cardiomyocytes, smooth muscle fibres, epithelial cells and astrocytes. Communication between the cytoplasm of adjacent cells is established through an aqueous pore. Each cell contributes half the intercellular channel. Each moiety is known as a connexon, a hemichannel or a gap junction hemichannel. Hemichannels can be found in the plasma membrane alone representing some intermediate step in the turnover of gap junctions. However, some authors suggest they may have a physiological role.

In astrocytes, experimental evidence suggests that hemichannels contribute to communication between cells by releasing ATP, which in turn activates purinergic receptors and eventually generates a propagating calcium wave.

Probably the most frequent cell pathway of ATP release is the exocytosis of vesicles or granules, although hemichannels are theoretically permeable to ATP. We have examined the direct role of connexins in the release of ATP, using Xenopus oocytes. They contain a particular kind of connexin called Cx38, which forms hemichannels that are activated by physiological solutions devoid of divalent cations. When Cx38 is activated, we find a release of ATP, which is dependent on the level of expression of Cx38 and is sensitive to gap junction blockers. The activity of Cx38 hemichannels can be modulated by other membrane proteins; for example, it is inhibited by syntaxin 1A, a SNARE protein. Syntaxin forms complexes that control vesicle exocytosis, but also modulates the activity of voltage-dependent calcium channels and CFTR-supported chloride currents.

Moreover, mutations in human connexins have been related to certain disorders. X-linked Charcot Marie Tooth disease has been related to mutations in Cx32, and a form of deafness is related to Cx26. By combining the two electrode voltage clamp technology and the use of luciferin-luciferase reaction in single Xenopus oocytes, we have simultaneously measured the activation of Cx32 and ATP release. In oocytes transfected with Cx32 cRNA, the release of ATP was associated with a tail current when oocytes were depolarized. In addition, Cx32 hemichannel activity is sensitive to the extracellular calcium concentration. Indeed, both the current and the amount of ATP released were higher when the oocytes were depolarized in solutions with low calcium concentration.

All these results strongly support the view that ATP is released through hemichannels in certain conditions. Changes in the permeability of hemichannels may raise the extracellular concentration of ATP, surpassing a toxic threshold that may induce cell death and pathological alterations.

Ministerio de Ciencia y Tecnologa, Spanish Government

Where applicable, the experiments described here conform with Physiological Society ethical requirements.