Protease-Activated Receptor-2 Peptides Activate Neurokinin-1 Receptors in the Mouse Isolated Trachea

Hugh T. Abey, David P. Fairlie, James D. Moffatt, Rowan W. Balzary, and Thomas M. Cocks

Department of Pharmacology, University of Melbourne, Parkville, Australia (H.T.A., R.W.B., T.M.C.); Institute for Molecular Bioscience, University of Queensland, Brisbane, Australia (D.P.F.); and Sackler Institute of Pulmonary Pharmacology, King’s College, London, United Kingdom (J.D.M.)

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ABSTRACT

Protective roles for protease-activated receptor-2 (PAR2) in the airways including activation of epithelial chloride (Cl−) secretion are based on the use of presumably PAR2-selective peptide agonists. To determine whether PAR2 peptide-activated Cl− secretion from mouse tracheal epithelium is dependent on PAR2, changes in ion conductance across the epithelium [short-circuit current (Isc)] to PAR2 peptides were measured in Ussing chambers under voltage clamp. In addition, epithelium-and endothelium-dependent relaxations to these peptides were measured in two established PAR2 bioassays, isolated ring segments of mouse trachea and rat thoracic aorta, respectively. Apical application of the PAR2 peptide SLIGRL caused increases in Isc, which were inhibited by three structurally different neurokinin receptor-1 (NK1R) antagonists and inhibitors of Cl− channels but not by capsaicin, the calcitonin gene-related peptide (CGRP) receptor antagonist CGRP8–37, or the nonselective cyclooxygenase inhibitor indomethacin. Only high concentrations of trypsin caused an increase in Isc but did not affect the responses to SLIGRL. Relaxations to SLIGRL in the trachea and aorta were unaffected by the NK1R antagonist nolpitantium (SR 140333) but were abolished by trypsin desensitization. The rank order of potency for a range of peptides in the trachea Isc assay was 2-furoyl-LIGRL > SLCGRL > SLIGRL = SLIGRT > LSIGRL compared with 2-furoyl-LIGRL > SLIGRL > SLIGRT > LSIGRL (LSIGRL inactive) in the aorta relaxation assay. In the mouse trachea, PAR2 peptides activate both epithelial NK1R coupled to Cl− secretion and PAR2 coupled to prostaglandin E2-mediated smooth muscle relaxation. Such a potential lack of specificity of these commonly used peptides needs to be considered when roles for PAR2 in airway function in health and disease are determined.

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ABBREVIATIONS: PAR2, protease-activated receptor(s)-2; PGE2, prostaglandin E2; SR 140333, nolpitantium; GR205171, 3(S)-(2-methoxy-5-(5-trifluoromethyltetrazol-1-yl)-(phenylethylamino)-2(S)-phenylpiperidine; GR82334, d-Pro[9]-[spiro-gamma-lactam]-Leu[10]-Trp[11]-physalaemin-(1-11); DIDS, 4,4-diisothio-cyanostilbene-2,2-disulfonic acid; NK1R, neurokinin-1 receptor(s); CGRP, calcitonin gene-related peptide; Isc, short-circuit current; ANOVA, analysis of variance.
Cl⁻ secretion would maintain the protective layer underneath the mucus and as such aid in the efficient removal of inhaled antigens and has been proposed as a possible protective role of epithelial PAR₂ (Cocks and Moffatt, 2000). In the mouse trachea, Kunzelmann et al. (2005) reported that PAR₂ located on the basolateral surface of epithelial cells couple to basolateral K⁺ channels and mucosal Cl⁻ channels to mediate net Cl⁻ secretion into the lumen of the airways. They also suggested that these basolateral PAR₂ as well as apical PAR₂ are coupled to the production of PGE₂ to mediate smooth muscle relaxation. As in our previous study (Cocks et al., 1999), Kunzelmann et al. (2005) similarly reported that PAR₂ are predominantly expressed on the apical surface of mouse tracheal epithelial cells. Activation of these apically expressed PAR₂, however, with either trypsin or SLIGRL, did not have any effect on ion conductance (Kunzelmann et al., 2005). In the present study we found an increase in Cl⁻ conductance to SLIGRL in the mouse trachea that was not PGE₂-dependent and was not mimicked by trypsin or affected by prior exposure to high concentrations of trypsin. In addition, the supposedly inactive PAR₂ peptide LSIGRL (Lindner et al., 2000; Fiorucci et al., 2001; Stenton et al., 2002) caused a similar increase in Cl⁻ conductance to SLIGRL. Furthermore, the increase in Cl⁻ conductance across the epithelium in response to SLIGRL was blocked by three structurally different NK₁R antagonists, SR 140333, GR205171, and GR82334. Thus, results from our study indicate that PAR₂-like peptides directly activate apical NK₁R coupled to Cl⁻ secretion and as such may need to be considered when roles for PAR₂ are claimed, particularly in inflammatory diseases of the airways.

Materials and Methods

Materials. The following drugs were used: 4,4-diisothio-cyanostilbene-2,2-disulfonic acid (DIDS), acetylcholine chloride, ATP, capsaicin, carbachol, carbachol chloride, carbachol hydrochloride, trypsin, phenylephrine hydrochloride, DMSO, ethanol, acetic acid, L-glutamic acid, D-glucose, glycine, 1 M Na₂CO₃, 1 M NaHCO₃, 1 M NaCl, 1 M MgCl₂, 1 M CaCl₂, 1 M KCl, 1 M SO₄, 1 M H₂PO₄, and 1 M HEPES. Materials and methods were followed as described in previous studies (Mages et al., 2003). All cells were plated in 96-well cell culture plates (American Type Culture Collection, Manassas, VA) and grown to confluence. Cells were then loaded with 2 µM Fura-2/acetoxymethyl ester (Molecular Probes, Eugene, OR) with 0.1% Fluoronic F127 to facilitate more even dispersion of the dye for 30 min at 37°C and 5% CO₂. After this incubation, cells were rinsed twice and resuspended in a Krebs-HEPES buffer with 1 mM probenecid (Sigma Chemical) and rinsed with Dulbecco’s modified Eagle’s medium with HEPES (Gibco, Grand Island, NY). Pelleted cells were then incubated with the specific antagonist at room temperature for the final 10 min of de-esterification. At the completion of this procedure, all cells were placed on ice until use to reduce leakage of dye. Cells were plated in black 96-well plates (BMG LabTechnologies) at a density of 2 × 10⁵ cells · ml⁻¹. Microplates were then inserted into the preincubated plate reader (37°C). Estimation of [Ca²⁺], was achieved by exposure of the cells to rapidly alternating 340- and 380-nm wavelengths and measuring the resulting 510-nm emission from the Fura-2/acetoxymethyl ester. After a 30-s baseline reading, test compounds were injected via the plate reader’s prefitted injectors into each specified well.

Statistics. All responses are expressed as means ± S.E.M. of 3-6 independent experiments. The pEC₅₀ values were determined by nonlinear regression analysis using Prism 4.0a (GraphPad Software, San Diego, CA). Statistical comparisons were performed with either paired or nonpaired Student’s t tests or analysis of variance (ANOVA) as appropriate (Snedecor and Cochran, 1989).
Results

Tension and $I_{SC}$ Responses to Trypsin and PAR Peptides. In isometric tension studies of mouse airways, trypsin (0.01–3 U ml$^{-1}$) caused concentration-dependent relaxations of mouse trachea (Fig. 1). Trypsin is known to cleave the N terminus of PAR receptors, creating tethered agonist ligands that activate PARs, leading to tracheal relaxation (Cocks et al., 1999; Chow et al., 2000; Lan et al., 2001). Similar relaxant responses produced by the PAR$_2$ peptide agonist, the isolated N-terminal hexapeptide of rodent PAR$_2$ SLIGRL (0.1–30 μM), were prevented if the preparation was initially desensitized to trypsin (3 U ml$^{-1}$) (Fig. 1). However, in the Ussing chamber assay, using an identical protocol, trypsin failed to either elicit a response or prevent $I_{SC}$ responses to SLIGRL (Fig. 1) when added to either side of the preparation (not shown) at concentrations 10-fold in excess of those required for 50% relaxation. SLIGRL was only active if applied to the mucosal surface of the preparations (Fig. 2). A 33-fold higher concentration of trypsin (100 U ml$^{-1}$) caused a small, slowly developing increase in $I_{SC}$ (Fig. 1), again only if applied to the mucosal surface (Fig. 2). However, at the plateau of this response, the addition of SLIGRL elicited a further, sharp increase in $I_{SC}$ that was not different in magnitude to the response to SLIGRL alone, suggesting that trypsin and SLIGRL do not act via the same mechanism (Fig. 1).

Epithelium-dependent smooth muscle relaxations to trypsin and SLIGRL in the tracheal mechanical assay (Figs. 1 and 6) were within the previously reported range (Cocks et al., 1999) with EC$_{50}$ of 0.5 ± 0.1 U ml$^{-1}$ and pEC$_{50}$ of 5.6 ± 0.1, respectively. Two novel PAR$_2$ peptide agonists, SLIGRT (pEC$_{50}$ 5.9 ± 0.2) and SLCGRL (pEC$_{50}$ 5.3 ± 0.2), were approximately 6- and 20-fold less potent, respectively, than SLIGRL (pEC$_{50}$ 6.4 ± 0.1) in causing endothelium-dependent relaxation of the rat aorta preparation (Fig. 3), which has previously been shown to relax in response to SLIGRL via PAR$_2$ in an endothelium-dependent manner (Hwa et al., 1996; Saifeddine et al., 1998). In addition, we confirmed that a recently reported stable PAR$_2$ peptidomimetic agonist, 2-furoyl-LIGRL, was more potent (pEC$_{50}$ 7.3 ± 0.1) than SLIGRL in this assay (Kawabata et al., 2004). By contrast, another novel PAR$_2$ peptide agonist, SLSGRT, and the commonly used partial scramble peptide for SLIGRL, LSIGRL (Lindner et al., 2000; Fiorucci et al., 2001; Stenton et al., 2002), were both inactive in the aorta up to 100 μM (Fig. 3). Thus, an estimated rank order of potency for these peptides in the rat aortic endothelium-dependent relaxation assay was 2-furoyl-LIGRL > SLIGRL > SLIGRT > SLCGRL, with LSIGRL and SLSGRT inactive. Also in the aorta, responses to 2-furoyl-LIGRL, SLIGRL, SLIGRT, and SLCGRL were abolished if preparations were desensitized to trypsin (3 U ml$^{-1}$; data not shown), suggesting that all peptides activate PAR$_2$. In the Ussing chamber mouse tracheal assay, however, SLCGRL, SLIGRT, and LSIRGL, but not SLSGRT, induced $I_{SC}$ increases over concentration ranges similar to those of SLIGRL. 2-Furoyl-LIGRL, however, seemed to be more potent than any of the other active peptides but with a maximal increase in $I_{SC}$ of only 9.0 ± 3.0 μA cm$^{-2}$ at a concentration of 3 μM (Fig. 3). Therefore, an apparent rank order of potency of the peptides was 2-furoyl > SLCGRL >
SLIGRT = SLIGRL > LSIGRL and SLSGRT inactive (Fig. 3), with 2-furoyl-LIGRL appearing to act as a partial agonist.

**Involvement of Cl⁻ Conductance and Prostanoids in I_{SC} Responses to SLIGRL.** Replacement of Cl⁻ with gluconate abolished I_{SC} responses to SLIGRL (Fig. 4). In addition, of glibenclamide (100 μM) and DIDS (100 μM), inhibitors of cystic fibrosis transmembrane conductance regulator and Ca^{2+}-activated Cl⁻ channels, respectively, only glibenclamide inhibited the response to SLIGRL by approximately 50%. In addition, combined glibenclamide and DIDS reduced the response to SLIGRL by 80% (Fig. 4). Indomethacin (3 μM) had no significant effect on the I_{SC} response curve to SLIGRL (Fig. 5).

**Effects of Substance P, Capsaicin, Tachykinin, and CGRP Antagonists.** Substance P caused increases in I_{SC} in the mouse trachea over two concentration ranges. The threshold concentration of the first range varied from 0.01 to 1 μM with the maximum occurring at 0.1 to 1.0 μM. Threshold concentration for the second phase of the I_{SC} curve to substance P usually occurred at ~10 μM and was maximum at approximately 1 mM (Fig. 5). The selective NK₁ antagonist SR 140333 (0.1 μM) abolished the first phase of the substance P curve and caused an estimated approximate 1000-fold shift to the right in the second component (Fig. 5). SR 140333 (0.1 μM) had no effect on the increase in I_{SC} to substance P by approximately 50%. In addition, the selective NK₁ antagonist SR 140333 (100 nM) had no inhibitory effect on the relaxation curve to SLIGRL, whereas the biphasic response curve to substance P was abolished by SR 140333 (Fig. 6). In addition, in rat thoracic aorta, in which substance P failed to cause any mechanical responses (either endothelium-dependent relaxation or contraction; data not shown), SR 140333 had no effect on the relaxation curve to SLIGRL and L(SLIGRL was inactive (Fig. 6).

**Ca^{2+} Measurement in A549 Cells.** Substance P (100 nM) caused little if any increase in intracellular Ca^{2+} in surface of the tracheal preparation (data not shown). In addition, treatment of the luminal side with the mast cell-degranulating agent, compound 48/80 (10 μM for 20 min), had no effect on I_{SC} nor did it inhibit increases in I_{SC} to SLIGRL (data not shown). Surprisingly, perhaps, indomethacin (3 μM) caused a significant ~10-fold rightward shift in the concentration-dependent I_{SC} curve to substance P (Fig. 5). As for SLIGRL (Fig. 5), similar indomethacin treatment failed to have any effect on the increases in I_{SC} to ATP (data not shown).

In tension assays in the mouse trachea, the NK₁ antagonist SR 140333 (100 nM) had no inhibitory effect on the relaxation curve to SLIGRL, whereas the biphasic response curve to substance P was abolished by SR 140333 (Fig. 6). In addition, in rat thoracic aorta, in which substance P failed to cause any mechanical responses (either endothelium-dependent relaxation or contraction; data not shown), SR 140333 had no effect on the relaxation curve to SLIGRL and L(SLIGRL was inactive (Fig. 6).
A549 cells, and the response to SLIGRL was unaffected by SR 140333 but was inhibited after desensitization with trypsin (Fig. 7), confirming the above tension data in the rat thoracic aorta. Clearly, SR 140333 had no inhibitory effect at PAR2.

Discussion

The results of this study indicate that the supposed selective PAR2 peptide agonist SLIGRL is an agonist of NK1R located on the apical surface of epithelial cells of the mouse trachea. Therefore, given the accepted role of substance P and NK1R in asthma (Barnes, 1998), any claims for roles of PAR2 in inflammatory airway disease that are based solely on data from experiments using SLIGRL or other similar PAR2-activating peptides may need to be reconsidered in light of our study.

Unlike Kunzelmann et al. (2005), we found no change in Isc to the PAR2 activators trypsin and SLIGRL when they were applied to the basolateral surface of mouse tracheal epithelial cells. In addition, contrary to our findings here, Kunzelmann et al. (2005) found no response to apically applied trypsin or SLIGRL, even though, as we reported earlier (Cocks et al., 1999), they found that PAR2 was predominantly expressed on the apical surface of mouse tracheal epithelial cells. Both the inability of Kunzelmann et al. (2005) to demonstrate responses to apically applied SLIGRL and our inability to similarly show responses to basolaterally applied SLIGRL most likely reflect a lack of NK1R expressed on the mucosal surface of airway epithelial cells in the strain of mice used by Kunzelmann et al. (2005) and the inability of basolateral PAR2 to couple to Cl− secretion in the strain of mouse (BALB/c) used in our study.

The increase in Isc to SLIGRL in the trachea was most likely due to NK1R-mediated stimulation of luminal Cl− secretion as it was inhibited by removal of Cl− from the bathing Krebs’ solution, by inhibitors of cystic fibrosis transmembrane conductance regulator (glibenclamide) (Schultz et al., 1999) and Ca2+−activated (DIDS) (Kidd and Thorn, 2000) Cl− channels, and by three potent, selective and structurally dissimilar NK1R antagonists (Emonds-Alt et al., 1993; Maggi et al., 1994; Gardner et al., 1996; Moriarty et al., 2001). In addition, the increase in Isc to SLIGRL only occurred when the peptide was applied to the luminal surface of the trachea.
carbachol (A and B) and phenylephrine (C) contractions. Data points are means ± S.E.M. Number of experiments for control and SR 140333 curves were (A) n = 6, (B) n = 14, and (C) n = 3. Number of experiments for LSIGRL and substance P were n = 3 (A and C, respectively).

Fig. 6. The effect of SR 140333 (●, 0.1 μM) on relaxations to SLIGRL (●) or substance P (▲) in smooth muscle relaxation assays of mouse trachea (A and B) and rat aorta (C). LSIGRL (●) was inactive in both preparations. Responses are expressed as percentage of relaxations of 6.5/4.5/3/2/1 M)-induced increases in free intracellular calcium as measured by changes in the emission wavelength ratio 340/380 nm of Fura-2) in A549 cells. Also shown is the near-zero average number of experiments for LSIGRL and substance P were n = 3 (A and C, respectively).

and was unaffected by indomethacin. Together, these results indicate that SLIGRL causes an increase in luminal Cl⁻ secretion in the mouse trachea via activation of NK₁R located on the apical surfaces of epithelial cells and that the resultant increase in I_sc is not due to concomitant PAR₂-dependent abluminal PGE₂ release (Asokananthan et al., 2002), which has been shown previously to mediate epithelium-dependent relaxation in this tissue (Cocks et al., 1999; Lan et al., 2001; De Campo and Henry, 2005).

Fortner et al. (2001b) have claimed that substance P causes similar increases in luminal Cl⁻ conductance in the mouse airway but that this activity is linked in some way to smooth muscle relaxation, which the same group showed was due to concomitant abluminal release of PGE₂ from the epithelium (Fortner et al., 2001). A possible simple explanation for this rather confusing concept is that some of the PGE₂ released contraluminally into the wall of the airway activates basolateral EP receptors to increase luminal ion conductance. This idea is supported by our current finding that part of the increase in ion conductance to luminal substance P was inhibited by indomethacin, which abolished the concomitant relaxation and which, like that to ATP, is epithelium-dependent (Kao et al., 1999). The lack of effect of indomethacin on increases in I_sc to SLIGRL observed here is most likely due to the relatively weak efficacy of SLIGRL at luminal NK₁R, such that levels of PGE₂ released abluminally are insufficient to stimulate basolateral epithelial EP receptors to increase I_sc. Likewise, the failure of indomethacin to block relatively large increases in I_sc to ATP indicates poor coupling of the purinoceptor involved in PGE₂ synthesis. This possibility is supported by ATP only being able to cause weak epithelium-dependent relaxations in the mouse trachea which, like those for SLIGRL and substance P, are abolished by indomethacin (J. D. Moffatt and T. M. Cocks, unpublished data).

Because SLIGRL, 2-furoyl-LIGRL, LSIGRL, and SLCGRL are all cationic with arginine at position 5, it is possible that these and other peptides increase I_sc indirectly via nonreceptor-dependent degranulation of mast cells (Stenton et al., 2002). That the response is blocked by three different NK₁R antagonists, however, still implies a role for NK₁R, which is possible as rat colonic mast cells have been shown to release substantial amounts of substance P in response to anti-IgE, which was inhibited by SR 140333 (Moriarty et al., 2001). However, such a nonspecific action on mast cells is unlikely to explain the current findings for two reasons. First, we found that the mast cell degranulating agent compound 48/80 failed to cause an increase in I_sc or affect subsequent I_sc responses to SLIGRL (H. T. Abey and T. M. Cocks, unpublished data). The second reason that mast cells are unlikely to be involved in the increase in I_sc to PAR₂ peptides is that capsaicin, which failed to affect I_sc increases to SLIGRL in the present study, has been shown to deplete mast cells of substance P (Moriarty et al., 2001), most likely via activation of vanilloid VR₁ receptors (Biro et al., 1998). Furthermore, another novel R² cationic peptide, SLSGRT, failed to cause either an increase in I_sc in the mouse trachea or smooth muscle relaxation in both the mouse trachea and rat thoracic aorta. That single amino acid substitutions can markedly modify the binding characteristics of these peptides further argues against a nonspecific action of these molecules such as their gross charge.

Substance P and capsaicin both caused increases in I_sc in the mouse trachea. Therefore, it is possible that the similar
responses to PAR2 peptides in this preparation were due to their ability to cause release of substance P from sensory nerves, either directly through activation of PAR2 on sensory nerves (Fiorucci et al., 2001; Kawabata et al., 2001; Su et al., 2005) or indirectly via a mechanism similar to that reported for mast cells (Stenton et al., 2002). This seems unlikely, however, because sensory neuropeptide depletion with prolonged exposure to capsaicin had no inhibitory effect on the ISC response to SLIGRL. In addition, any role for sensory nerves in the ISC response to SLIGRL was further ruled out by the lack of effect of an antagonist (CGRPα,α,α) of the other major sensory neurotransmitter in the airways, CGRP (Barnes, 1998). Rather, combined with the different rank order of potencies of 2-furoyl-LIGRL, SLIGRL, LSIGRL, SLIGRT, and SLCGRL for ion secretion compared with that for endothelium-dependent nitric oxide production in the rat aorta (Hwa et al., 1996), the lack of activity of LSIGRL in both the mouse trachea and rat aortic smooth muscle assays, and the selectivity of SR140333 for substance P in the ISC and airway relaxation assays, our data suggest that SLIGRL directly activates NK1R located on the epithelium.

The biphasic concentration-ISC curve to substance P may reflect two molecular isoforms of NK1R that have been shown in molecular reconstruction studies to be determined by the type of G-protein to which each NK1R form couples (Holst et al., 2001). In addition, the high potency of substance P in this assay may reflect high affinity with both isoforms of the receptor. If all of the PAR2 peptides activated the same isoform of NK1R to increase ISC, then some structure activity can be deduced. Thus, their rough rank order of potency (2-furoyl-LIGRL > SLCGRL > SLIGRL = SLIGRT > LSIGRL with SLSGRL inactive) indicates that reversal of the first two amino acids diminishes NK1R activity, S3 for F3 substitution abolishes it, C4 for I4 substitution appears to increase it, T4 for L6 substitution has little effect, and substitution of 2-furyl for S1 increases potency but markedly reduces efficacy. This apparent weak partial agonist activity of 2-furoyl-LIGRL at NK1R in the mouse trachea compared with its potent, full agonist profile at PAR2 in the rat aorta makes it an attractive alternative to SLIGRL for further research into the biology of PAR2, particularly if complicating effects of NK1R are possible.

The results presented here allow a closer examination of some recent studies in which PAR2 has been suggested to mediate effects in the airways but in which the pharmacological data presented are not always consistent with classic PAR2 pharmacology (Moffatt, 2004). In some studies, the nonspecific mechanism of action may account for the findings rather than PAR2 per se. For example, high micromolar concentrations of PAR2-activating peptides have been shown to increase ISC in human airway epithelial cells (Danahay et al., 2001), act as a mitogen for airway smooth muscle (Chambers et al., 2000), and activate eosinophils (Miike et al., 2001). Although trypsin may also elicit a response in these studies, it is often not practically possible to perform cross-desensitization studies to establish that trypsin and peptide activators act via a common mechanism. Furthermore, concentrations of trypsin that are supramaximal for PAR2 activation, as we found here in the ISC response to trypsin, are often required to elicit a response. Therefore, in the absence of PAR2 antagonists and the ability to cross-desensitize with trypsin, PAR2 knockout animals provide the only certain information regarding the role of this receptor in airway inflammation. Thus, Schmidlin et al. (2002) showed that the early but not the late phase of immune cell trafficking into the airway lumen after ovalbumin challenge in ovalbumin-sensitized wild-type mice was reduced and increased in PAR2 knockout and PAR2 overexpressing transgenic mice, respectively, suggesting that PAR2 was indeed involved in this early-phase allergic response in the airways. However, unexplained effects of PAR2 deletion on the production of IgE in response to sensitization (Schmidlin et al., 2002) may account for this difference.

In conclusion, roles for PAR2 in airway barrier defense, inflammation, and other processes will only be unequivocally resolved with the development of more selective PAR2 ligands (agonists and antagonists). Although reported PAR2-binding peptides are typically selective for PAR2 over PAR1, they may not be selective for PAR2 over other GPCRs (e.g., NK1R), and, as such, receptor specificity beyond simple PAR2 subtypes clearly needs to be considered when one draws conclusions regarding the roles of PAR2 in airway inflammation.

References


Address correspondence to: Dr. Thomas M. Cocks, Department of Pharmacology, The University of Melbourne, Victoria, 3010, Australia. E-mail: thomasmc@unimelb.edu.au

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