Characterization of mouse urea transporters UT-A1 and UT-A2

R. A. FENTON,1 G. S. STEWART,1 B. CARPENTER,1 A. HOWORTH,1 E. A. POTTER,1 G. J. COOPER,2 AND C. P. SMITH1

1School of Biological Sciences, University of Manchester, Manchester, M13 9PT; and
2Department of Biomedical Science, University of Sheffield, Sheffield, S10 2TN United Kingdom

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Fenton, R. A., G. S. Stewart, B. Carpenter, A. Howorth, E. A. Potter, G. J. Cooper, and C. P. Smith. Characterization of mouse urea transporters UT-A1 and UT-A2. Am J Physiol Renal Physiol 283: F817–F825, 2002. First published June 4, 2002; 10.1152/ajprenal.00263.2001.—Specialized transporter proteins that are the products of two closely related genes, UT-A (Slc14a2) and UT-B (Slc14a1), modulate the movement of urea across cell membranes. The purpose of this study was to characterize the mouse variants of two major products of the UT-A gene, UT-A1 and UT-A2. Screening a mouse kidney inner medulla cDNA library yielded 4,047- and 2,876-bp cDNAs, the mouse homologues of UT-A1 and UT-A2. Northern blot analysis showed high levels of UT-A mRNAs in kidney medulla. UT-A transcripts were also present in testes, heart, brain, and liver. Immunoblots with an antiserum raised to the 19 COOH-terminal amino acids of rat UT-A1 (L194) identified immunoreactive proteins in kidney, testes, heart, brain, and liver and showed a complex pattern of differential expression. Relative to other tissues, kidney and brain had the highest levels of UT-A protein expression. In kidney sections, immunostaining with L194 revealed immunoreactive proteins in type 1 (short) and type 3 (long) thin descending limbs of the loop of Henle and in the middle and terminal inner medullary collecting ducts. Expression in Xenopus laevis oocytes showed that, characteristic of UT-A family members, the cDNAs encoded phloretin-inhibitable urea transporters. Acute application of PKA agonists (cAMP/forskolin/IBMX) caused a significant increase in UT-A1- and UT-A3-, but not UT-A2-mediated, urea transport.

Significant amounts of UT-A3 are expressed in the cytoplasm of rat IMCD cells (29), and UT-A3 mRNA levels increase in response to thirsting (2, 6). In addition, a 24-h treatment with forskolin of HEK-293 cells heterologously expressing UT-A3 caused an increase in urea uptake (11). Together, these observations suggest that UT-A3 may play a role in the urinary concentrating mechanism and may also be regulated by PKA agonists. However, in contrast to the stimulatory effects of PKA agonists on UT-A3 regulation reported by Karakashian et al. (11), Shayakul and colleagues (26) characterized the function of UT-A3 by using Xenopus laevis oocyte expression and found that a mixture of CAMP agonists (dibutyryl CAMP, IBMX, and forskolin) did not increase UT-A3 activity. At present, the reason for this discrepancy between the two studies is unknown.

Compared with other members of the UT-A family, relatively little is known about UT-A4. UT-A4 mRNA has been detected in the outer medulla, and a protein proposed to be UT-A4 has been detected in the outer medulla by immunoblotting (11, 30), but the nephron location of UT-A4 has not been determined.

Although the rat remains the species of choice for many renal studies, the mouse is the preferred species for gene deletion or disruption studies. This has led to a demand for basic information regarding murine biology and, in particular, information that adds functional context to molecular data. Compared with rats,
relatively little is known about mouse (m)UT-A isoforms. The molecular characteristics of mUT-A3 and a novel isoform, mUT-A5, have been reported (8). mUT-A3 has a high degree of identity with rat UT-A3, and, as in the rat kidney, mUT-A3 mRNA localizes to the kidney inner medulla. mUT-A5 is an NH₂ terminally truncated variant of mUT-A3. It lacks the first 139 amino acids of mUT-A3 and is expressed within seminiferous tubules in the testes.

Given the need for information pertaining to mouse urea transporters, we have isolated two cDNAs from mouse kidney encoding mUT-A1 and mUT-A2. Here, we report their molecular characteristics and show that PKA agonists acutely stimulate urea transport meditated by UT-A1 or UT-A2 but not UT-A2, and, furthermore, we resolve the discrepancy between previous studies on PKA stimulation of UT-A3 (11, 26).

**METHODS**

**Clone isolation.** cDNA clones were isolated from an unamplified, size-selected (> 1.5 kb) MF1 mouse inner medulla λ (λGT10) μgt10 cDNA library (8). Initially, ~300,000 clones were screened using the following gel-purified, 32P-labeled (Rediprime II; Amersham Pharmacia Biotech) probes: nucleotides 1–219 of mUT-A3 (GenBank accession no. AF258602) probe 1 (8), or a 427-bp PCR product corresponding to bp 281–708 of mUT-A2 (characterized in Ref. 7). Hybridization was at 48°C in a solution containing 10% (wt/vol) dextran sulfate and 50% formamide. Final washes were carried out at 65°C in 0.1× SSC, 0.1% SDS. Resultant clones from these screens were subcloned into the pBluescript SK(–) vector (Stratagene), and both strands were sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems).

**Xenopus oocyte expression experiments.** These were performed as previously described (27). Briefly, plasmids containing mUT-A1, mUT-A2, or mUT-A3 were linearized, and cRNA was prepared using the T7 mMessage mMachine (Ambion). Defolliculated oocytes were injected with cRNA or deionized H2O and incubated for 3 days at 18°C. cRNA was isolated from MF1 mouse inner medulla RNA (3 μg/lane) was separated in a 1% agarose gel. Autoradiography was performed using a Fuji FLA-3000 PhosphorImager.

**Northern blot analysis.** RNA was isolated from MF1 mouse tissues by the guanidine isothiocyanate method. Either poly-A⁺ RNA (3 μg/lane, enriched as described previously) (8) or total RNA (15 μg/lane) was separated in a 1% agarose gel in the presence of 2.2 M formaldehyde and transferred to Hybond-N nylon membranes (Amersham Pharmacia Biotech). Filters were probed using either 32P-labeled probe 1, nucleotides 1–739 of mouse UT-A2 (probe 2), or full-length mUT-A1. Hybridization was for 16 h at 42°C (50% formamide) and washing at 65°C (high) or 60°C (medium) in 0.1× SSC, 0.1% SDS.

**Protein preparation and immunoblotting.** Tissues were homogenized in 5 ml of ice-cold isolation solution in a handheld Douse homogenizer. Isolation solution contained 300 mM mannitol plus 12 mM HEPES adjusted to pH 7.6. The protease inhibitors, pepstatin (1 μg/ml final concentration, Sigma), leupeptin (2 μg/ml, Sigma), and phenylmethylsulfonyl (1 μg/ml, Sigma) were added before homogenization. Homogenates were centrifuged at 2,000 g for 30 min at 4°C. The supernatants were saved and centrifuged at 200,000 g for 1 h at 42°C (50% formaldehyde) and washing at 65°C (high) or 60°C (medium) in 0.1× SSC, 0.1% SDS.

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**Primer extension analysis.** Plasmids containing the UT-Aα (MUT9) or UT-Aβ (MUT1) promoter regions (6) and part of the first exon of UT-A1 or UT-A2, respectively, were used in an in vitro transcription assay as described previously (16) to produce an enriched population of UT-A1 and UT-A2 cRNA. Primer extension was carried out using AMV-reverse transcriptase (Roche) and 5 μg of cRNA using [γ-32P]P-labeled oligonucleotides corresponding to nucleotides 61–82 of mUT-A1 (5’-CTCAAGGAAGACTGCAAGTAC) or nucleotides 50–74 of mouse UT-A2 (5’-GGTCGAGATTCTCCACAAGATTCC) at 42°C. These primers were also used to sequence MUT9 and MUT1 using 32P dCTP and the Sequenase Quick Denatured Plasmid Sequencing Kit (Amersham Pharmacia Biotech). Sequencing reactions and extension products were separated by electrophoresis on a 6% polyacrylamide gel. Autoradiography was performed using a Fuji FLA-3000 PhosphorImager.
embedded in paraffin wax. Tissue was sectioned at 5 μm, mounted on Superfrost Plus slides (BDH) and allowed to dry overnight at 37°C. After xylene treatment and rehydration in a descending series of ethanol concentrations (100–70%), endogenous peroxidase was blocked by incubating sections for 30 min in 3% hydrogen peroxide in methanol. Antigen retrieval was performed by boiling sections for 10 min in a solution containing 25 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 50 mM glucose before overnight incubation at 4°C with affinity-purified antiserum L194 diluted in 1% BSA, 0.3% Triton X-100 in PBS. Labeling was visualized with a 1:200 dilution of P448 goat-anti-rabbit horseradish peroxidase-conjugated secondary antibody (Dako), followed by incubation with dianaminobenzidine.

Statistics. Quantitative data were compared using one-way ANOVA. The Student-Newman-Keuls post hoc test was used to detect significant differences between groups. Values are quoted as means ± SE, and statistical significance was assumed at the 5% level.

RESULTS

Homology screening experiments. High-stringency screening of a mouse kidney inner medulla cDNA library with probe 1, corresponding to nucleotides 1–219 of mUT-A3 (8), yielded a 4,047-bp cDNA (Fig. 1A). Analysis of the nucleotide sequence revealed a putative open reading frame (ORF) from nucleotide 449 to 3,241, a polyadenylation signal (ATTAAA) at position 4,014, and a poly (A) tail. The ORF predicts a 930-residue protein, the mouse homologue of rat UT-A1 (25). mUT-A1 (GenBank accession no. AF366052) has 93 and 85% amino acid identity with rat UT-A1 (25) and human UT-A1 (1), respectively. There are three putative N-glycosylation sites (N-I/N-T-W/G) at N-280, N-424, and N-743; seven potential PKC sites at S24, S88, S206, T448, S495, T546, and S669; and five potential PKA sites at S85, S92, S487, S564 and S919 (Fig. 2).

High-stringency screening of a mouse kidney inner medulla cDNA library with a 427-bp PCR product corresponding to bp 281–708 of mUT-A2 yielded a 2,876-bp cDNA (Fig. 1B). Analysis of the nucleotide sequence revealed a putative ORF from nucleotide 449 to 3,241, a polyadenylation signal (ATTAAA) at position 4,014, and a poly (A) tail. The ORF predicts a 930-residue protein, the mouse homologue of rat UT-A2 (27). mUT-A2 (GenBank accession no. AF367359) has 95 and 91% amino acid identity with rat UT-A2 (27) and human UT-A1 (18), respectively. There is one putative N-glycosylation site (N-I/N-T-W/G) at N-280, two potential PKC sites at T13 and S136, and one putative N-glycosylation site (N-I/N-T-W/G) at N-210, two potential PKC sites at T13 and S136, and one putative N-glycosylation site (N-I/N-T-W/G) at N-424, and N-743; seven potential PKC sites at S24, S88, S206, T448, S495, T546, and S669; and five potential PKA sites at S85, S92, S487, S564 and S919 (Fig. 2).

X. laevis oocyte expression. Oocytes injected with mUT-A1 or mUT-A2 cRNA showed threefold and twofold increases, respectively. Inclusion of 0.5 mM phloretin in the uptake solution did not significantly affect urea uptake in H2O-injected oocytes. However, consistent with previously characterized UT-A transporters (8, 11, 25, 27), phloretin reduced urea influx into oocytes injected with mUT-A1 or mUT-A2 (data not shown). Phloretin completely inhibited mUT-A1- or mUT-A2-mediated urea influx. Titration of mUT-A1 cRNA (data not shown) revealed that urea transport by oocytes injected with ≤1 ng cRNA was increased by cAMP (Fig. 3). Injection of molar equivalent amounts of mUT-A2 or mUT-A3 showed that urea transport via UT-A2 was not affected by cAMP; however, urea transport was significantly increased in oocytes expressing mUT-A3 (Fig. 3). These findings agree, in part, with those previously reported by others for UT-A orthologs. However, our result that mUT-A3 acutely responds to PKA agonists is in contrast to the observations reported by Shayakul et al. (26) using rat UT-A3 but does agree with those reported by Karakashian and colleagues (11). This acute effect of PKA agonists may have implications for AVP-stimulated urea transport in the IMCD.

Identification of transcriptional start site for UT-A1 and UT-A2. The transcriptional start of UT-A1 and UT-A3 was identified using 5′ rapid amplification of cDNA ends (RACE) and primer extension. 5′RACE identified a UT-A transcript that was shorter than mUT-A1 cDNA (corresponding to bp 43 of the UT-A1 cDNA) but 49 bp longer than mUT-A3 cDNA (8). This transcript generated by 5′-RACE had an m7G cap structure at its 5′-end so was likely to represent a full-length transcript (22). With the use of primer extension, three transcriptional start sites were identified 5, 8, and 13 bp 3′ to the 5′-terminus of mUT-A1 cDNA and a further start site 12 bp 5′ to the 5′-terminus of mUT-A1 cDNA (Fig. 4B). Together, these data indicated that UT-A1 and UT-A3 mRNA transcripts have multiple transcriptional start sites that are driven by the same promoter. In contrast, 5′-RACE identified 39 additional nucleotides at the 5′-end of the mUT-A2 cDNA that are different from the region identified upstream of the mUT-A1 cDNA (Fig. 4B). Independently, primer extension analysis gave the same result. These data show that transcription of the mUT-A2 mRNA transcript is initiated from a single start site, which is distinct from the transcription start site of UT-A1 and UT-A3.

Northern blot analysis and tissue distribution. High-stringency Northern blot analysis of kidney inner medulla poly-A+ RNA (3 μg/lane) using probe 1, the unique 5′-untranslated region (UTR) of UT-A1 (see METHODS), resulted in strong signals of 4.1 and 2.1 kb (Fig. 5A). These signals correspond to mUT-A1 and mUT-A3 (8), respectively. No other transcripts were detected in this tissue segment using this probe. High-stringency Northern blot analysis of mRNA using probe 2, the unique 5′-UTR of UT-A2 (see METHODS), resulted in a strong signal of 3.1 kb in kidney inner medulla (Fig. 5B). This signal corresponds to the mUT-A2 transcript and confirmed that the 5′-UTR of UT-A2 is a unique sequence not present in any other major urea transporter isoform. No other signals were apparent, even after prolonged exposure of up to 6 days.

Medium-stringency (60°C) Northern blot analysis of total RNA (15 μg/lane) isolated from mouse kidney inner medulla, kidney outer medulla, kidney cortex, testes, heart, brain, and liver, using a full-length
A: mouse (m)UT-A1 urea transporter cDNA and amino acid sequence (GenBank accession no. AF366052).

B: mUT-A2 cDNA and amino acid sequence (GenBank accession no. AF367359).
mUT-A1 probe, showed UT-A transcripts were present in all tissues tested (Fig. 5C). Strong signals of 4.1 and 3.1 kb were present in the kidney inner medulla and a weaker band at 2.1 kb. In the kidney outer medulla, a strong 3.1-kb signal was distinguishable, and weak 3.4- and 2.9-kb signals were detected in the kidney cortex. UT-A transcripts were also detected in extrarenal tissues. In testes, signals of 3.4, 2.9, and 1.5 kb were observed. After prolonged exposure (3 days; not shown), signals of 3.4 and 2.9 kb were also detected in heart, and a 3.1-kb transcript was detected in the brain and liver. These results were regarded as semiquantitative because total RNA was analyzed and lanes were equally loaded. From this viewpoint, it was evident that the kidney medulla contains the highest concentration of UT-A mRNA compared with the other tissues analyzed. Weak bands at 1.8 and 4.4 kb evident in total RNA samples, but not in poly-A+ RNA samples, indicated that the mUT-A1 bound weakly to rRNA despite the inclusion of yeast tRNA in the hybridization solution.

Western blot analysis. Antiserum L194 has been used extensively in rats to detect UT-A1 and UT-A2 (17, 30). In mouse kidney outer medulla, it has been reported to detect UT-A2 and, putatively, UT-A4 (30). Immunoblots of the 200,000-g fraction of mouse tissue, containing plasma membranes and subcellular vesicular membranes, showed that antiserum L194 labeled several proteins of various molecular weights (Fig. 6A). Compared with the other tissues we tested, excluding the brain, the kidney inner medulla showed the strongest protein signals. In the kidney inner medulla, L194 detected strong protein bands of 44, 47, 53, 72, 77, 98, 105, and 120 kDa. The predominant protein band in the kidney outer medulla was 53 kDa. Several weaker protein bands were also observed in the kidney outer medulla at 44, 47, 94, and 105 kDa. Surprisingly, the 53-kDa signal was equally strong in kidney cortex, and the 94- and 105-kDa protein bands, although weaker, were also detected. In testes, protein bands of 51, 72, 100, 110, and 120 kDa were detected, along with a protein band at 145 kDa. In the heart, weak protein bands of 53, 70, and 105 kDa were detected, whereas in the liver the predominant signals were at 98, 115, and 154 kDa. Very strong protein bands were observed in the brain at 45, 53, 60, and 154 kDa, and weaker protein bands were detected at 45, 72, 105, and 120 kDa. All signals were absent from immunoblots when L194 was preincubated with immunizing peptide before analysis (Fig. 6B). No signals were observed when L194 was excluded and only the secondary antiserum was applied (not shown).

Immunocytochemistry. Immunostaining with antisemur L194 strongly labeled structures in the renal medulla (Fig. 7, A–C). Preincubation of L194 with the immunizing peptide abolished immunostaining by L194 (Fig. 7D). In the inner stripe of the outer medulla, at the junction with the inner medulla, the radial pattern of labeling was very similar to that previously reported in rats and mice (30). This labeling corresponds to type 1 tDLs contained in vascular bundles. In the initial part of the inner medulla, L194 labeled tubules of a similar dimension to those labeled in the outer medulla, but the
pattern of labeling was very different. Labeled tubules were not clustered in bundles but were sparsely distributed among unlabeled tubules. Previous RT-PCR studies in rat showed that UT-A2 mRNA was present in type 3 long tDLs, proximal to the bend in the loop of Henle and thin ascending limbs (23). On the basis of these data and the pattern of distribution of the L194-labeled tubules in the mouse, we conclude that the structures labeled by L194 in the initial inner medulla are type 3 tDLs. As previously observed in the rat, L194 strongly labeled mouse IMCD in the middle inner medulla and papillary tip. Interestingly, both apical and basolateral membranes were strongly labeled, suggesting that UT-A proteins were present in both membranes of the epithelium.

**DISCUSSION**

The proteins UT-A1, UT-A2, and UT-A3 (reviewed in Ref. 28) represent the major urea transporter proteins expressed in the rat kidney. Knowledge of their molecular structure has greatly added to our understanding of renal urea handling and its regulation. A prerequisite to studies incorporating UT-A knockout mice is knowledge of the characteristics and distribution of the target proteins in wild-type animals. Toward this end, we have previously isolated a cDNA encoding mUT-A3 (8). The purpose of this study was to characterize the mouse homologues of two other major UT-A gene products, UT-A1 and UT-A2, and to resolve the controversy between the effects of PKA agonists on UT-A3-mediated urea transport reported by two other groups (11, 26).

Screening a mouse kidney inner medulla cDNA library yielded cDNAs encoding mUT-A1 and mUT-A2. Both proteins share a high degree of identity with their rodent and human homologues. However, mUT-A1 is one amino acid longer than rat UT-A1 due to an extra histidine residue at position 4. The consensus glycosylation sites present in rat and human UT-A1 are also
we can account for the molecular identity of four of these transcripts: 1.5 kb (UT-A5), 2.1 kb (UT-A3), 3.1 kb (UT-A2), and 4.1 kb (UT-A1). At present we do not know the molecular identity of the proteins encoded by the 2.9- or 3.4-kb transcripts. In comparison, the rat UT-A gene gives rise to at least seven transcripts of 1.7, 2.1, 2.8, 3.0, 3.1, 3.7, and 4.0 kb (2, 11, 25, 27). In the mouse kidney, like in the rat, the predominant transcripts are UT-A1 and UT-A2, and these transcripts are differentially expressed with respect to kidney region. In the mouse, UT-A1 mRNA is predominant in the kidney inner medulla, whereas levels of UT-A2 mRNA are approximately equal between the kidney inner medulla and outer medulla. UT-A3 mRNA is expressed in low abundance in the kidney inner medulla, similar to the distribution of UT-A3 mRNA in the rat (2).

As previously reported in the rat, UT-A mRNA transcripts were also evident in low abundance in the liver, where urea transporters probably mediate the movement of urea out of hepatocytes (12). In heart, brain, and testes, tissues not associated with urea metabolism, the physiological role of UT-A urea transporters is far from clear and further studies are needed.

In previous studies, antisera L194/L403 has provided an effective tool for immunolocalizing UT-A1 and UT-A2 in rat kidney (17, 30). Western blot analysis of mouse tissues using L194 showed that L194 recognized multiple protein bands in all tissues tested. Preincubation of L194 with the immunizing peptide abolished these signals and indicated that L194 was recognizing mouse UT-A proteins containing all or part of the COOH terminus of UT-A1. Of the known UT-A proteins, this would include UT-A1, UT-A2, and UT-A4, although we have so far been unable to isolate a cDNA encoding mouse UT-A4. Further analysis is clearly needed with antisera targeted to different UT-A regions before definitive conclusions can be drawn. It should also be considered that some bands may represent isoforms in different states of glycosylation, as has been previously shown in the rat (3), or potentially be the result of other posttranslational modification.

Immunocytochemistry of mouse kidney using L194 revealed strong staining of type 1 and type 3 tDLs in the kidney medulla region. Stained tubules in the kidney outer medulla occurred in groups, characteristic of the special arrangement of type 1 tDLs in mouse kidney outer medulla (13, 14) and very similar to the pattern of labeling using antisera L403 reported by Wade et al. (30). Interspersed within the initial part of the inner medulla were signals from type 3 tDLs. In the rat, the pattern of distribution is very similar to that seen in the mouse, and RT-PCR of rat nephron segments has shown the presence of UT-A2 mRNA in type 1 and type 3 tDLs (23). On the basis of these data and data from Western blot analysis, we suggest that the pattern of staining in mice, like in rats, represents UT-A2 expression. Strong staining was also seen in the middle IMCD and papillary tip. In the rat, this staining corresponds to UT-A1 in the apical membrane and cytosolic compartment of principal cells. Interestingly, we observed staining of both apical and basolateral membranes of...
This suggests that UT-A proteins mediate translocation of urea not only across the apical membrane but also across the basolateral membrane in some IMCD cells.

Acute application of a “cocktail” of PKA agonists significantly increased urea transport in *X. laevis* oocytes expressing UT-A1 and UT-A3 but not when applied to oocytes that expressed UT-A2. The actions of AVP on IMCD urea permeability occur within 10 min of AVP application and continue to increase over a time frame of ~60 min (21). Over a comparable time frame, application of PKA agonists has been shown to increase urea transport by rat and human UT-A1 in *X. laevis* oocytes (1, 25). Two previous studies have reported conflicting data regarding the effects of PKA agonists on UT-A3-mediated urea uptake. One study by Shayakul and colleagues (26) reported that rat UT-A3, which has the same complement of PKA sites as mUT-A3, did not respond to treatment with a cocktail of cAMP agonists. However, 24-h treatment of HEK-293 cells expressing rat UT-A3 with forskolin was reported to increase urea uptake (11). This stim-
ularatory effect can be considered to be due to an acute regulation of the UT-A3 protein, and not to possible interference by transcriptional regulation, because the transfecting construct did not include a promoter sequence. Our findings with mUT-A3 agree with those of Karakashian et al. (11) and provide strong evidence that UT-A3 is stimulated by PKA. Furthermore, our result suggests that UT-A3 may be involved in the AVP-stimulated increase in IMCD urea permeability. One possible explanation for the conflicting results observed between our data and that of Shayakul et al. (26) is that both we and other groups (1) have found that a titration of the amount of UT-A cRNA injected into oocytes is necessary before the cAMP effect can be observed. In the light of our findings, knowledge of the cellular location of UT-A3 in mIMCD would represent a major step toward understanding the role this protein plays in renal urea handling and in the urinary concentrating mechanism.

In summary, we have isolated cDNAs encoding the mouse homologues of UT-A1 and UT-A2 and begun to characterize the proteins they encode. We have shown that mUT-A1 and mUT-A3 have a different transcriptional start site to that driving mUT-A2 transcription and that mUT-A proteins are differentially expressed in the kidney, testes, heart, brain, and liver. Finally, we report urea transport mediated via UT-A1 or UT-A3 is increased by PKA agonists.

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