[38] X. Bossuyt, M. Muller, P.J. Meier, Multispecific amphipathic substrate transport by an organic anion transporter of human liver, J. Hepatol. 25 (1996) 733–738.
[39] T. Abe, M. Kakyo, T. Tokui, R. Nakagomi, T. Nishio, D. Nakai, H. Nomura, M. Unno, M. Suzuki, T. Naitoh, S. Matsuno, H. Yawo, Identification of a novel gene family encoding human liver-specific organic anion transporter LST-1, J. Biol. Chem. 274 (1999) 17159–17163.
[40] G.A. Kullak-Ublick, B. Hagenbuch, B. Stieger, C.D. Schteingart, A.F. Hofmann, A.W. Wolkoff, P.J. Meier, Molecular and functional characterization of an organic anion transporting polypeptide cloned from human liver, Gastroenterology 109 (1995) 1274–1282.
[41] G.A. Kullak-Ublick, T. Fisch, M. Oswald, B. Hagenbuch, P.J. Meier, U. Beuers, G. Paumgartner, Dehydroepiandrosterone sulfate (DHEAS): identification of a carrier protein in human liver and brain, FEBS Lett. 424 (1998) 173–176.
[42] T. Nishio, H. Adachi, R. Nakagomi, T. Tokui, E. Sato, M. Tanemoto, K. Fujiwara, M. Okabe, T. Onogawa, T. Suzuki, D. Nakai, K. Shiiba, M. Suzuki, H. Ohtani, Y. Kondo, M. Unno, S. Ito, K. Iinuma, K. Nunoki, S. Matsuno, T. Abe, Molecular identification of a rat novel organic anion transporter moat1, which transports prostaglandin D(2), leukotriene C(4), and taurocholate, Biochem. Biophys. Res. Commun. 275 (2000) 831–838.
[43] I. Tamai, J. Nezu, H. Uchino, Y. Sai, A. Oku, M. Shimane, A. Tsuji, Molecular identification and characterization of novel members of the human organic anion transporter (OATP) family, Biochem. Biophys. Res. Commun. 273 (2000) 251–260.
[44] G.A. Kullak-Ublick, M.G. Ismair, B. Stieger, L. Landmann, R. Huber, F. Pizzagalli, K. Fattinger, P.J. Meier, B. Hagenbuch, Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver, Gastroenterology 120 (2001) 525–533.
[45] D. Kobayashi, T. Nozawa, K. Imai, J. Nezu, A. Tsuji, I. Tamai, Involvement of human organic anion transporting polypeptide OATP-B (SLC21A9) in pH-dependent transport across intestinal apical membrane, J. Pharmacol. Exp. Ther. 306 (2003) 703–708.
[46] K. Sato, J. Sugawara, T. Sato, H. Mizutamari, T. Suzuki, A. Ito, T. Mikkaichi, T. Onogawa, M. Tanemoto, M. Unno, T. Abe, K. Okamura, Expression of organic anion transporting polypeptide E (OATP-E) in human placenta, Placenta 24 (2003) 144–148.
[47] J.Y. Li, R.J. Boado, W.M. Pardridge, Blood–brain barrier genomics, J. Cereb. Blood Flow Metab. 21 (2001) 61–68.
[48] F. Pizzagalli, B. Hagenbuch, B. Stieger, U. Klenk, G. Folkers, P.J. Meier, Identification of a novel human organic anion transporting polypeptide as a high affinity thyroxine transporter, Mol. Endocrinol. 16 (2002) 2283–2296.
[49] A. Enomoto, M.F. Wempe, H. Tsuchida, H.J. Shin, S.H. Cha, N. Anzai, A. Goto, A. Sakamoto, T. Niwa, Y. Kanai, M.W. Anders, H. Endou, Molecular identification of a novel carnitine transporter specific to human testis. Insights into the mechanism of carnitine recognition, J. Biol. Chem. 277 (2002) 36262–36271.
[50] A. Enomoto, H. Kimura, A. Chairoungdua, Y. Shigeta, P. Jutabha, S.H. Cha, M. Hosoyamada, M. Takeda, T. Sekine, T. Igarashi, H. Matsuo, Y. Kikuchi, T. Oda, K. Ichida, T. Hosoya, K. Shimokata, T. Niwa, Y. Kanai, H. Endou, Molecular identification of a renal urate anion exchanger that regulates blood urate levels, Nature 417 (2002) 447–452.
[51] T. Sekine, S.H. Cha, H. Endou, The multispecific organic anion transporter (OAT) family, Pflugers Arch. 440 (2000) 337–350.
[52] D.H. Sweet, K.T. Bush, S.K. Nigam, The organic anion transporter family: from physiology to ontogeny and the clinic, Am. J. Physiol., Renal Physiol. 281 (2001) F197–F205.
[53] H. Koepsell, H. Endou, The SLC22 drug transporter family, Pflugers Arch. 447 (2004) 666–676.
[54] D.H. Sweet, D.S. Miller, J.B. Pritchard, Y. Fujiwara, D.R. Beier, S.K. Nigam, Impaired organic anion transport in kidney and choroid plexus of organic anion transporter 3 (Oat3 (Slc22a8)) knockout mice, J. Biol. Chem. 277 (2002) 26934–26943.
[55] Y. Nagata, H. Kusuhara, H. Endou, Y. Sugiyama, Expression and functional characterization of rat organic anion transporter 3 (rOat3) in the choroid plexus, Mol. Pharmacol. 61 (2002) 982–988.
[56] M. Alebouyeh, M. Takeda, M.L. Onozato, A. Tojo, R. Noshiro, H. Hasannejad, J. Inatomi, S. Narikawa, X.L. Huang, S. Khamdang, N. Anzai, H. Endou, Expression of human organic anion transporters in the choroid plexus and their interactions with neurotransmitter metabolites, J. Pharmacol. Sci. 93 (2003) 430–436.
[57] H. Kusuhara, T. Sekine, N. Utsunomiya-Tate, M. Tsuda, R. Kojima, S.H. Cha, Y. Sugiyama, Y. Kanai, H. Endou, Molecular cloning and characterization of a new multispecific organic anion transporter from rat brain, J. Biol. Chem. 274 (1999) 13675–13680.
[58] S.H. Cha, T. Sekine, J.I. Fukushima, Y. Kanai, Y. Kobayashi, T. Goya, H. Endou, Identification and characterization of human organic anion transporter 3 expressing predominantly in the kidney, Mol. Pharmacol. 59 (2001) 1277–1286.
[59] M. Hasegawa, H. Kusuhara, D. Sugiyama, K. Ito, S. Ueda, H. Endou, Y. Sugiyama, Functional involvement of rat organic anion transporter 3 (rOat3; Slc22a8) in the renal uptake of organic anions, J. Pharmacol. Exp. Ther. 300 (2002) 746–753.
[60] M. Hasegawa, H. Kusuhara, H. Endou, Y. Sugiyama, Contribution of organic anion transporters to the renal uptake of anionic compounds and nucleoside derivatives in rat, J. Pharmacol. Exp. Ther. 305 (2003) 1087–1097.
[61] D. Sugiyama, H. Kusuhara, Y. Shitara, T. Abe, P.J. Meier, T. Sekine, H. Endou, H. Suzuki, Y. Sugiyama, Characterization of the efflux transport of 17beta-estradiol-D-17beta-glucuronide from the brain across the blood–brain barrier, J. Pharmacol. Exp. Ther. 298 (2001) 316–322.

[62] S. Mori, H. Takanaga, S. Ohtsuki, T. Deguchi, Y.S. Kang, K. Hosoya, T. Terasaki, Rat organic anion transporter 3 (rOAT3) is responsible for brain-to-blood efflux of homovanillic acid at the abluminal membrane of brain capillary endothelial cells, J. Cereb. Blood Flow Metab. 23 (2003) 432–440.

[63] T. Deguchi, S. Ohtsuki, M. Otagiri, H. Takanaga, H. Asaba, S. Mori, T. Terasaki, Major role of organic anion transporter 3 in the transport of indoxyl sulfate in the kidney, Kidney Int. 61 (2002) 1760–1768.

[64] D.H. Sweet, L.M. Chan, R. Walden, X.P. Yang, D.S. Miller, J.B. Pritchard, Organic anion transporter 3 (Slc22a8) is a dicarboxylate exchanger indirectly coupled to the Na+ gradient, Am. J. Physiol. Renal Physiol. 284 (2003) F763–F769.

[65] A. Bakhiya, A. Bahn, G. Burckhardt, N. Wolff, Human organic anion transporter 3 (hOAT3) can operate as an exchanger and mediate secretory urate flux, Cell. Physiol. Biochem. 13 (2003) 249–256.

[66] T. Sekine, N. Watanabe, M. Hosoyamada, Y. Kanai, H. Endou, Expression cloning and characterization of a novel multispecific organic anion transporter, J. Biol. Chem. 272 (1997) 18526–18529.

[67] D.H. Sweet, N.A. Wolff, J.B. Pritchard, Expression cloning and characterization of ROAT1. The basolateral organic anion transporter in rat kidney, J. Biol. Chem. 272 (1997) 30088–30095.

[68] G.D. Simonson, A.C. Vincent, K.J. Roberg, Y. Huang, V. Iwanij, Molecular cloning and characterization of a novel liver-specific transport protein, J. Cell. Sci. 107 (1994) 1065–1072.

[69] T. Sekine, S.H. Cha, M. Tsuda, N. Apiwattanakul, N. Nakajima, Y. Kanai, H. Endou, Identification of multispecific organic anion transporter 2 expressed predominantly in the liver, FEBS Lett. 429 (1998) 179–182.

[70] N. Morita, H. Kusuhara, T. Sekine, H. Endou, Y. Sugiyama, Functional characterization of rat organic anion transporter 2 in LLC-PK1 cells, J. Pharmacol. Exp. Ther. 298 (2001) 1179–1184.

[71] D.H. Sweet, D.S. Miller, J.B. Pritchard, Ventricular choline transport: a role for organic cation transporter 2 expressed in choroid plexus, J. Biol. Chem. 276 (2001) 41611–41619.

[72] D. Grundemann, V. Gorboulev, S. Gambaryan, M. Veyhl, H. Koepsell, Drug excretion mediated by a new prototype of polyspecific transporter, Nature 372 (1994) 549–552.

[73] M. Okuda, H. Saito, Y. Urakami, M. Takano, K. Inui, cDNA cloning and functional expression of a novel rat kidney organic cation transporter, OCT2, Biochem. Biophys. Res. Commun. 224 (1996) 500–507.

[74] R. Kekuda, P.D. Prasad, X. Wu, H. Wang, Y.J. Fei, F.H. Leibach, V. Ganapathy, Cloning and functional characterization of a potential-sensitive, polyspecific organic cation transporter (OCT3) most abundantly expressed in placenta, J. Biol. Chem. 273 (1998) 15971–15979.

[75] V. Gorboulev, J.C. Ulzheimer, A. Akhoundova, I. Ulzheimer-Teuber, U. Karbach, S. Quester, C. Baumann, F. Lang, A.E. Busch, H. Koepsell, Cloning and characterization of two human polyspecific organic cation transporters, DNA Cell Biol. 16 (1997) 871–881.

[76] L. Zhang, M.J. Dresser, A.T. Gray, S.C. Yost, S. Terashita, K.M. Giacomini, Cloning and functional expression of a human liver organic cation transporter, Mol. Pharmacol. 51 (1997) 913–921.

[77] J.W. Jonker, A.H. Schinkel, Pharmacological and physiological functions of the polyspecific organic cation transporters OCT1, 2 and 3 (SLC22A1–3), J. Pharmacol. Exp. Ther. 308 (2004) 2–9.

[78] X. Wu, R. Kekuda, W. Huang, Y.J. Fei, F.H. Leibach, J. Chen, S.J. Conway, V. Ganapathy, Identity of the organic cation transporter OCT3 as the extraneuronal monoamine transporter (uptake2) and evidence for the expression of the transporter in the brain, J. Biol. Chem. 273 (1998) 32776–32786.

[79] J.W. Jonker, E. Wagenaar, C.A. Mol, M. Buitelaar, H. Koepsell, J.W. Smit, A.H. Schinkel, Reduced hepatic uptake and intestinal excretion of organic cations in mice with a targeted disruption of the organic cation transporter 1 (Oct1 [Slc22a1]) gene, Mol. Cell. Biol. 21 (2001) 5471–5477.

[80] J.W. Jonker, E. Wagenaar, S. Van Eijl, A.H. Schinkel, Deficiency in the organic cation transporters 1 and 2 (Oct1/Oct2 [Slc22a1/Slc22a2]) in mice abolishes renal secretion of organic cations, Mol. Cell. Biol. 23 (2003) 7902–7908.

[81] R. Zwart, S. Verhaagh, M. Buitelaar, C. Popp-Snijders, D.P. Barlow, Impaired activity of the extraneuronal monoamine transporter system known as uptake-2 in Orct3/Slc22a3-deficient mice, Mol. Cell. Biol. 21 (2001) 4188–4196.

[82] H. Yabuuchi, I. Tamai, J. Nezu, K. Sakamoto, A. Oku, M. Shimane, Y. Sai, A. Tsuji, Novel membrane transporter OCTN1 mediates multispecific, bidirectional, and pH-dependent transport of organic cations, J. Pharmacol. Exp. Ther. 289 (1999) 768–773.

[83] I. Tamai, R. Ohashi, J. Nezu, H. Yabuuchi, A. Oku, M. Shimane, Y. Sai, A. Tsuji, Molecular and functional identification of sodium ion-dependent, high affinity human carnitine transporter OCTN2, J. Biol. Chem. 273 (1998) 20378–20382.

[84] T. Sekine, H. Kusuhara, N. Utsunomiya-Tate, M. Tsuda, Y. Sugiyama, Y. Kanai, H. Endou, Molecular cloning and characterization of high-affinity carnitine transporter from rat intestine, Biochem. Biophys. Res. Commun. 251 (1998) 586–591.

[85] I. Tamai, R. Ohashi, J.I. Nezu, Y. Sai, D. Kobayashi, A. Oku, M. Shimane, A. Tsuji, Molecular and functional characterization of organic cation/carnitine transporter family in mice, J. Biol. Chem. 275 (2000) 40064–40072.

[86] J. Nezu, I. Tamai, A. Oku, R. Ohashi, H. Yabuuchi, N. Hashimoto, H. Nikaido, Y. Sai, A. Koizumi, Y. Shoji, G. Takada, T. Matsuishi, M. Yoshino, H. Kato, T. Ohura, G. Tsujimoto, J. Hayakawa, M. Shimane, A. Tsuji, Primary systemic carnitine deficiency is caused by mutations in a gene encoding sodium ion-dependent carnitine transporter, Nat. Genet. 21 (1999) 91–94.

[87] A. Inano, Y. Sai, H. Nikaido, N. Hasimoto, M. Asano, A. Tsuji, I. Tamai, Acetyl-L-carnitine permeability across the blood–brain barrier and involvement of carnitine transporter OCTN2, Biopharm. Drug Dispos. 24 (2003) 357–365.

[88] Y. Kido, I. Tamai, A. Ohnari, Y. Sai, T. Kagami, J. Nezu, H. Nikaido, N. Hashimoto, M. Asano, A. Tsuji, Functional relevance of carnitine transporter OCTN2 to brain distribution of L-carnitine and acetyl-L-carnitine across the blood–brain barrier, J. Neurochem. 79 (2001) 959–969.

[89] K. Yokogawa, Y. Higashi, I. Tamai, M. Nomura, N. Hashimoto, H. Nikaido, J. Hayakawa, K. Miyamoto, A. Tsuji, Decreased tissue distribution of L-carnitine in juvenile visceral steatosis mice, J. Pharmacol. Exp. Ther. 289 (1999) 224–230.

[90] R. Ohashi, I. Tamai, Ji J. Nezu, H. Nikaido, N. Hashimoto, A. Oku, Y. Sai, M. Shimane, A. Tsuji, Molecular and physiological evidence for multifunctionality of carnitine/organic cation transporter OCTN2, Mol. Pharmacol. 59 (2001) 358–366.

[91] C. Shu, H. Shen, N.S. Teuscher, P.J. Lorenzi, R.F. Keep, D.E. Smith, Role of PEPT2 in peptide/mimetic trafficking at the blood–cerebrospinal fluid barrier: studies in rat choroid plexus epithelial cells in primary culture, J. Pharmacol. Exp. Ther. 301 (2002) 820–829.

[92] T. Zhu, X.Z. Chen, A. Steel, M.A. Hediger, D.E. Smith, Differential recognition of ACE inhibitors in *Xenopus laevis* oocytes expressing rat PEPT1 and PEPT2, Pharm. Res. 17 (2000) 526–532.

[93] M. Boll, M. Herget, M. Wagener, W.M. Weber, D. Markovich, J. Biber, W. Clauss, H. Murer, H. Daniel, Expression cloning and functional characterization of the kidney cortex high-affinity proton-coupled peptide transporter, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 284–289.

[94] M.E. Ganapathy, P.D. Prasad, B. Mackenzie, V. Ganapathy, F.H. Leibach, Interaction of anionic cephalosporins with the intestinal and renal peptide transporters PEPT1 and PEPT2, Biochim. Biophys. Acta 1324 (1997) 296–308.

[95] T. Terada, H. Saito, M. Mukai, K. Inui, Recognition of beta-lactam antibiotics by rat peptide transporters, PEPT1 and PEPT2, in LLC-PK1 cells, Am. J. Physiol. 273 (1997) F706–F711.

[96] M.E. Ganapathy, W. Huang, H. Wang, V. Ganapathy, F.H. Leibach, Valacyclovir: a substrate for the intestinal and renal peptide transporters PEPT1 and PEPT2, Biochem. Biophys. Res. Commun. 246 (1998) 470–475.

[97] H. Shen, D.E. Smith, R.F. Keep, J. Xiang, F.C. Brosius III, Targeted disruption of the PEPT2 gene markedly reduces dipeptide uptake in choroid plexus, J. Biol. Chem. 278 (2003) 4786–4791.

[98] S.M. Ocheltree, H. Shen, Y. Hu, J. Xiang, R.F. Keep, D.E. Smith, Mechanisms of cefadroxil uptake in the choroid plexus: studies in wild type and PEPT2 knockout mice, J. Pharmacol. Exp. Ther. 308 (2004) 462–467.

[99] H.H. Kao, J.D. Huang, M.S. Chang, cDNA cloning and genomic organization of the murine MRP7, a new ATP-binding cassette transporter, Gene 286 (2002) 299–306.

[100] E. Hopper, M.G. Belinsky, H. Zeng, A. Tosolini, J.R. Testa, G.D. Kruh, Analysis of the structure and expression pattern of MRP7 (ABCC10), a new member of the MRP subfamily, Cancer Lett. 162 (2001) 181–191.

[101] M. Kool, M. de Haas, G.L. Scheffer, R.J. Scheper, M.J. van Eijk, J.A. Juijn, F. Baas, P. Borst, Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines, Cancer Res. 57 (1997) 3537–3547.

[102] Y. Guo, E. Kotova, Z.S. Chen, K. Lee, E. Hopper-Borge, M.G. Belinsky, G.D. Kruh, MRP8, ATP-binding cassette C11 (ABCC11), is a cyclic nucleotide efflux pump and a resistance factor for fluoropyrimidines 2′,3′-dideoxycytidine and 9′-(2′-phosphonylmethoxyethyl)adenine, J. Biol. Chem. 278 (2003) 29509–29514.

[103] A.H. Schinkel, The roles of P-glycoprotein and MRP1 in the blood–brain and blood–cerebrospinal fluid barriers, Adv. Exp. Med. Biol. 500 (2001) 365–372.

[104] P. Borst, R. Evers, M. Kool, J. Wijnholds, A family of drug transporters: the multidrug resistance-associated proteins, J. Natl. Cancer Inst. 92 (2000) 1295–1302.

[105] T. Ishikawa, M.T. Kuo, K. Furuta, M. Suzuki, The human multidrug resistance-associated protein (MRP) gene family: from biological function to drug molecular design, Clin. Chem. Lab. Med. 38 (2000) 893–897.

[106] Y.M. Qian, W.C. Song, H. Cui, S.P. Cole, R.G. Deeley, Glutathione stimulates sulfated estrogen transport by multidrug resistance protein 1, J. Biol. Chem. 276 (2001) 6404–6411.

[107] J. Nishino, H. Suzuki, D. Sugiyama, T. Kitazawa, K. Ito, M. Hanano, Y. Sugiyama, Transepithelial transport of organic anions across the choroid plexus: possible involvement of organic anion transporter and multidrug resistance-associated protein, J. Pharmacol. Exp. Ther. 290 (1999) 289–294.

[108] V.V. Rao, J.L. Dahlheimer, M.E. Bardgett, A.Z. Snyder, R.A. Finch, A.C. Sartorelli, D. Piwnica-Worms, Choroid plexus epithelial expression of MDR1 P glycoprotein and multidrug resistance-associated protein contribute to the blood–cerebrospinal-fluid drug-permeability barrier, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 3900–3905.

[109] S.P. Cole, G. Bhardwaj, J.H. Gerlach, J.E. Mackie, C.E. Grant, K.C. Almquist, A.J. Stewart, E.U. Kurz, A.M. Duncan, R.G. Deeley, Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line, Science 258 (1992) 1650–1654.

[110] I. Leier, G. Jedlitschky, U. Buchholz, S.P.C. Cole, R.G. Deeley, D. Keppler, The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates, J. Biol. Chem. 269 (1994) 27807–27810.

[111] M. Muller, C. Meijer, G.J. Zaman, P. Borst, R.J. Scheper, N.H. Mulder, E.G. de Vries, P.L.M. Jansen, Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione *S*-conjugate transport, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 13033–13037.

[112] D.W. Loe, R.G. Deeley, S.P. Cole, Biology of the multidrug resistance-associated protein, MRP, Eur. J. Cancer 32A (1996) 945–957.

[113] J. Wijnholds, E.C. deLange, G.L. Scheffer, D.J. van den Berg, C.A. Mol, M. van der Valk, A.H. Schinkel, R.J. Scheper, D.D. Breimer, P. Borst, Multidrug resistance protein 1 protects the choroid plexus epithelium and contributes to the blood–cerebrospinal fluid barrier, J. Clin. Invest. 105 (2000) 279–285.

[114] Y.J. Lee, H. Kusuhara, Y. Sugiyama, Do multidrug resistance-associated protein-1 and -2 play any role in the elimination of estradiol-17 beta-glucuronide and 2,4-dinitrophenyl-S-glutathione across the blood-cerebrospinal fluid barrier? J. Pharm. Sci. 93 (2004) 99–107.

[115] R.A. van Aubel, P.H. Smeets, J.G. Peters, R.J. Bindels, F.G. Russel, The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP, J. Am. Soc. Nephrol. 13 (2002) 595–603.

[116] J.D. Schuetz, M.C. Connelly, D. Sun, S.G. Paibir, P.M. Flynn, R.V. Srinivas, A. Kumar, A. Fridland, MRP4: a previously unidentified factor in resistance to nucleoside-based antiviral drugs, Nat. Med. 5 (1999) 1048–1051.

[117] Z.S. Chen, K. Lee, G.D. Kruh, Transport of cyclic nucleotides and estradiol 17-beta-D-glucuronide by multidrug resistance protein 4. Resistance to 6-mercaptopurine and 6-thioguanine, J. Biol. Chem. 276 (2001) 33747–33754.

[118] G. Reid, P. Wielinga, N. Zelcer, I. Van Der Heijden, A. Kuil, M. De Haas, J. Wijnholds, P. Borst, The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs, Proc. Natl. Acad. Sci. U. S. A. (2003) 9244–9249.

[119] P.R. Wielinga, I. van der Heijden, G. Reid, J.H. Beijnen, J. Wijnholds, P. Borst, Characterization of the MRP4- and MRP5-mediated transport of cyclic nucleotides from intact cells, J. Biol. Chem. 278 (2003) 17664–17671.

[120] N. Zelcer, G. Reid, P. Wielinga, A. Kuil, I. van der Heijden, J.D. Schuetz, P. Borst, Steroid and bile acid conjugates are substrates of human multidrug-resistance protein (MRP) 4 (ATP-binding cassette C4), Biochem. J. 371 (2003) 361–367.

[121] G. Jedlitschky, B. Burchell, D. Keppler, The multidrug resistance protein 5 functions as an ATP-dependent export pump for cyclic nucleotides, J. Biol. Chem. 275 (2000) 30069–30074.

[122] J. Wijnholds, C.A.A.M. Mol, L. van Deemter, M. de Haas, G.L. Scheffer, F. Baas, J.H. Beijnen, R.J. Scheper, S. Hatse, E. De Clercq, J. Balzarini, P. Borst, Multidrug-resistance protein 5 is a multispecific organic anion transporter able to transport nucleotide analogs., Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 7476–7481.

[123] A.H. Schinkel, J.J. Smit, O. van Tellingen, J.H. Beijnen, E. Wagenaar, L. van Deemter, C.A. Mol, M.A. van der Valk, E.C. Robanus-Maandag, H.P. te Riele, et al., Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood–brain barrier and to increased sensitivity to drugs, Cell 77 (1994) 491–502.

[124] G.R. Lankas, M.E. Cartwright, D. Umbenhauer, P-glycoprotein deficiency in a subpopulation of CF-1 mice enhances avermectin-induced neurotoxicity, Toxicol. Appl. Pharmacol. 143 (1997) 357–365.

[125] R.B. Kim, Drugs as P-glycoprotein substrates, inhibitors, and inducers, Drug Metab. Rev. 34 (2002) 47–54.

[126] M. Cvetkovic, B. Leake, M.F. Fromm, G.R. Wilkinson, R.B. Kim, OATP and P-glycoprotein transporters mediate the cellular uptake and excretion of fexofenadine, Drug Metab. Dispos. 27 (1999) 866–871.

[127] M.F. Fromm, The influence of MDR1 polymorphisms on P-glycoprotein expression and function in humans, Adv. Drug Deliv. Rev. 54 (2002) 1295–1310.

[128] L. Huang, T. Hoffman, M. Vore, Adenosine triphosphate-dependent transport of estradiol-17beta(beta-D-glucuronide) in membrane vesicles by MDR1 expressed in insect cells, Hepatology 28 (1998) 1371–1377.

[129] A.H. Schinkel, P-glycoprotein, a gatekeeper in the blood–brain barrier, Adv. Drug Deliv. Rev. 36 (1999) 179–194.

[130] G. Lee, S. Dallas, M. Hong, R. Bendayan, Drug transporters in the central nervous system: brain barriers and brain parenchyma considerations, Pharmacol. Rev. 53 (2001) 569–596.

[131] I. Tamai, A. Tsuji, Transporter-mediated permeation of drugs across the blood–brain barrier, J. Pharm. Sci. 89 (2000) 1371–1388.

[132] T. Kitazawa, K. Hosoya, T. Takahashi, Y. Sugiyama, T. Terasaki, In-vivo and in-vitro evidence of a carrier-mediated efflux transport system for oestrone-3-sulphate across the blood–cerebrospinal fluid barrier, J. Pharm. Pharmacol. 52 (2000) 281–288.

[133] R. Spector, A.V. Lorenzo, The effects of salicylate and probenecid on the cerebrospinal fluid transport of penicillin, aminosalicyclic acid and iodide, J. Pharmacol. Exp. Ther. 188 (1974) 55–65.

[134] A. Hakvoort, M. Haselbach, H.J. Galla, Active transport properties of porcine choroid plexus cells in culture, Brain Res. 795 (1998) 247–256.

[135] T. Nohjoh, H. Suzuki, Y. Sawada, Y. Sugiyama, T. Iga, M. HananoTransport of cefodizime, a novel third generation cephalosporin antibiotic, in isolated rat choroid plexus, J. Pharmacol. Exp. Ther. 250 (1989) 324–328.

[136] T. Ooie, H. Suzuki, T. Terasaki, Y. Sugiyama, Characterization of the transport properties of a quinolone antibiotic, fleroxacin, in rat choroid plexus, Pharm. Res. 13 (1996) 523–527.

[137] H. Suzuki, Y. Sawada, Y. Sugiyama, T. Iga, M. Hanano, Transport of benzylpenicillin by the rat choroid plexus in vitro, J. Pharmacol. Exp. Ther. 242 (1987) 660–665.

[138] R. Spector, Ceftriaxone pharmacokinetics in the central nervous system, J. Pharmacol. Exp. Ther. 236 (1986) 380–383.

[139] H. Suzuki, Y. Sawada, Y. Sugiyama, T. Iga, M. Hanano, R. Spector, Transport of imipenem, a novel carbapenem antibiotic, in the rat central nervous system, J. Pharmacol. Exp. Ther. 250 (1989) 979–984.

[140] H. Suzuki, Y. Sawada, Y. Sugiyama, T. Iga, M. Hanano, Anion exchanger mediates benzylpenicillin transport in rat choroid plexus, J. Pharmacol. Exp. Ther. 243 (1987) 1147–1152.

[141] N. Strazielle, M.F. Belin, J.F. Ghersi-Egea, Choroid plexus controls brain availability of anti-HIV nucleoside analogs via

pharmacologically inhibitable organic anion transporters. Aids 17 (2003) 1473–1485.
[142] J.B. Pritchard, D.H. Sweet, D.S. Miller, R. Walden, Mechanism of organic anion transport across the apical membrane of choroid plexus, J. Biol. Chem. 274 (1999) 33382–33387.
[143] C.M. Breen, D.B. Sykes, G. Fricker, D.S. Miller, Confocal imaging of organic anion transport in intact rat choroid plexus, Am. J. Physiol. Renal. Physiol. 282 (2002) F877–F885.
[144] T.B. Miller, C.R. Ross, Transport of organic cations and anions by choroid plexus, J. Pharmacol. Exp. Ther. 196 (1976) 771–777.
[145] K.J. Ullrich, G. Rumrich, C. David, G. Fritzsch, Bisubstrates: substances that interact with renal contraluminal organic anion and organic cation transport systems: I. Amines, piperidines, piperazines, azepines, pyridines, quinolines, imidazoles, thiazoles, guanidines and hydrazines, Pflugers Arch. 425 (1993) 280–299.
[146] H. Suzuki, Y. Sawada, Y. Sugiyama, T. Iga, M. Hanano, Saturable transport of cimetidine from cerebrospinal fluid to blood in rats, J. Pharmacobio-Dyn. 8 (1985) 73–76.
[147] H. Suzuki, Y. Sawada, Y. Sugiyama, T. Iga, M. Hanano, Transport of cimetidine by the rat choroid plexus in vitro, J. Pharmacol. Exp. Ther. 239 (1986) 927–935.
[148] A.R. Villalobos, J.T. Parmelee, J.B. Pritchard, Functional characterization of choroid plexus epithelial cells in primary culture, J. Pharmacol. Exp. Ther. 282 (1997) 1109–1116.
[149] A.R. Villalobos, J.T. Parmelee, J.L. Renfro, Choline uptake across the ventricular membrane of neonate rat choroid plexus, Am. J. Physiol. 276 (1999) C1288–C1296.
[150] T. Kitazawa, K. Hosoya, M. Watanabe, T. Takashima, S. Ohtsuki, H. Takanaga, M. Ueda, N. Yanai, M. Obinata, T. Terasaki, Characterization of the amino acid transport of new immortalized choroid plexus epithelial cell lines: a novel in vitro system for investigating transport functions at the blood–cerebrospinal fluid barrier, Pharm. Res. 18 (2001) 16–22.
[151] T. Terasaki, K. Hosoya, Conditionally immortalized cell lines as a new in vitro model for the study of barrier functions, Biol. Pharm. Bull. 24 (2001) 111–118.
[152] D.M. Dykxhoorn, C.D. Novina, P.A. Sharp, Killing the messenger: short RNAs that silence gene expression, Nat. Rev., Mol. Cell Biol. 4 (2003) 457–467.
[153] M. Sasaki, H. Suzuki, K. Ito, T. Abe, Y. Sugiyama, Transcellular transport of organic anions across a double-transfected Madin–Darby canine kidney II cell monolayer expressing both human organic anion-transporting polypeptide (OATP2/SLC21A6) and Multidrug resistance-associated protein 2 (MRP2/ABCC2), J. Biol. Chem. 277 (2002) 6497–6503.
[154] Y. Cui, J. Konig, D. Keppler, Vectorial transport by double-transfected cells expressing the human uptake transporter SLC21A8 and the apical export pump ABCC2, Mol. Pharmacol. 60 (2001) 934–943.

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24. 肝シヌソイド側における異物排泄トランスポーター（MRP4）の機能解析

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Functional Characterization of MRP4

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はじめに

肝臓は異物排泄臓器として重要な組織である。薬物の肝胆系輸送には種々のトランスポーターが関与していることが明らかにされている。胆管側膜での排泄過程では，細胞質側ドメインにヌクレオチド結合ドメインをもつABCトランスポーターが種々同定されている[1,2]。これらは，ATPの加水分解と共役した一次性能動輸送により，基質化合物を胆汁中へと排泄する。グルクロン酸，グルタチオン抱合体，両親媒性の有機アニオンを基質とするmultidrug resistance associated protein 2（MRP2/*ABCC2*），脂溶性の高いカチオン・中性薬物を基質とするP-糖タンパク（P-gp/*ABCB1*）が同定されている。MRP2は異物排泄のほか，グルタチオンの胆汁排泄にも関与しており，胆汁酸を分泌するBile Sale Export Pump（BSEP/*ABCB11*）と並んで，胆汁流を生成する重要な役割を果たす。

近年，Breast Cancer Resistance protein（BCRP/*ABCG2*）も同じく胆管側膜に局在することが示されている[3,4]。BCRPはP-gpやMRP2，BSEPとは異なり，N末側にヌクレオチド結合ドメインを有し，6回膜貫通領域で構成されるが，生理的にはホモダイマーを構成して，薬物排出ポンプとして機能する[5]。BCRPは抗癌剤のほか，硫酸抱合体，シメチジンや食品中に含まれる発癌剤PhIPも基質とする[5~7]。遺伝子欠損マウスではPhIP[8]や抗がん剤methotrexate[9]の胆汁排泄が低下することから，MRP2，P-gpとともに胆管側の異物排泄ポンプとしての重要性が明らかにされつつある。

肝シヌソイド側膜にもABCトランスポーターは発現していることが見いだされており，MRP familyに属する分子種であるMRP3，-4，-6が同定されている。MRP3は，MRP2を先天的に欠損した変異動物（Eisai Hyperbilirubinemic rat：EHBR）で，肝臓での発現が誘導されていることから見いだされたABCトランスポーターである[10]。MRP3は正常ラットの肝臓ではほとんど発現していないが，EHBRや肝内性胆汁うっ滞を引き起こすα-naphthylisothiocyanateやCytochrome P-450を誘導するphenobarbital，bilirubinを投与したラットではその発現が誘導される[11]。MRP3の基質にはグルクロン酸抱合体が含まれ，さらに胆汁酸も基質とすることが明らかにされている[12,13]。phenobarbital投与時，またEHBRでシヌソイド側でのtaurocholateの排出能力とMRP3の発現量には相関があり[14]，誘導時にMRP3

がシヌソイド側における排出ポンプとして働き，抱合代謝物や胆汁酸など異物の蓄積を防いでいることが示唆される。MRP6は，皮膚真皮における弾性線維の変性とカルシウム沈着で特徴づけられる弾性線維性仮性黄色種（pseudoxanthoma elasticum：PXE）の原因遺伝子と考えられている[15]。PXEは視野狭窄，皮膚障害，血管でのアテローム硬化症などの病変を伴うことが知られていたが，その原因遺伝子は長きにわたり明らかとなっていなかった。ポジショナルクローニング法によりその原因遺伝子が探索され，PXEの患者では，MRP6に変異がはいっていることが見いだされた。エンドセリンA受容体拮抗薬であるBQ-123やグルタチオン抱合体のLTC4，N-ehylmaleimide S-glutathione，グルクロン酸抱合体estradiol 17β glucuronideを基質とするものの[16,17]，病態と直接関連のある内因性基質の同定には至っていない。

MRP4は核酸アナログである抗ウィルス薬である9-(2-phosphonylmethoxyethul) adenineの排泄に関与し，MRP4過剰発現細胞はこれら核酸アナログに対し耐性を示す[18]。核酸アナログ以外に，DHEASやestradiol 17β glucuronideなどステロイドの抱合体やprostaglandinなども基質として輸送する[19~21]。さらに，グルタチオン存在下では，taurocholateも輸送基質とすることが報告されている[22]。肝臓ではシヌソイド側膜に局在していることから[22]，正常時にtaurocholateをはじめとする基質化合物のシヌソイド側における排出ポンプとして機能していることが示唆されている。

本研究では，肝胆管側膜と肝シヌソイド側膜に発現していることが近年見いだされたMRP4とBCRPについてアデノウィルスを用いた遺伝子発現系を構築し，その基質選択性について検討を加えた。

I 実験方法

1 化合物

[^{3}H]Dehydroepiandrosterone sulfate（DHEAS；79.1 Ci/mmol），[^{3}H]p-aminohippuric acid（PAH；4.22 Ci/mmol）および[^{3}H]estrone sulfate（E_1S；46 Ci/mmol）はPerkinElmer Life Science (Boston, MA)から購入した。[^{3}H]ochratoxinA（21.3 Ci/mmol），[^{14}C]uric acid（53 mCi/mmol），[^{3}H]xanthine（20 Ci/mmol），[^{3}H]hypoxanthine（20 Ci/mmol），[^{3}H]cAMP（17 Ci/mmol）および[^{3}H]cGMP（2.4 Ci/mmol）はMoravek（Brea, CA）から購入した。[^{3}H]Pravastatin（45.5 Ci/mmol）およびその非標識体は三共㈱に供与していただいた。

2 Western Blot Analyses

Anti-hMRP4（PC-063）およびanti-hBCRP（BXP-21）モノクローナル抗体はKamiya Biomedicalから購入した。膜ベシクルを3×SDSサンプルバッファー（New England BioLabs, Beverly, MA）に溶解後，3分間ボイルし，10% SDS-polyacrylamideゲル電気泳動を行った。泳動後，蛋白質をゲルからニトロセルロース膜（Millipore, Bedford, MA）に転写した（15 V for 1時間）。転写後，PBS+5%スキムミルクで1時間振盪後，一次抗体を添加し，さらに1時間室温で振盪した。ニトロセルロース膜をPBS+0.1% Tween-20で洗浄後，Alexa Fluor 680 anti-mouse IgG（Molecular Probes, Inc. Eugene, OR）により検出した。

3 hMRP4 cDNAのクローニング

hMRP4 cDNAは，ヒト腎臓RNA（STRATAGENE）からRT-PCRにより単離した。PCRプライマーは，GenBank accession number AF071202の配列に基づいて設計し，センス鎖5′-AAGATGCTGCCCGTGT-ACCA-3′とし，アンチセンス鎖は5′-TGTCAAGT-CCGTTCCGAAGG-3′とした。単離したcDNAは，3532番目のTがGであった。このことにより，1139番目のアミノ酸がAsnからLysに変異していた。Direct Sequenceにより，この変異は鋳型としたヒト腎臓RNA由来であった。

4 hMRP4を導入したアデノウィルスの作製

単離したヒトMRP4 cDNAをpShuttle vector plasmid（ApaI, KpnIサイト）に組込み，さらにAdeno-X™ Expression System（BD Biosciences, Palo Alto, CA）を用いて，Adeno-XTM Viral DNAへと組込んだ。hMRP4-組込みアデノウィルスを作製するため，pAd-hMRP4をPacIで処理後，HEK293 cellsにFuGENE6（Roche Diagnostics Corporation, Indianapolis, IN）を用いて導入した。Recombinant viruses of hMRP4 and hBCRPはCsCl密度勾配遠心法により精製し，ストック溶液（10 mM Tris（pH 7.5），1 mM

MgCl2 および 10% glycerol）に懸濁後，−80°C で保存した。ヒト BCRP については，既報[23]であるので，詳細はそちらを参照していただきたい。

5 膜ベシクルの調製

常法に従って，膜ベシクルを調製した[23]。ヒト胎児腎由来細胞（HEK293，2×10^6）を，15 cm シャーレ上で培養した。72 時間後，細胞を hMRP4-or hBCRP 組換えアデノウィルスを，1×10^8 pfu per plate で感染させた。ネガティブコントロールとして，GFP 組換えアデノウィルス（pAd-GFP）を感染させた。感染後 48 時間に，細胞を回収した。細胞は，40 倍の低張バッファー（1 mM Tris-HCl, 0.1 mM EDTA, pH 7.4, 4°C）にて，1 時間振盪し，破砕した。ホモジネートを 100000 g，30 分間遠心後，ペレットを 10 mL 等張 TS バッファー（10 mM Tris-HCl, 250 mM sucrose, pH 7.4 at 4°C）に懸濁し，Dounce B homogenizer（glass/glass, tight pestle, 30 strokes）でホモジナイズした。得られた粗膜分画を，38%（w/v）スクロース溶液（5 mM Tris-HEPES, pH 7.4, 4°C）にのせ，280000 g で 45 分間遠心した。境界面を回収し，23 mL の TS バッファーに懸濁後，100000 g で 30 分間遠心した。ペレットを 400 μL の TS バッファーに懸濁し，27-gauge の注射針を 30 回通すことでベシクル化した。ベシクルは−80°C にて保存した。

6 膜ベシクルを用いた輸送実験

トランスポートバッファー（10 mM Tris, 250 mM sucrose and 10 mM $MgCl_2$. $6H_2O$, pH 7.4）に，基質，ATP（5 mM）およびその再生系（10 mM creatine phosphate and 100 g/L creatine phosphokinase）を添加した。37°C にて，3 分間インキュベーションした後，膜ベシクルを添加し，実験開始とした。一定時間インキュベーション後，氷中においた 1 mL バッファー（250 mM sucrose, 100 mM NaCl, 10 mM Tris-HCl, pH 7.4）を加え反応を停止させ，そのうち 900 μL を 0.45 μm HA フィルター（Millipore Corp., Bedford, MA）にアプライし，濾過する。さらに，5 mL のバッファーで 2 回洗浄する。フィルター上の放射活性（LS 6000SE, Beckman Instruments, Fullerton, CA）を用いて測定した。ベシクルへの取込みは，トランスポートバッファー中の放射活性で補正した。

II 結果および考察

Western blot 法により，MRP4 組換え型アデノウィルスを感染させた HEK293 から調製したベシクル（MRP4-HEK）に，約 190 kDa の位置に MRP4 の発現を確認した。このベシクルを用いて，種々化合物について ATP 依存的な輸送活性の比較を行った。BCRP 発現ベシクルについても，BCRP 組換え型アデノウィルスを感染させた HEK293 から調製した[23]。

BCRP-HEK では，DHEAS（K_m=30 μM），estrone sulfate（E1S）などステロイド抱合体，pravastatin や，尿中に主として排泄される有機アニオンである ochratoxin A（K_m=270 μM），PAH（K_m=5.6 mM），cAMP（K_m=3.1 mM），cGMP（K_m=6.0 mM），methotrexate（K_m=5.2 mM）や，xanthine, hypoxanthine, urate（K_m=2.5 mM）など核酸代謝物など非常に広範な化合物の ATP 依存的な輸送活性を検出することができた。PAH をはじめとして腎排泄型の有機アニオンに対する K_m 値は，ステロイド抱合体に比べて大きい値を示し，E1S, ochratoxinA が DHEAS の輸送活性と同程度であるが，他の基質化合物については，20 分の 1 以下の輸送活性であった。

MRP4-HEK では，DHEAS（K_m=2.7 μM）のほか，PAH（K_m=738 μM），methotrexate（K_m=100 μM），pravastatin, cGMP, xanthine の輸送活性は検出できたのに対して，E_1S, ochratoxin A, cAMP, hypoxanthine では GFP-HEK に比較して，有意な取込みを検出できなかった。ochratoxin A が DHEAS の 6 分の 1 程度の輸送活性であるが，他の基質については DHEAS の 20 分の 1 以下であった。

DHEAS に比較すると輸送活性は低いながらも，MRP4 と BCRP が両親媒性の有機アニオンのほか，腎排泄型の有機アニオンも基質とするなど，幅広い基質選択性を有していることが明らかとなった。基質化合物には一部重複もみられたが，DHEAS や PAH, methotrexate は BCRP よりも MRP4 に対して高い親和性を示し，E_1S など BCRP のみで輸送が検出された化合物も見いだされた。

おわりに

本研究では，MRP4 と BCRP の遺伝子発現系を用いて，両トランスポーターの基質化合物の探索を行い，特に腎排泄型の有機アニオンも基質とすることを見いだした。MRP4 と BCRP は肝シヌソイド側，胆管側膜に発現しているが，今回見いだした基質の肝内動態にどのように関連しているのか，さらに検討を進める必要がある。最近，BCRP[3] と MRP4[24] を欠損したマウスも作出されており，こうしたマウスを用いた *in vivo/in situ* での解析により，それぞれのトランスポーターの役割は明確にされるであろう。

また，肝臓以外の臓器として，MRP4 は肝臓以外にも，腎近位尿細管（刷子縁膜）[21]，脳毛細血管内皮細胞（管腔側）[24] に，BCRP は小腸上皮細胞（刷子縁膜）[3,4]，脳毛細血管内皮細胞（管腔側）[25] のほか，マウスでは腎（刷子縁膜）[3] に発現していることから，これらの組織において異物排泄システムとしての役割についても今後検討していく必要がある。

BCRP については，141 番目のアミノ酸残基に遺伝子多型（Gln141Lys）が存在し，変異型（141Lys）では，BCRP のタンパクとしての発現量は低下する[23,26]。さらに，日本人では頻度高く，Allele 頻度としては約 30％に達する[26]。この変異をヘテロで有する患者では，抗がん剤 diflomotecan の静脈内投与後の血中消失の遅延が報告されており[27]，薬剤感受性の個人差を決定する要因として注目されている。MRP4 についても，体内動態における役割を明らかにするとともに，遺伝子多型を解析し，薬剤感受性における個人差の要因としてどの程度重要であるのか解析を進めていくことが必要である。

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文　献

1) Meier PJ, Stieger B. Bile salt transporters. Annu Rev Physiol 2002；64：635-61.
2) Faber KN, Muller M, Jansen PL. Drug transport proteins in the liver. Adv Drug Deliv Rev 2003；55：107-24.
3) Jonker JW, Buitelaar M, Wagenaar E, Van Der Valk MA, Scheffer GL, Scheper RJ, et al. The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. Proc Natl Acad Sci U S A 2002；99：15649-54.
4) Maliepaard M, Scheffer GL, Faneyte IF, van Gastelen MA, Pijnenborg AC, Schinkel AH, et al. Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. Cancer Res 2001；61：3458-64.
5) Doyle LA, Ross DD. Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). Oncogene 2003；22：7340-58.
6) Suzuki M, Suzuki, H, Sugimoto Y, Sugiyama Y. ABCG2 transports sulfated conjugates of steroids and xenobiotics. J Biol Chem 2003；278：22644-9.
7) Pavek P, Merino G, Wagenaar E, Bolscher E, Novotna M, Jonker JW, et al. Human Breast Cancer Resistance Protein (BCRP/ABCG2)：interactions with steroid drugs, hormones, the dietary carcinogen PhIP, and transport of cimetidine. J Pharmacol Exp Ther. (In press)
8) van Herwaarden AE, Jonker JW, Wagenaar E, Brinkhuis RF, Schellens JH, Beijnen JH, et al. The breast cancer resistance protein (Bcrp1/Abcg2) restricts exposure to the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine. Cancer Res 2003；63：6447-52.
9) Breedveld P, Zelcer N, Pluim D, Sonmezer O, Tibben MM, Beijnen JH, et al. Mechanism of the pharmacokinetic interaction between methotrexate and benzimidazoles：potential role for breast cancer resistance protein in clinical drug-drug interactions. Cancer Res 2004；64：5804-11.
10) Hirohashi T, Suzuki H, Ito K, Ogawa K, Kume K, Shimizu T, et al. Hepatic expression of multidrug resistance-associated protein-like proteins maintained in eisai hyperbilirubinemic rats. Mol Pharmacol 1998；53：1068-75.
11) Ogawa K, Suzuki H, Hirohashi T, Ishikawa T, Meier PJ, Hirose K, et al. Characterization of inducible nature of MRP3 in rat liver. Am J Physiol Gastrointest Liver Physiol 2000；278：G438-46.
12) Hirohashi T, Suzuki H, Takikawa H, Sugiyama Y. ATP-dependent transport of bile salts by rat multidrug resistance-associated protein 3 (Mrp3). J Biol Chem 2000；275：2905-10.
13) Hirohashi T, Suzuki H, Sugiyama Y. Characterization of the transport properties of cloned rat multidrug resistance-associated protein 3 (MRP3). J Biol Chem 1999；274：15181-5.
14) Akita H, Suzuki H, Sugiyama Y. Sinusoidal efflux of taurocholate correlates with the hepatic expression level of Mrp3. Biochem Biophys Res Commun 2002；299：681-7.
15) Le Saux O, Urban Z, Tschuch C, Csiszar K, Bacchelli B, Quaglino D, et al. Mutations in a gene encoding an ABC

transporter cause pseudoxanthoma elasticum. Nat Genet 2000 ; 25 : 223-7.

16) Ilias A, Urban Z, Seidl TL, Le Saux O, Sinko E, Boyd CD, et al. Loss of ATP-dependent transport activity in pseudoxanthoma elasticum-associated mutants of human ABCC6 (MRP6). J Biol Chem 2002 ; 277 : 16860-7.

17) Madon J, Hagenbuch B, Landmann L, Meier PJ, Stieger B. Transport function and hepatocellular localization of mrp6 in rat liver. Mol Pharmacol 2000 ; 57 : 634-41.

18) Schuetz JD, Connelly MC, Sun D, Paibir SG, Flynn PM, Srinivas RV, et al. MRP4 : A previously unidentified factor in resistance to nucleoside-based antiviral drugs. Nat Med 1999 ; 5 : 1048-51.

19) Reid G, Wielinga P, Zelcer N, van der Heijden I, Kuil A, de Haas M, et al. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. Proc Natl Acad Sci U S A 2003 ; 100 : 9244-9.

20) Zelcer N, Reid G, Wielinga P, Kuil A, van der Heijden I, Schuetz JD, et al. Steroid and bile acid conjugates are substrates of human multidrug-resistance protein (MRP) 4 (ATP-binding cassette C4). Biochem J 2003 ; 371 : 361-7.

21) van Aubel RA, Smeets PH, Peters JG, Bindels RJ, Russel FG. The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules : putative efflux pump for urinary cAMP and cGMP. J Am Soc Nephrol 2002 ; 13 : 595-603.

22) Rius M, Nies AT, Hummel-Eisenbeiss J, Jedlitschky G, Keppler D. Cotransport of reduced glutathione with bile salts by MRP4(ABCC4) localized to the basolateral hepatocyte membrane. Hepatology 2003 ; 38 : 374-84.

23) Kondo C, Suzuki H, Itoda M, Ozawa S, Sawada J, Kobayashi D, et al. Functional analysis of SNPs variants of BCRP/ABCG2. Pharm Res 2004 ; 21 : 1895-903.

24) Leggas M, Adachi M, Scheffer GL, Sun D, Wielinga P, Du G, et al. Mrp4 confers resistance to topotecan and protects the brain from chemotherapy. Mol Cell Biol 2004 ; 24 : 7612-21.

25) Lee YJ, Kusuhara H, Jonker JW, Schinkel AH, Sugiyama Y. Investigation of efflux transport of dehydroepiandrosterone sulfate and mitoxantrone at the mouse blood-brain barrier : a minor role of breast cancer resistance protein. J Pharmacol Exp Ther. (In press)

26) Kobayashi D, Ieiri I, Hirota T, Takane H, Maegawa S, Kigawa J, et al. Functional Assessment of Abcg2 (Bcrp) Gene Polymoephisms to Protein Expression in Human Placenta. Drug Metab Dispos. (In press)

27) Sparreboom A, Gelderblom H, Marsh S, Ahluwalia R, Obach R, Principe P, et al. Diflomotecan pharmacokinetics in relation to ABCG2 421C>A genotype. Clin Pharmacol Ther 2004 ; 76 : 38-44.

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Characterization of Multiple P2X Receptors in Cultured Normal Human Epidermal Keratinocytes

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ATP-gated ion channels (P2X) are expressed in human epidermis and cultured keratinocytes. The aim of this study was to characterize native P2X receptors in normal human epidermal keratinocytes (NHEK) using whole-cell patch clamp technique, RT-PCR, and determination of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Application of ATP resulted in an inward current with a reversal potential of 0 mV. Response to ATP showed two types of currents: the slowly desensitizing response and the rapidly desensitizing response. The slowly desensitizing response was blocked by iso-pyridocaphosphate-6-azophenyl-2′, 5′ disulfonic acid (PPADS), a P2X receptor antagonist. We found that the expression of multiple $P2X_2$, $P2X_3$, $P2X_5$, and $P2X_7$ receptor subtype mRNA was increased in differentiated cells. On the other hand, the expression of G-protein-coupled $P2Y_2$ mRNA was downregulated in differentiated cells. Increases in $[Ca^{2+}]_i$ evoked by αβ-methylene ATP (αβ-meATP) and 2′, 3′-O-(4-benzoylbenzoyl) ATP (BzATP) were elevated, whereas elevation of $[Ca^{2+}]_i$ evoked by uridine 5′-triphosphate (UTP) was decreased in differentiated cells. Application of ATP or UVB radiation increased the expression of $P2X_1$, $P2X_2$, $P2X_3$, and $P2X_7$ receptors in NHEK. Changes in the expression levels and cation influx via multiple P2X receptors might be involved in the regulation of differentiation and one of the epidermal external sensors.

Key words: ATP/channel/differentiation/intracellular calcium
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ATP is released from a variety of tissues (Milner *et al*, 1990; Hansen *et al*, 1993; Ferguson *et al*, 1997) and acts as one of the mediators to transmit signals to the central and peripheral nervous system (for reviews, see Burnstock and Wood, 1996; Thorne and Housley, 1996; Norenberg and Illes, 2000; North and Surprenant, 2000). ATP receptors (P2 receptors) are classified broadly within two families; ligand-gated ion channels (P2X) and G-protein-coupled metabotropic receptors (P2Y). P2X receptors have seven subtypes ($P2X_{1-7}$) and form heteromeric or homomeric channel assemblies.

Strong evidence has been accumulated, which states that ATP regulates the structure of skin systems by acting as an important messenger via the intermediary of P2 receptors (Pillai and Bikle, 1992; Dixon *et al*, 1999; Greig *et al*, 2003; Koizumi *et al*, 2004). For instance, ATP increases DNA synthesis (Pillai and Bikle, 1992) and cell number in keratinocytes (Greig *et al*, 2003). Keratinocytes constantly release ATP whether skin is damaged or not (Dixon *et al*, 1999; Cook and McCleskey, 2002). Koizumi *et al* (2004) reported that Ca^{2+} waves evoked by mechanical stimulation in cultured normal human epidermal keratinocytes (NHEK) were heavily dependent on release and diffusion of ATP. This extracelluar ATP is a dominant messenger in cell-to-cell communication and in turn activates $P2Y_2$ receptors. As for the expression of P2 receptors in human skin, $P2Y_1$ and $P2Y_2$ receptors are relatively expressed in the basal layer and their localization is associated with the proliferation stage (Dixon *et al*, 1999; Greig *et al*, 2003). The expression of $P2Y_2$ mRNA is downregulated in differentiating HaCaT keratinocytes (Koegel and Alzheimer, 2001; Burrell *et al*, 2003). On the other hand, $P2X_5$ and $P2X_7$ receptors are expressed in the suprabasal layer, spinosum layer, and granular layer and their localization is associated with the differentiation or terminal differentiation phases (Greig *et al*, 2003). Moreover, P2X receptors play a role in delayed barrier recovery in hairless mouse epidermis when topical ATP and αβ-methylene ATP (αβ-meATP) are applied (Denda *et al*, 2002). But, research has still not been carried out with regard to the functional roles of all P2X receptors in cultured NHEK.

The aim of our study is to clarify which P2X receptor subtypes are expressed in NHEK using electrophysiological methods. Furthermore, we also provide evidence that P2X receptor expression is affected by the differentiation phase, application of ATP and UVB radiation *in vitro*, using RT-PCR methods, and monitoring free intracellular calcium concentration ($[Ca^{2+}]_i$).

Abbreviations: αβ-meATP,α,β-methylene ATP; BzATP, 2′, 3′-O-(4-benzoylbenzoyl) ATP; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; 2MeSADP, 2-methylthioadenosine 5′-diphosphate; NHEK, normal human epidermal keratinocytes; PPADS, iso-pyridocaphosphate-6-azophenyl-2′, 5′, disulfonic acid

Results

Characterization of ATP-activated inward currents ATP evoked inward currents in keratinocytes (67 of 168 cells) loaded with 2 mM GDPβ. Response to ATP showed two

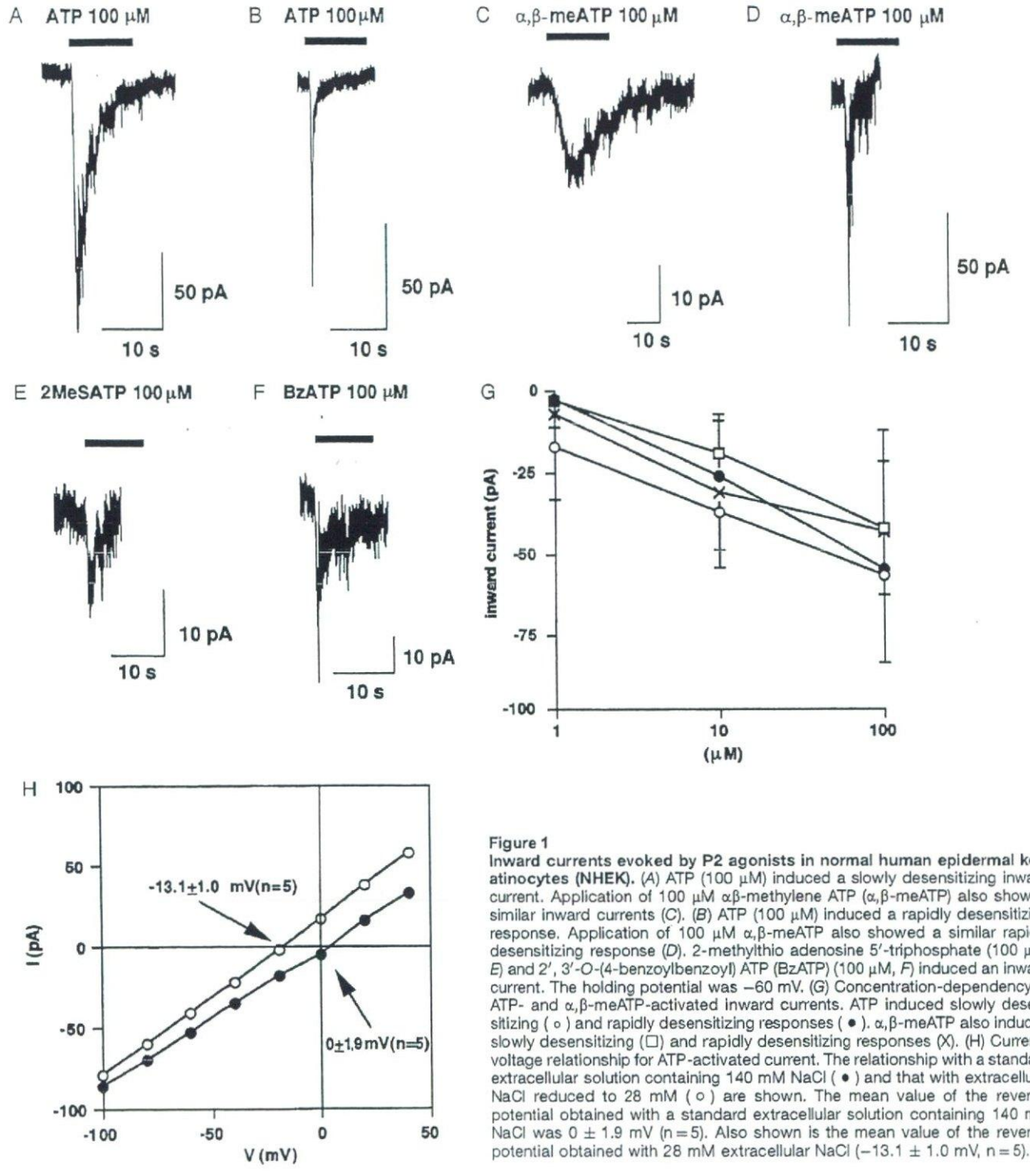

Figure 1
Inward currents evoked by P2 agonists in normal human epidermal keratinocytes (NHEK). (*A*) ATP (100 μM) induced a slowly desensitizing inward current. Application of 100 μM αβ-methylene ATP (α,β-meATP) also showed similar inward currents (*C*). (*B*) ATP (100 μM) induced a rapidly desensitizing response. Application of 100 μM α,β-meATP also showed a similar rapidly desensitizing response (*D*). 2-methylthio adenosine 5′-triphosphate (100 μM, *E*) and 2′, 3′-*O*-(4-benzoylbenzoyl) ATP (BzATP) (100 μM, *F*) induced an inward current. The holding potential was −60 mV. (*G*) Concentration-dependency of ATP- and α,β-meATP-activated inward currents. ATP induced slowly desensitizing (○) and rapidly desensitizing responses (●). α,β-meATP also induced slowly desensitizing (□) and rapidly desensitizing responses (X). (H) Current–voltage relationship for ATP-activated current. The relationship with a standard extracellular solution containing 140 mM NaCl (●) and that with extracellular NaCl reduced to 28 mM (○) are shown. The mean value of the reversal potential obtained with a standard extracellular solution containing 140 mM NaCl was 0 ± 1.9 mV (n = 5). Also shown is the mean value of the reversal potential obtained with 28 mM extracellular NaCl (−13.1 ± 1.0 mV, n = 5).

types of currents, that is, a slowly desensitizing (Fig 1*A*) and a rapidly desensitizing response (Fig 1*B*). The fraction of ATP-responding cells with a rapidly desensitizing current was 36% (24 of 67). The remaining cells, approximately 64%, showed a slowly desensitizing response. Of 88 cells tested, 14 cells responded to ATP and αβ-meATP (100 μM), 23 cells responded only to ATP, and the remaining 51 cells responded to neither ATP nor αβ-meATP. The values of the peak amplitudes by ATP and αβ-meATP (100 μM), a $P2X_1$, $P2X_3$, and $P2X_{2/3}$ receptors agonist, with rapidly desensitizing responses were −54.7 ± 32.8 pA (n = 11) and −43.3 ± 19.4 pA (n = 6), respectively (Fig 1*B*, *D*, and *G*). The responses to the second application by these agonists were not observed. The peak values of the slowly desensitizing response to ATP and αβ-meATP (100 μM) were −56.8 ± 26.8 pA (n = 10) and −42.3 ± 30.3 pA (n = 13) (Fig 1*A*, *C*, and *G*). ATP and αβ-meATP evoked inward currents concentration-dependently in both types of responses (Fig 1*G*). The slowly desensitizing responses to αβ-meATP (10 μM) were inhibited by iso-pyridocaphosphate-6-

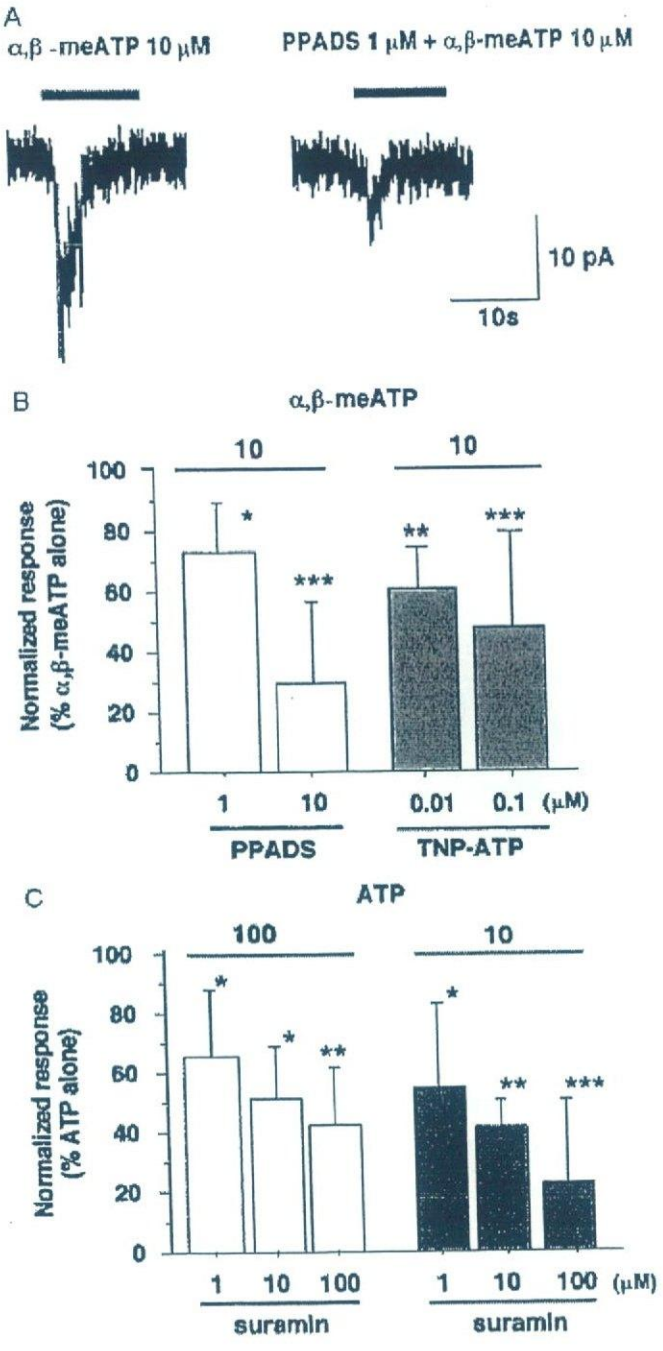

Figure 2
Inhibitory effects because of antagonists in response to ATP and αβ-methylene ATP (α,β-meATP) with slowly desensitizing currents. (*A*) Suppression of ATP-activated current because of P2X antagonist iso-pyridocaphosphate-6-azophenyl-2′, 5′ disulphonic acid (PPADS). (*B*) Concentration-dependency of inhibitory effects of PPADS (1 and 10 μM) and 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate (10 and 100 nM) on α,β-meATP-activated currents (*$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with the response of 10 μM α,β-meATP alone). Antagonists were applied to the cells 2 min before and during the α,β-meATP application. (*C*) Concentration-dependency of inhibitory effects of suramin on ATP-activated currents (*$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with the response of ATP alone). Suramin was applied to the cells 2 min before and during the ATP application. The holding potential was −60 mV.

azopheny l-2′, 5′ disulfonic acid (PPADS; 1 and 10 μM; 72.3% ± 14.9% and 29.4% ± 25.4%; n = 3–7, Fig 2*A* and *B*) and 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP; 10 and 100 nM; 62.5% ± 12.1% and 47.9% ± 32.0%; n = 4–11, Fig 2*B*), a $P2X_1$, $P2X_{2/3}$, and $P2X_3$ antagonist. This indicates that an inward current was evoked by the activation of $P2X_{2/3}$ receptors. The non-specific P2 receptor antagonist suramin concentration-dependently inhibited the ATP-activated current (Fig 2*C*). ATP-activated current (10 μM; 32.7 ± 20.5 pA, n = 3) in αβ-meATP-insensitive cells was blocked by PPADS (1 μM; 32.7% ± 9.8%, n = 3, $p>0.001$). This indicates that an inward current was evoked by the activation of $P2X_2$ and $P2X_5$ receptors. The response to 2′, 3′-*O*-(4-benzoylbenzoyl) ATP (BzATP; 10 μM, −38.1 ± 24.2 pA, n = 6) was equal to ATP (−39.0 ± 17.1 pA, n = 6) and BzATP-activated current was inhibited by brilliant blue G (BBG; 1 μM; 41.5% ± 29.8%; n = 6, $p>0.001$). BBG blocks rat $P2X_2$ receptors (Jiang *et al*, 2000), human $P2X_5$ receptors (Bo *et al*, 2003), and human $P2X_7$ receptors (Jiang *et al*, 2000). Each IC_{50} value on the ATP-activated current is 1370 nM in rat $P2X_2$ receptors and is 530 nM in human $P2X_5$ receptors. The IC_{50} value on the BzATP-activated current is 265 nM in human $P2X_7$ receptors (Jiang *et al*, 2000). Although ATP and BzATP were equipotent with respect to current responses, these responses seem to be evoked by the activation of $P2X_2$, $P2X_5$, and/or $P2X_7$ receptors in the present study. The slowly desensitizing response to 2-methylthio adenosine 5′-triphosphate (2MeSATP; 100 μM, −22.6 ± 8.4 pA, n = 6) was also smaller than the ATP-evoked current. This response was also insensitive to αβ-meATP and not inhibited by PPADS. This indicates that an inward current was evoked by the activation of $P2X_4$.

Figure 1*H* shows the measurement of the reversal potential of the ATP-activated current. To determine the ionic selectivity of the ATP-activated conductance, the reversal potential was measured in the presence of decreased extracellular NaCl in the cells loaded with 2 mM GDPβ. The reversal potential became more negative when the extracellular NaCl was decreased to 28 mM (Fig 1*H*). A negative shift in the reversal potential with a decreased NaCl concentration indicates that the ATP-activated conductance via P2X receptors is selective to cations. On the other hand, in the case of the ATP-activated conductance via P2Y receptors, the mean value of the reversal potential obtained with a standard extracellular solution was −18.5 ± 3.4 mV (n = 5) in the cells loaded with 0.3 mM GTP. A positive shift in reversal potential with a decreased NaCl concentration (−6.0 ± 3.0 mV, n = 5) indicates that the ATP-activated conductance via P2Y receptors conductance is selective to anions.

Comparison of the $[Ca^{2+}]_i$ response by P2 agonists between proliferating and differentiating keratinocytes Next, we investigated whether the differentiation stage in NHEK affects functional P2 receptor expression using a Ca^{2+} imaging method. Increase in $[Ca^{2+}]_i$ by ATP was not influenced by the absence of external Ca^{2+} ($0Ca^{2+}$) in proliferating subconfluent cells (Fig 3*A*). This indicates that the increase of $[Ca^{2+}]_i$ was dependent on the intracellular Ca source, suggesting the involvement of

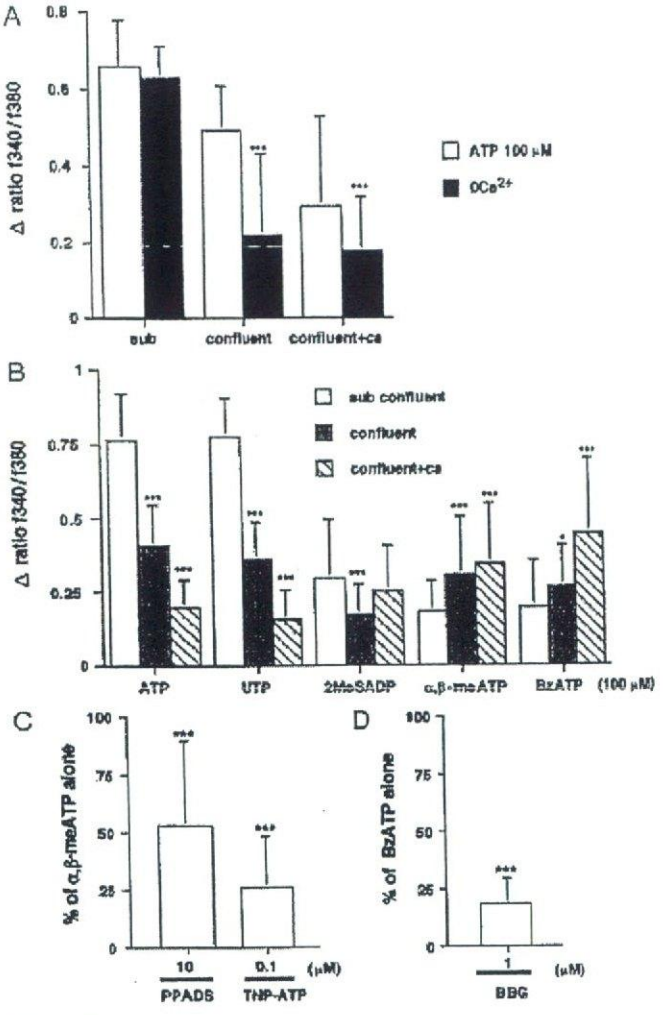

Figure 3
Characterization of P2 receptor-mediated Ca^{2+} responses in proliferated or differentiated keratinocytes. (*A*) Effect of extracellular Ca^{2+} on ATP-evoked increases in $[Ca^{2+}]_i$ in proliferating subconfluent cells (subconfluent) or differentiating over-confluent cells (confluent and confluent + Ca). ATP was applied to normal human epidermal keratinocytes (NHEK) for 20 s. Results were obtained from all cells (n = 62–113), which were tested from two different strains of NHEK. These histograms show a comparison of significant differences from the responses evoked by 100 μM ATP in the absence ($0Ca^{2+}$) or presence of extracellular Ca^{2+} (***$p < 0.001$). (*B*) Pharmacological characterization of Ca^{2+} responses in the different culture conditions. Results were obtained from all cells (n = 109–273), which were tested from two different strains of NHEK. *$p < 0.05$ and ***$p < 0.001$ compared with the response evoked by ATP analogues in subconfluent cells. (*C*) Isopyridocaphosphate-6-azophenyl-2′, 5′ disulphonic acid (10 μM) and 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate (100 nM) inhibited the α,β-meATP-evoked increases in $[Ca^{2+}]_i$ in differentiating over-confluent cells. Antagonists were applied to the cells 5 min before and during the α,β-meATP application. Results were obtained from 59 to 160 tested (at least two independent experiments using two strain of NHEK). ***$p < 0.001$ compared with the response evoked by 100 μM α,β-meATP alone. (*D*) Brilliant blue G (BBG) (1 μM) inhibited the BzATP-evoked increases in $[Ca^{2+}]_i$ in differentiating over-confluent cells. BBG was applied to the cells 5 min before and during the BzATP application. Results were obtained from 124 to 210 tested (at least two independent experiments using two strain of NHEK). ***$p < 0.001$ compared with the response evoked by 100 μM BzATP alone.

G-protein-coupled P2Y receptors. On the other hand, increase in $[Ca^{2+}]_i$ by ATP was influenced by the absence of external Ca^{2+} in differentiating over-confluent cells (Fig 3*A*). This indicates that the increase of $[Ca^{2+}]_i$ was dependent on the extracellular Ca^{2+}, suggesting the involvement of P2X receptors. The rank order of the Ca^{2+} response was ATP = uridine 5′-triphosphate (UTP) > 2-methylthioadenosine 5′-diphosphate (2MeSADP) > α,β-meATP = BzATP in proliferating subconfluent cells (Fig 3*B*). On the other hand, the rank order of the Ca^{2+} response in differentiating over-confluent cells (confluent + ca) was BzATP > α,β-meATP > 2MeSADP > ATP = UTP (Fig 3*B*). PPADS (10 μM) and TNP-ATP (100 nM) inhibited the α,β-meATP-evoked $[Ca^{2+}]_i$ increase (53.0% ± 36.1%; n = 73, 25.7% ± 22.6%; n = 60, Fig 3*C*). BBG (1 μM) inhibited the BzATP-evoked $[Ca^{2+}]_i$ increase (18.2% ± 10.8%; n = 124, Fig 3*D*). These results suggest that $P2X_1$, $P2X_{2/3}$, or $P2X_3$, and $P2X_7$ receptors were responsible for the responses in differentiating over-confluent cells.

Changes in P2X and P2Y receptor subtypes mRNA expression P2X and P2Y receptors have a different pattern of localization in the skin (Greig *et al*, 2003). We investigated whether their expression was influenced by culture conditions. Figure 4 shows the expression patterns of mRNA for P2 receptors under the different conditions. We detected the expression of $P2X_1$, $P2X_4$, $P2X_5$, $P2X_7$ (weak signal or not), $P2Y_1$, and $P2Y_2$ in subconfluent proliferating cells. The expression of $P2X_2$, $P2X_3$, $P2X_5$, and $P2X_7$ receptor subtypes was upregulated in differentiated cells whereas $P2X_1$ was downregulated at the stage of differentiation. The expression of $P2X_4$ and $P2Y_1$ receptor subtypes was not changed by any culture conditions. On the other hand, the expression of $P2Y_2$ mRNA was downregulated in differentiated cells. $P2X_6$ receptors were not expressed under these culture conditions (data not shown).

A large amount of ATP was released from NHEK because of damage or mechanical stimulation (Cook and McCleskey, 2002; Koizumi *et al*, 2004). It is well known that UVB causes skin inflammation. NHEK will be exposed to ATP in irritated skin. We also investigated whether autocrine stimulation (application of ATP) and a type of external stimulation such as UVB radiation changed P2X and P2Y receptor subtype expression. Application of ATP (300 μM) increased the expression of $P2X_1$, $P2X_2$, $P2X_3$, and $P2X_7$ receptor subtypes. UVB radiation (30 or 60 mJ per cm^2) specifically increased the expression of $P2X_1$, $P2X_3$, and $P2X_7$ receptor subtypes. The expression of $P2X_4$, $P2X_5$, and $P2Y_1$ receptor subtypes was not changed by the application of ATP or UVB radiation. The expression of $P2Y_2$ receptor was downregulated under both conditions. Cytotoxicity was not observed in any case of the conditions for 6 h (legend of Fig 4).

Discussion

This study is an analysis of the electrophysiological properties of P2X receptors in NHEK. We found that the expression of multiple P2X receptor subtypes was influenced during the differentiation phase. NHEK was stimulated by ATP and UVB treatments, which in turn affected P2X receptor expression. We determined that the P2X receptors are present in NHEK and are subject to the following conditions. The reversal potential of ATP-evoked current was

0 mV and the conductance is selective to cations in GDPβs-loaded cells. P2X agonists produced a rapidly desensitizing response in NHEK as well as in DRG neurons (Grubb and Evans, 1999). Furthermore, the ATP-evoked increase of $[Ca^{2+}]_i$ was influenced by the absence of extracellular Ca^{2+} in differentiating over-confluent cells.

P2X receptors were classified within several subtypes, based on their sensitivity to agonists and antagonists, or the time course of their desensitization because of currents (Evans and Surprenant, 1996). $P2X_1$ and $P2X_3$ receptors are characterized by their sensitivity to αβ-meATP and a rapidly desensitizing current. $P2X_2$ and $P2X_{4-7}$ receptors are characterized by insensitivity to αβ-meATP and a slowly desensitizing current. Although homomeric $P2X_2$ receptors are insensitive to αβ-meATP, heteromeric $P2X_{2/3}$ receptors are characterized by their sensitivity to αβ-meATP and a slowly desensitizing current (Lewis *et al*, 1995; Ueno *et al*, 1998). Additionally, the responses of the currents are categorized by their sensitivity to the P2X antagonist PPADS. PPADS antagonized $P2X_1$, $P2X_2$, $P2X_3$, $P2X_{2/3}$, $P2X_5$, and $P2X_7$, but not $P2X_4$ or $P2X_6$. In this study, αβ-meATP-activated currents have the following features: their responses may be rapidly or slowly desensitizing currents; the slowly desensitizing currents were inhibited by PPADS and TNP-ATP. These results suggest that $P2X_1$, $P2X_{2/3}$, and $P2X_3$ receptors were responsible for the responses. ATP-activated currents that yield slowly desensitizing responses have the following features: these responses were insensitive to αβ-meATP, and inhibited by PPADS. ATP-activated current with a slowly desensitizing response however was not inhibited by PPADS in some of cells. Furthermore, the current responses attained because of BzATP and 2MeSATP support our characterization of $P2X_2$, $P2X_4$, $P2X_5$, and/or $P2X_7$. Although it is evident from the results of current responses and RT-PCR that $P2X_1$, $P2X_{2/3}$, $P2X_3$, $P2X_4$, and $P2X_5$ receptors are functional in proliferating subconfluent cells, their contribution seems minimal as the ATP-evoked increases of $[Ca^{2+}]_i$ were not influenced by the absence of extracellular Ca^{2+} in proliferating subconfluent cells (Fig 3). Furthermore, UTP and ATP evoked the same increases of $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}. These results coincide with previous researches that indicated that it is the $P2Y_2$ receptors that play a functional role in the proliferated phase (Dixon *et al*, 1999; Lee *et al*, 2001; Burrell *et al*, 2003; Greig *et al*, 2003). Although UTP activates $P2Y_2$ and $P2Y_4$ receptors, the $P2Y_4$ subtype is a functional receptor in HaCaT keratinocytes but not in NHEK (Burrell *et al*, 2003).

$P2Y_2$ receptors respond to ATP in the proliferated phase; however, in the differentiated phase, it is the P2X receptors that mediate a greater response from ATP (Fig 3). Only differentiated over confluent cells were affected by the absence of extracellular Ca^{2+}. There were higher increases

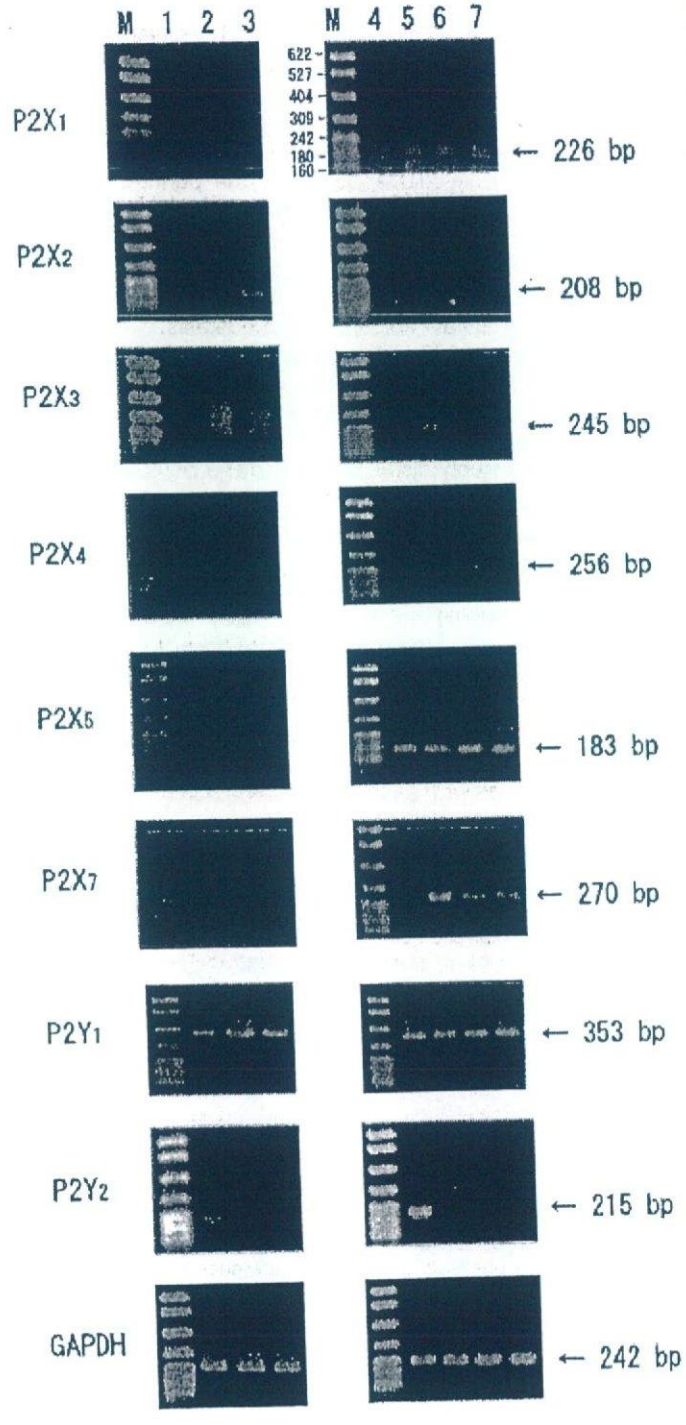

Figure 4
Changes in P2X and P2Y receptor subtype mRNA expressions in different conditions of normal human epidermal keratinocytes (NHEK). The left panels indicate that P2 receptor mRNA expression is affected by each culture condition. The right panels indicate that P2 receptor mRNA expression is affected while exposed to ATP and UVB radiation. Arrows indicate the PCR amplification products corresponding to P2X and P2Y receptor subtypes. M, DNA size markers; lane 1, proliferating subconfluent keratinocytes; lane 2, differentiating over-confluent keratinocytes; lane 3, differentiating over-confluent keratinocytes on addition of Ca (1.8 mM); lane 4, proliferating subconfluent keratinocytes; lane 5, proliferating subconfluent keratinocytes exposed to ATP (300 μM) for 6 h; lane 6, proliferating subconfluent keratinocytes exposed to UVB (60 mJ per cm^2); lane 7, proliferating subconfluent keratinocytes exposed to UVB (30 mJ per cm^2). Cytotoxicity was not found in any of the cell conditions (proliferating subconfluent keratinocytes; lanes 1 and 4, 1017 ± 52, n = 6, differentiating over-confluent keratinocytes; lane 2, 1152 ± 29, n = 6, differentiating over-confluent keratinocytes on addition of Ca; lane 3, 1173 ± 47, n = 6, proliferating subconfluent keratinocytes exposed to ATP for 6 h; lane 5, 1027 ± 43, n = 6, proliferating subconfluent keratinocytes exposed to UVB (60 mJ per cm^2); lane 6, 1025 ± 64, n = 6, proliferating subconfluent keratinocytes exposed to UVB (30 mJ per cm^2); lane 7, 1003 ± 41, n = 6).

of response to αβ-meATP and BzATP in differentiating overconfluent cells. On the other hand, the responses to ATP, UTP, and 2MeSADP decreased in the differentiated phase. Although the 2MeSADP-evoked $[Ca^{2+}]_i$ decreased in confluent cells, $P2Y_1$ expression remained unchanged. At this point however, we cannot distinguish the inconsistency between the expression level and Ca^{2+} response. Although 2MeSADP is also an agonist for $P2Y_{12}$, and $P2Y_{13}$, ADP, an agonist for $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$, elevated $[Ca^{2+}]_i$ in HaCaT keratinocytes but not in NHEK (Burrell *et al*, 2003). Thus, it is unlikely that these receptors are functional. Although the expression of $P2Y_2$ mRNA was downregulated at the differentiated phase, the expression of multiple $P2X_2$, $P2X_3$, $P2X_5$, and $P2X_7$ receptor subtype mRNA increased (Fig 4). Judging from the results of Ca^{2+} responses and RT-PCR, $P2X_3$ and $P2X_7$ receptor subtypes mainly function in the differentiated phase. The variation of multiple P2X receptor expression in cultured keratinocytes supports the notion that the $P2X_5$ and $P2X_7$ receptors are localized in the differentiated or terminal differentiated skin (Greig *et al*, 2003).

$P2X_3$ receptors are known to be selectively expressed in a subpopulation of small diameter sensory neurons (Chen *et al*, 1995; Lewis *et al*, 1995). $P2X_3$ receptors however, have been observed in nonneuronal cells, such as thymus (Glass *et al*, 2000) and urothelial cells (Sun and Chai, 2004). Stretching in bladder urothelial cells increased $P2X_3$ receptor expression and their expression was increased more in urothelial cells from patients with interstitial cystitis than that in control subjects (Sun and Chai, 2004). The epidermis could be an interface of the body and environment; hence, the P2X receptors may play a role as some kind of sensor against multiple environmental factors such as barrier disruption and UV radiation. $P2X_7$ receptors are known to be involved in ATP-induced apoptosis (Ferrari *et al*, 1997). $P2X_7$ receptors are likely to be part of the machinery of the end-stage terminal differentiation of keratinocytes (Greig *et al*, 2003). Extracellular ATP increased $P2X_1$, $P2X_2$, $P2X_3$, and $P2X_7$ receptors but not $P2X_4$, $P2X_5$ expression (Fig 4). UVB radiation also induces apoptosis in keratinocytes (Schwarz *et al*, 1995). In this study, $P2X_1$, $P2X_3$, and $P2X_7$ receptor expression, but not $P2X_2$ receptor expression, was augmented by UVB radiation. $P2X_4$, $P2X_5$, and $P2Y_1$ receptors expression however, was unaffected by ATP or UVB radiation. $P2Y_2$ receptor expression was downregulated by the application of ATP and UVB radiation. The downregulation of $P2Y_2$ receptors expression shows that extracellular ATP and UVB radiation inhibited proliferation. A high concentration of ATP inhibits proliferation and a low concentration of ATP promotes proliferation (Dixon *et al*, 1999; Greig *et al*, 2003).

We demonstrated that P2X receptors were nonselective cationic channels; on the other hand, the response to ATP mediated through P2Y receptors activated Cl^- conductance. Ca^{2+}-activated Cl channel and K channel contributed to the hyperpolarization induced by ATP, bradykinin, and histamine in HaCaT keratinocytes (Koegel and Alzheimer, 2001). Mauro *et al*, (1990) described that Cl^- conductance, increased by elevating extracellular Ca^{2+}, plays a role in the initiation of differentiation. Increases in $[Ca^{2+}]_i$ and phosphatidylinositol turnover because of the elevation of extracellular Ca^{2+} were important components of the signal for differentiation (Jaken and Yuspa, 1988; Hennings *et al*, 1989). These studies suggest the possibility that the intracellular Ca^{2+} released from IP_3-sensitive stores affects Cl^- conductance and resultantly leads to keratinocyte differentiation. With these studies as a background, it shall be assumed that Cl^- conductance via P2Y receptors also contributes to the initiation of differentiation. This ionic selectivity of P2 receptor subtypes may be associated with the localization in skin and contribute to the maintenance of homeostasis in skin.

Furthermore, a difference in the amount of released ATP or the localization of P2 receptors between normal healthy subjects and patients would be expected. ATP released from uroepithelial cells was higher in patients with interstitial cystitis than in controls (Sun *et al*, 2001). Since mechanical scratching has the potential to induce the release of a large amount of ATP release in atopic or psoriatic skin and leads to skin inflammation, it would appear that the purinergic signaling is clinically significant. The stimulation of ATP occurs throughout all stages, through proliferation, differentiation, and apoptosis. Regulation of P2 receptor subtypes is necessary in order to control ion influx and membrane potential, which helps maintain epidermal homeostasis.

In summary, we demonstrated the presence of functional multiple P2X receptors in NHEK, suggesting their important physiological role as an initial sensor for external stimuli. P2 receptor subtypes in keratinocytes would provide a basis to study the regulatory mechanisms underlying the differentiation and proliferation of keratinocytes.

Materials and Methods

Cells and cell culture NHEK (10 Strains of NHEK) were purchased from Kurabo (Osaka, Japan). NHEK were cultured in serum-free keratinocyte growth medium, consisting of Humedia-KB2 (Kurabo) supplemented with bovine pituitary extract (0.4% vol/vol), human recombinant epidermal growth factor (0.1 ng per mL), insulin (10 μg per mL), and hydrocortisol (0.5 μg per mL). The medium was replaced every 2–3 d. For the electrophysiological experiments, NHEK (passage 1–3 cells) were seeded onto collagen-coated glass coverslips and used within 4 d.

Electrophysiological recordings Membrane currents were measured using whole-cell clamp techniques (Hamill *et al*, 1981). Cells that were grown on collagen coated-cover slips were transferred to an experimental chamber of about 1 mL volume. The chamber was continuously perfused with an extracellular solution containing (in mM) NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, 10.0 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 11.1 D-glucose (adjusted with NaOH to pH 7.4). Heat-polished patch pipettes had a tip resistance of 3–5 MΩ when filled with an intracellular solution containing 150 mM CsCl, 1 mM $MgCl_2$, 10 mM HEPES, and 5 mM-glycoletherdiamine *N,N,N′,N′*-tetraacetic acid (pH 7.2 with CsOH). Intracellular solution was supplemented with 0.3 mM guanosine 5′triphosphate (GTP) or 2 mM guanosine 5′-*O*-(2-thiodiphosphate) trilitium salt (GDPβS). To exclude the P2Y-activated current, GDPβS, an inhibitor of GTP-binding protein, was applied to the cells (Nakazawa, 1994). Three hundred millimolar KCl-agar bridge electrode was used as the reference electrode. Cell capacitance was compensated after the whole-cell mode was obtained. Cells were clamped at −60 mV. A step pulse between −100 and +40 mV was applied to the cell. Membrane currents were recorded with a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Union City, California). Electrical signals were filtered

at 1 kHz. Current signals were stored in a personal computer and analyzed using pCLAMP 6.0 and Clampfit 6.0 software (Axon Instruments). The drugs were dissolved in the extracellular solution and applied to the cells by perfusion. The experiments were performed at room temperature (~25°C). TNP-ATP was purchased from Molecular Probes (Eugene, Oregon). All other chemicals were purchased from Sigma-Aldrich (St Louis, Missouri).

Ca^{2+} imaging in single keratinocyte NHEK were grown to approximately 60%–80% confluency (subconfluent), 100%–120% confluency (confluent), and 100%–120% confluency at 48 h post-treatment with 1.8 mM Ca^{2+} (confluent + Ca) on collagen-coated cover glass chambers (Nalge Nunc, Naperville, Illinois). Changes in $[Ca^{2+}]_i$ in single cell were measured by the fura-2 method as described by Grynkiewicz *et al* (1985) with minor modifications (Koizumi and Inoue, 1997). In brief, the culture medium was replaced with a balanced salt solution (BSS) of the following composition (mM): NaCl 150, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1.2, HEPES 25, and D-glucose 10 (pH = 7.4). Cells were loaded with 5 μM fura-2 acetoxymethylester (fura-2AM) (Molecular Probes) at room temperature (~ 25°C) in BSS for 45 min, followed by a BSS wash and a further 15 min incubation to allow de-esterification of the loaded dye. The coverslip was mounted on an inverted epifluorescence microscope (IX70, TS Olympus, Tokyo, Japan), equipped with a 75 W xenon-lamp and band-pass filters of 340 and 380 nm wavelengths. The image data, recorded by a high-sensivity CCD (charge-coupled-device) camera (ORCA-ER, Hamamatsu Photonics, Hamamatsu, Japan) were regulated by a Ca^{2+} analyzing system (AQUACOSMOS/RATIO, Hamamatsu Photonics). In the Ca^{2+}-free experiments, Ca^{2+} was removed from the BSS and 1 mM EGTA was added. Nucleotides were dissolved in the BSS and the cells were exposed to it by method of perfusion. Data were represented as the ratio of fluorescence intensities of 340 and 380 nm.

The preparation for total RNA extraction and synthesis cDNA For RT-PCR studies, NHEK were grown in 10 cm collagen-coated dish (Asahi Techno Glass, Tokyo, Japan) to 60%–80% confluency (subconfluent), 100%–120% confluency (confluent), and 100%–120% confluency at 48 h post-treatment with 1.8 mM Ca^{2+} (confluent + Ca). Sixty to eighty percent confluency cells collected at 6 h post-treatment with UVB (30 and 60 mJ per cm^2) and ATP (300 μM). While using UV radiation, the medium was replaced by PBS (–). NHEK were exposed to UVB radiation from a bank of two Toshiba FL 20 SE sunlamps (Toshiba Electric, Tokyo, Japan). These tubes emit wavelengths between 280 and 340 nm, with a peak of 304 nm. Radiance was measured by a UV-Radiometer (Topcon, Tokyo, Japan). After exposing NHEK to radiation, the medium was added back to the dishes and NHEK were incubated at 37°C in 5% CO_2 for 6 h. ATP was applied with medium for 6 h at 37°C in 5% CO_2. Total RNA was isolated from all individual samples using ISOGEN (Nippon Gene, Osaka, Japan) according to the manufacturer's protocol. We synthesized cDNA from 1 μg of total RNA by the use of 200 U of M-MLV RT (Invitrogen, Carisbad, California) in 20 μL of reaction mixture containing 0.5 μg of oligo (dT) primer (Invitrogen), 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.25 mM dATP, 0.25 mM dTTP, 0.25 mM dGTP, 0.25 mM dCTP (Takara, Japan), and 50 U of ribonuclease inhibitor (Takara, Otsu, Japan) at 37°C for 1 h.

RT-PCR The amounts of P2 receptors and human GAPDH cDNA in samples were amplified by using an ABI PRISM 7700 sequence detector (Applied BioSystems, Foster City, California). The reaction mixture was as follows: PCR buffer, 3.5 mM $MgCl_2$, 0.2 μM forward primer, 0.2 μM reverse primer, 0.2 mM dNTP, and 1.25 U of AmpliTaq Gold DNA polymerase (Applied BioSystems). The PCR conditions were: 50°C for 2 min; 95°C for 10 min; 35 cycles of 95°C for 15 s; and 60°C for 1 min. Obtained DNA fragments by PCR

Table I. Primers list of P2 receptors and glyceraldehydes-3-phosphate dehydrogenase (GAPDH)

Gene	Primers (forward and reverse)	Accession number in GenBank	Product size (bp)
$P2X_1$	5′-CCAGCTTGGCTACGTGGTGCAAGA-3′	U45448	226
	5′-ACGGTAGTTGGTCCCGTTCTCCACAA-3′		
$P2X_2$	5′-CCCGAGAGCATAAGGGTCCACAAC-3′	AF190823	208
	5′-AATTTGGGGCCATCGTACCCAGAA-3′		
$P2X_3$	5′-CCCCTCTTCAACTTTGAGAAGGGA-3′	NM002559	245
	5′-GTGAAGGAGTATTTGGGGATGCAC-3′		
$P2X_4$	5′-CCTTCCCAACATCACCACTACTTACC-3′	U85975	256
	5′-AGGAGATACGTTGTGCTCAACGTC-3′		
$P2X_5$	5′-AGCACGTGAATTGCCTCTGCTTAC-3′	AF016709	183
	5′-ATCAGACGTGGAGGTCACTTTGCTC-3′		
$P2X_6$	5′-ATGGCCCTGTCCAAGTTCTGACAC-3′	AF065385	140
	5′-TGTTGCCTCATCCTTGCTTTCGCT-3′		
$P2X_7$	5′-CTGCTCTCTTGAACAGTGCCGAAA-3′	Y09561	270
	5′-AGTGATGGAACCAACGGTCTAGGT-3′		
$P2Y_1$	5′-ACCTCAGACGAGTACCTGCGAAGT-3′	NM002563	353
	5′-AGAATGGGGTCCACACAACTGTTGAG-3′		
$P2Y_2$	5′-GTGTCTGGGCGTCTTACGACCTCT-3′	NM176072	215
	5′-GCATGACTGAGCTGTAGGCCACGAA-3′		
GAPDH	5′-GAAGGTGAAGGTCGGAGTC-3′	NM002046	242
	5′-GAAGATG GTGATGGGATTTC-3′		

were separated in 1% agarose in Tris-borate buffer containing 0.25 μg per mL ethidium bromide. The gel was visualized by ultraviolet B radiation. PCR primers were designed using Genetyx Software program (GENETYX, Japan). The primers (forward, reverse, accession number, and product size) are shown in Table I.

Cell viability The applied condition of ATP for 6 h (300 μM) and the condition of 6 h post-treatment with UVB (30 and 60 mJ per cm^2) on the cytotoxicity were assessed using an AlamarBlue assay (Alamar Biosciences, Camarillo, California), according to the manufacturer's protocol. The fluorescence intensities were determined at 544 and 590 nm.

Statistics Data represent the mean ± SD. Statistical differences between two groups were determined by a two-tailed Student's test. In the case of more than two groups, differences were analyzed by analysis of variance (ANOVA test) and Scheffe's test. $p < 0.05$ was considered to be statistically significant.

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References

Bo X, Jiang L-H, Wilson HL, Kim M, Burnstock G, Surprenant A, North RA: Pharmacological and biophysical properties of the human $P2X_5$ receptor. Mol Pharmacol 63:1407–1416, 2003

Burnstock G, Wood JN: Purinergic receptors: Their role in nociception and primary afferent neurotransmission. Curr Opin Neurobiol 6:526–532, 1996

Burrell HE, Bowler WB, Gallagher JA, Sharpe GR: Human keratinocytes express multiple P2Y-receptors: Evidence for functional P2Y1, P2Y2, and P2Y4 receptors. J Invest Dermatol 120:440–447, 2003

Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G, Wood JN: A P2X purinoceptor expressed by a subset of sensory neurons. Nature 377: 428–431, 1995

Cook SP, McCleskey EW: Cell damage excites nociceptors through release of cytosolic ATP. Pain 95:41–47, 2002

Denda M, Inoue K, Fuziwara S, Denda S: P2X purinergic receptor antagonist accelerates skin barrier repair and prevents epidermal hyperplasia induced by skin barrier disruption. J Invest Dermatol 119:1034–1040, 2002

Dixon CJ, Bowler WB, Littlewood-Evans A, Dillon JP, Bilbe G, Sharpe GR, Gallagher JA: Regulation of epidermal homeostasis through $P2Y_2$ receptors. Br J Pharmacol 127:1680–1686, 1999

Evans RJ, Surprenant A: P2X receptors in autonomic and sensory neurons. Semin Neurosci 8:217–223, 1996

Ferguson DR, Kennedy I, Burton TJ: ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes—a possible sensory mechanism? J Physiol 505:503–511, 1997

Ferrari D, Chiozzi P, Falzoni S, Dal Susino M, Collo G, Buell G, Di Virgilio F: ATP-mediated cytotoxicity in microglial cells. Neuropharmacol 36:1295–1301, 1997

Glass R, Townsend-Nichols A, Burnstock G: P2 receptors in the thymus: Expression of P2X and P2Y receptors in adult rats, an immunohistochemical and *in situ* hybridisation study. Cell Tissue Res 300:295–306, 2000

Greig AVH, Linge C, Terenghi G, McGrouther A, Burnstock G: Purinergic receptors are part of a functional signaling system for proliferation and differentiation of human epidermal keratinocytes. J Invest Dermatol 120: 1007–1015, 2003

Grubb BD, Evans RJ: Characterization of cultured dorsal root ganglion neuron P2X receptors. Eur J Neurosci 11:149–154, 1999

Grynkiewicz G, Poenie M, Tsien RY: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450, 1985

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ: Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 391:85–100, 1981

Hansen M, Boitano S, Dirksen ER, Sanderson MJ: Intercellular calcium signaling induced by extracellular adenosine 5′-triphosphate and mechanical stimulation in airway epithelial cells. J Cell Sci 106:995–1004, 1993

Hennings H, Kruszewski FH, Yuspa SH, Tucker RW: Intracellular calcium alterations in response to increased external calcium in normal and neoplastic keratinocytes. Carcinogenesis 10:777–780, 1989

Jaken Y, Yuspa SH: Early signals for keratinocyte differentiation: Role of Ca^{2+}-mediated inositol lipid metabolism in normal and neoplastic epidermal cells. Carcinogenesis 9:1033–1038, 1988

Jiang L-H, Mackenzie AB, North RA, Surprenant A: Brilliant Blue G selectively blocks ATP-gated rat $P2X_7$ receptors. Mol Pharmacol 58:82–88, 2000

Koegel H, Alzheimer C: Expression and biological significance of Ca^{2+}-activated ion channels in human keratinocytes. FASEB 15:145–154, 2001

Koizumi S, Fujishita K, Inoue K, Shigemoto-Mogami Y, Tsuda M, Inoue K: Ca^{2+} waves in keratinocytes are transmitted to sensory neurons: The involvement of extracellular ATP and P2Y2 receptor activation. Biochem J 380: 329–338, 2004

Koizumi S, Inoue K: Inhibition by ATP of calcium oscillations in rat cultured hippocampal neurones. Br J Pharmacol 122:51–58, 1997

Lee WK, Choi SW, Lee HR, Lee EJ, Lee KH, Kim HO: Purinoceptor-mediated calcium mobilization and proliferation in HaCaT keratrinocytes. J Dermatol Sci 25:97–105, 2001

Lewis C, Neidhart S, Holy C, North RA, Buell G, Surprenant A: Coexpression of $P2X_2$ and $P2X_3$ receptor subunits can account for ATP-gated currents in sensory neurons. Nature 377:432–435, 1995

Mauro TM, Pappone PA, Isseroff RR: Extracellular calcium affects the membrane currents of cultured human keratinocytes. J Cell Physiol 143:13–20, 1990

Milner P, Bodin P, Loesch A, Burnstock G: Rapid release of endothelin and ATP from isolated aortic endothelial cells exposed to increased flow. Biochem Biophys Res Commun 170:649–656, 1990

Nakazawa K: Modulation of the inhibitory action of ATP on acetylcholine-activated current by protein phosphorylation in rat sympathetic neurons. Pflugers Arch 427:129–135, 1994

Norenberg W, Illes P: Neuronal P2X receptors: Location and functional properties. Naunyn-Schmiedeberg's Arch Pharmacol 362:324–339, 2000

North RA, Surprenant A: Pharmacology of cloned P2X receptors. Annu Rev Pharmacol Toxicol 40:563–580, 2000

Pillai S, Bikle DD: Adenosine triphosphate stimulates phosphoinositide metabolism, mobilizes intracellular calcium, and inhibits terminal differentiation of human epidermal keratinocytes. J Clin Invest 90:42–51, 1992

Schwarz A, Bhardwaj R, Aragane Y, *et al*: Ultraviolet-B-induced apoptosis of keratinocytes: Evidence for partial involvement of tumor necrosis factor-α in the formation of sunburn cells. J Invest Dermatol 104:922–927, 1995

Sun Y, Chai TC: Up-regulation of $P2X_3$ receptor during stretch of bladder urothelial cells from patients with interstitial cystitis. J Urol 171:448–452, 2004

Sun Y, Keay S, De Deyne PG, Chai TC: Augmented stretch activated adenosine triphosphate release from bladder uroepithelial cells in patients with interstitial cystitis. J Urol 166:1951–1956, 2001

Thorne PR, Housley GD: Purinergic signaling in sensory systems. Semin Neurosci 8:233–246, 1996

Ueno S, Koizumi S, Inoue K: Characterization of Ca^{2+} influx through recombinant P2X receptor in C6BU-1 cells. Br J Pharmacol 124:1484–1490, 1998

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Long-lasting change in brain dynamics induced by methamphetamine: enhancement of protein kinase C-dependent astrocytic response and behavioral sensitization

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It is well known that long-term exposure to psychostimulants induces neuronal plasticity. Recently, accumulating evidence suggests that astrocytes may actively participate in synaptic plasticity. In this study, we found that *in vitro* treatment of cortical neuron/glia co-cultures with either methamphetamine (METH) or morphine (MRP) caused the activation of astrocytes via protein kinase C (PKC). Purified astrocytes were markedly activated by METH, whereas MRP had no such effect. METH, but not MRP, caused a long-lasting astrocytic activation in cortical neuron/glia co-cultures. Furthermore, MRP-induced behavioral sensitization to hyper-locomotion was reversed by 2 months of withdrawal following intermitted MRP administration, whereas behavioral sensitization to METH-induced hyper-locomotion was maintained even after 2 months of withdrawal. Consistent with this cell culture study, *in vivo* treatment with METH, which was associated with behavioral sensitization, caused a PKC-dependent astrocytic activation in the cingulate cortex and nucleus accumbens of mice. These findings provide direct evidence that METH induces a long-lasting astrocytic activation and behavioral sensitization through the stimulation of PKC in the rodent brain. In contrast, MRP produced a reversible activation of astrocytes via neuronal PKC and a reversibility of behavioral sensitization. This information can break through the definition of drugs of abuse and the misleading of concept that morphine produces a long-lasting neurotoxicity.

Keywords: astrocyte, synaptic plasticity, psychostimulant, opioid, protein kinase C, neuron–glia communication.
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Glial cells, including astrocytes, microglia and oligodendrocytes, are the most numerous type of brain cells, and their roles in providing structural, metabolic and trophic support to neurons are well established (Kettenmann and Ransom 1995; Bezzi and Volterra 2001). Over the past decade, an increasing number of observations have progressively challenged the classical view that glial cells only serve passive supportive functions in mammalian CNS. For example, one glial type, oligodendrocyte precursor cells has been shown to receive direct synaptic input from neurons in the hippocampus (Bergles *et al.* 2000), and another glial cell type, astrocyte, releases glutamate rapidly in response to physiological increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Parpura and Haydon 2000).

Astrocytes are a subpopulation of glial cells that control brain homeostasis to meet neuronal metabolic demands. Furthermore, astrocytes have a large variety of receptors for neurotransmitters and hormones, including dopamine receptor (Khan *et al.* 2001) and glutamate receptor (Nedergaard *et al.* 2002), which are coupled to various intracellular signaling cascades (Haydon 2001). Astrocytes are known to exhibit a form of excitability and communication based on changes in $[Ca^{2+}]_i$, which can be stimulated by neuronal synaptic activity (Parri *et al.* 2001). More recently, astrocytes have been reported to promote axonal extension and neuronal

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Abbreviations used: NPC-15437, *S*-2,6-diamino-*N*-[(1-[1-oxotridecyl]-2-piperidinyl)methyl]hexamide dihydorochloride; BSS, basal salt saline; CHE, chelerythrine chloride; DA, dopamine; GFAP, glial fibrillary acidic protein; GLU, glutamate; METH, methamphetamine; MRP, morphine; PFA, paraformaldehyde; PBS, phosphate-buffered saline; PKC, protein kinase C; p-PKC, phosphorylated-protein kinase C.

