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In situ hybridization studies of prostacyclin receptor mRNA expression in various mouse organs

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1 Expression of prostacyclin receptor (IP receptor) mRNA was examined in various mouse organs, and the cells expressing IP receptor mRNA were identified by *in situ* hybridization studies. Co-localization of mRNA for the IP receptor with that for preprotachykinin A (PPTA), a precursor protein for substance P, with mRNA for the prostaglandin E receptor subtypes (EP_1, EP_3 and EP_4), and with renin mRNA, was examined by double *in situ* hybridization studies in the dorsal root ganglion and kidney, respectively.

2 IP receptor mRNA was expressed in the thymus and spleen. Expression in the thymus was found exclusively in the medulla, where mature thymocytes expressed transcripts for the IP receptor. Expression in the spleen was found as scattered signals over the white pulp and as punctate signals in the red pulp. The former was found in splenic lymphocytes and the latter in megakaryocytes.

3 IP receptor mRNA was also expressed in the vascular tissues of various organs such as the aorta, coronary arteries, pulmonary arteries and the cerebral arteries, where its expression was confined to smooth muscle cells. No expression was found in veins. In the kidney, IP receptor mRNA was detected in the interlobular arteries and glomerular arterioles but not in the juxtaglomerular (JG) cells which were labelled with the renin mRNA probe.

4 IP receptor mRNA was expressed in about 40% of the neurones in the dorsal root ganglion. Both small- and large-sized neurones were labelled but no labelling was found in the glia. Expression of PPTA mRNA was found in about 30% of total neurones. About 70% of these neurones expressed IP receptor mRNA, and about half of the IP receptor-positive neurones expressed PPTA mRNA. In addition to IP mRNA, mRNAs for EP_1, EP_3 and EP_4 receptors were expressed in about 30%, 50% and 20%, respectively, of the dorsal root ganglion neurones. About 25%, 41% and 24% of the IP receptor-positive neurones co-expressed the EP_1, EP_3 and EP_4 receptor, respectively.

5 These results not only verified IP receptor expression in various cells and tissues known to be sensitive to prostacyclin, but also revealed its expression in other systems, which urges the study of the actions of prostacyclin in these tissues. They also indicated that the actions of prostacyclin on blood vessels and platelets are mediated by the same type of receptor. Absence of IP receptor mRNA in the JG cells suggests that the action of prostacyclin on renin release may be indirect.

Keywords: *In situ* hybridization; IP receptor; thymic lymphocytes; megakaryocyte; vascular smooth muscle cells; kidney; dorsal root ganglion; renin; preprotachykinin A; substance P

Introduction

Prostanoids, including various prostaglandins (PGs) and thromboxanes (TXs) exert a wide variety of actions to maintain local homeostasis in the body (Moncada *et al.*, 1985). These compounds are released immediately after synthesis and act on the cell-surface receptors to elicit their actions. These receptors have been characterized pharmacologically by comparing the actions and potencies of various prostanoids and their analogues in a number of systems. They have also been studied biochemically, by evaluating radioligand binding activities in many types of cells and tissues (Halushka *et al.*, 1989). Based on these analyses, Coleman *et al.* (1990) proposed a classification system for the prostanoid receptors, in which they suggested that each prostanoid has its own receptor and that there are four subtypes of the PGE receptor. They named these receptors DP, EP_1, EP_2, EP_3, EP_4, FP, IP and TP. While this proposal greatly facilitated studies of the prostanoid receptors, the exact properties and the tissue and cell localization of each receptor have remained unclarified. This is partly because most tissues contain more than one type of prostanoid receptor but also because a prostanoid ligand selectivity is limited. It is also difficult to detect receptor expression by ligand binding studies, unless the receptors are expressed at high densities. In any event, identification of the cell type expressing the receptor in tissue has been very difficult without the availability of specific histochemical probes. In addition, some pharmacological studies have suggested that there is more than one molecular form of the TP and IP receptors, and that these receptor forms are differentially distributed in tissues such as blood vessels and platelets (Lefer *et al.*, 1980; Mais *et al.*, 1985; Corsini *et al.*, 1987; Morinelli *et al.*, 1989; Takahara *et al.*, 1990). However, such an issue cannot be clarified by pharmacological experiments alone.

Recently, we have cloned several types of prostanoid receptors (Hirata *et al.*, 1991; 1994; Namba *et al.*, 1992; 1994; Sugimoto *et al.*, 1992; 1994b; Honda *et al.*, 1993; Watabe *et al.*, 1993). These studies together with a study by another group (Regan *et al.*, 1994) have identified all of the pharmacologically-defined prostanoid receptors described above. There was initial confusion over the identity of the cloned PGE receptor which positively coupled to adenylate cyclase. Honda *et al.* (1993) first cloned this receptor from a cDNA library derived from the mouse mastocytoma cell line P815, and reported it to be the EP_2 receptor subtype. Receptors homologous with it have since been cloned from various species and have also been described as EP_2 receptors (An *et al.*, 1993; Bastien *et al.*, 1994;

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Sando *et al.*, 1994). However, these receptors do not respond to one of the EP_2 agonists, butaprost, raising the possibility that they correspond to some other PGE receptor subtype. Recent reports by Regan *et al.* (1994) and Nishigaki *et al.* (1995) have now revealed that the receptor initially cloned by Honda *et al.* (1993) was indeed of the EP_4 subtype, and that cloned by Regan *et al.* (1994) was of the EP_2 subtype. Therefore, the mouse receptor cloned originally by Honda *et al.* (1993) is referred to as EP_4 in this paper.

These types and subtypes of the prostanoid receptors are encoded by different genes and, as a whole, constitute a family of homologous receptors. Expression of cDNAs for the cloned receptors in cultured cells devoid of any prostanoid receptor has made it possible to study the properties of each receptor in a homogeneous population, and to identify corresponding receptors which have been pharmacologically characterized in the body (Coleman *et al.*, 1994). These studies have also enabled us to study the distribution and cellular localization of each receptor by *in situ* hybridization. Using this method, we have already studied the distribution of the three EP receptor subtypes in the kidney, and the EP_3 receptor in the brain (Sugimoto *et al.*, 1994a,c). In the following study, the distribution of IP receptor mRNA in mouse organs, and the identification of cells expressing its mRNA, were examined in each tissue. This study not only verified IP receptor expression in those cells and tissues known to be sensitive to prostacyclin,

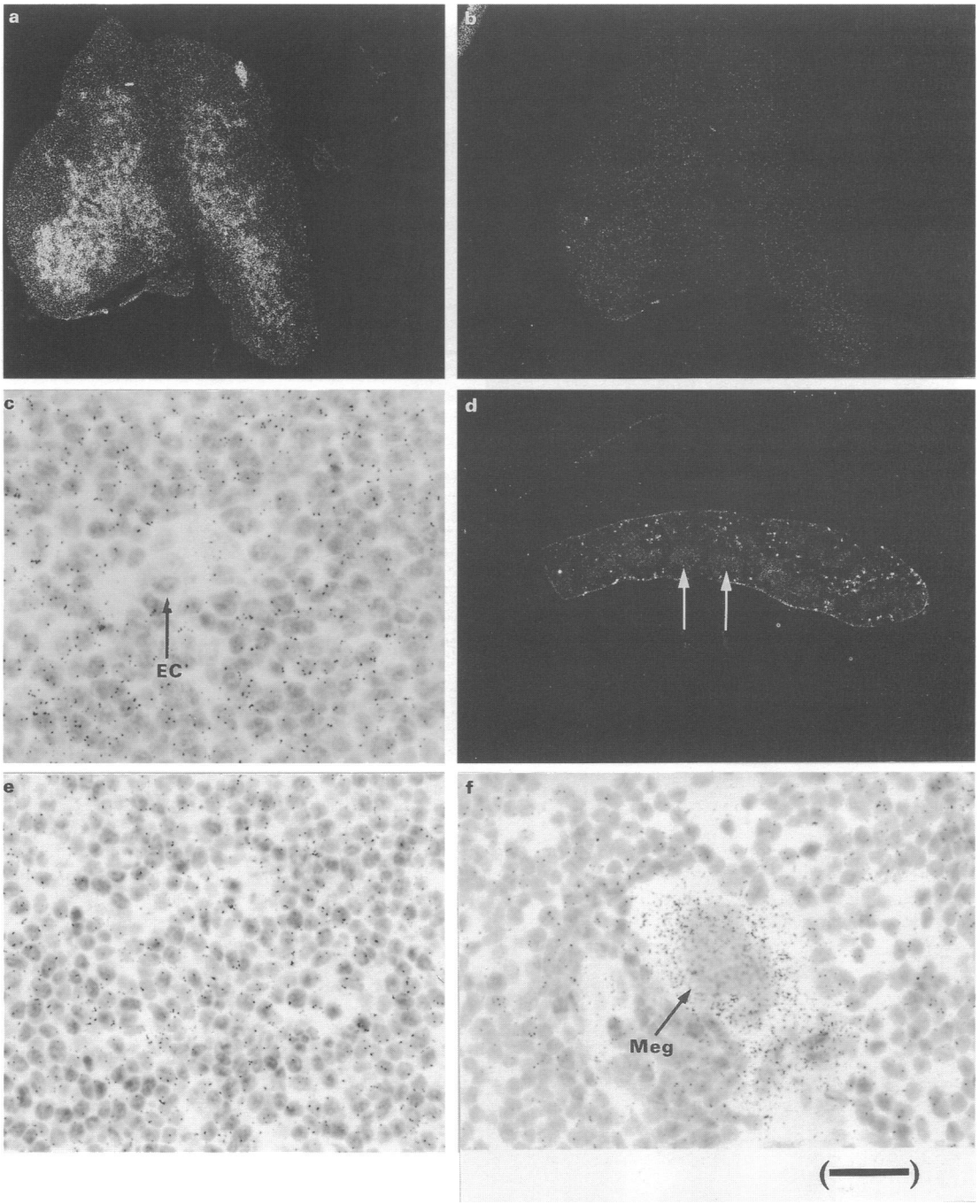

Figure 1 Regional and cellular localization of IP receptor mRNA in the thymus and spleen. Sections were hybridized with ^{35}S-labelled antisense riboprobe in the absence (a, c, d, e and f) or presence (b), of a 100 fold excess of the unlabelled probe. (a b) Autoradiograms from sections of the thymus. Med, thymic medulla; (c), a bright-field photomicrograph of the thymic medulla; EC, epithelial cells of thymus; (d), an autoradiogram from a section of the spleen, the white pulp is indicated by arrows; (e f), bright-field photomicrographs of the white and red pulp, respectively. Meg, splenic megakaryocytes. Bar, 1.5 mm for (a) (b) and (d), and 75 μm for (c), (e) and (f).

but also disclosed receptor expression in tissues where little is known about the action of this compound. It also presents evidence that the same receptor molecule is expressed in both platelets and blood vessels.

Methods

Synthesis of cRNA probes

An antisense cRNA probe for the mouse IP receptor was prepared as follows. A 1.7 kilobasepair 5′-fragment was prepared by deleting approximately 1.6 kilobasepairs from the 3′-end of CP302 (Namba *et al.*, 1994). This fragment was subcloned into pBluescript (Stratagene), linearized with Xbal, and used as a template for riboprobe synthesis. An antisense riboprobe was prepared by transcription with T3 RNA polymerase (Promega) in the presence of [α-^{35}S]-CTP to a specific activity of 1.0×10^9 d.p.m. μg^{-1}. The cold antisense riboprobe and the digoxigenin (Dig)-labelled antisense probe were synthesized by the same procedure using unlabelled nucleotides, and in the presence of 11-Dig-uridine triphosphate, respectively. cRNA probes for the EP_1, EP_3 and EP_4 receptors were prepared from the EP_1, EP_3 and EP_4 cDNAs in the presence of either [α-^{35}S]-CTP to a specific activity of 1.0×10^9 d.p.m. μg^{-1}, or unlabelled nucleotides as described previously (Sugimoto *et al.*, 1994a). Dig-labelled antisense probes and sense RNA probes for preprotachykinin (PPTA) mRNA and for renin mRNA were synthesized in the presence of 11-Dig-uridine triphosphate, using rat PPTA cDNA (Kawaguchi *et al.*, 1986) and mouse renin cDNA (Masuda *et al.*, 1982), respectively, as templates. After unincorporated nucleotides were removed, riboprobes were degraded to ~150 bases by alkaline-hydrolysis.

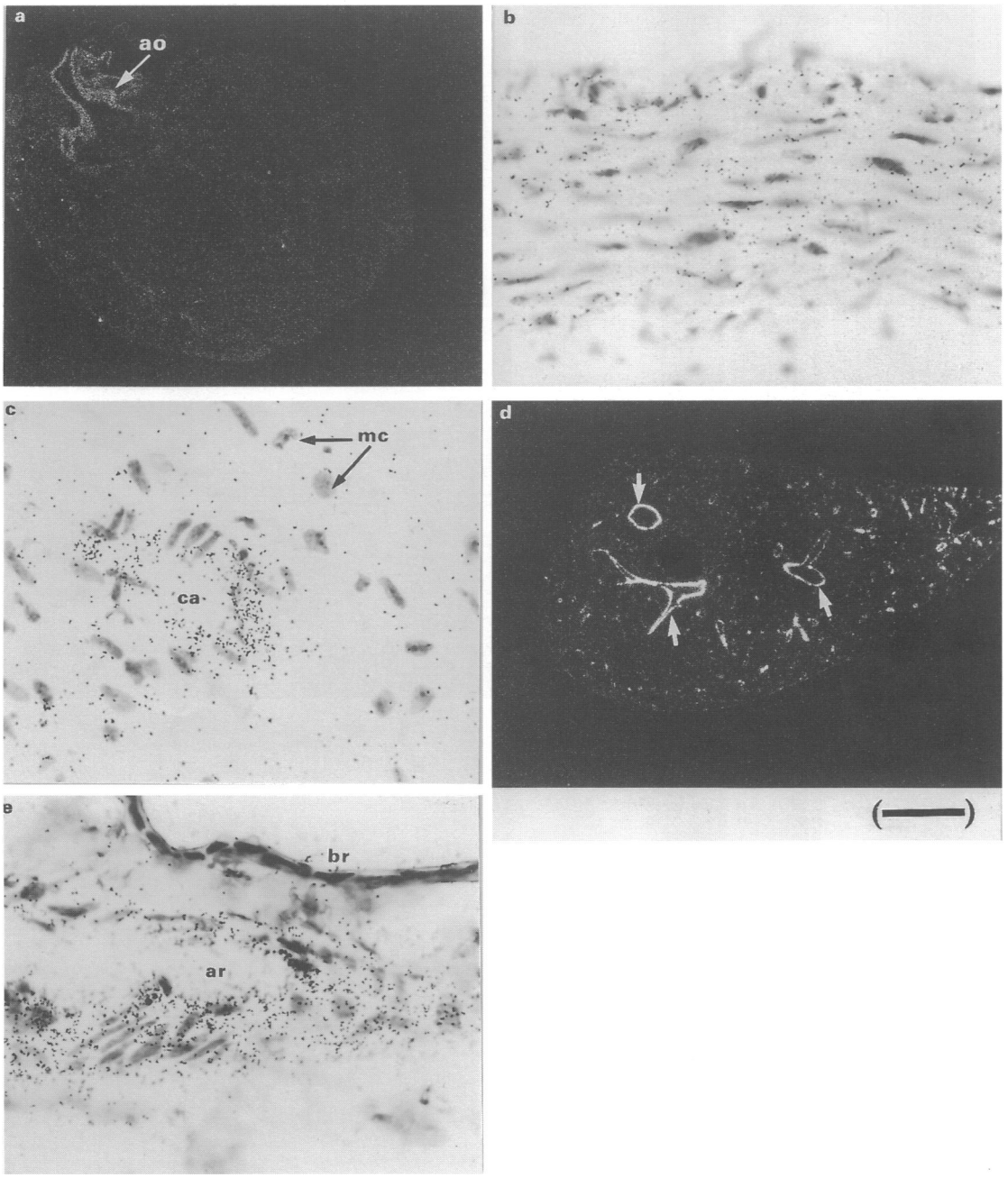

Figure 2 Regional and cellular localization of IP receptor mRNA in the heart and lung. (a) An autoradiogram of the heart; ao, aorta. Signals associated with a vessel-like structure are indicated by an arrow. (b c) Bright-field photomicrographs of the aorta and heart, respectively; ca, coronary artery; mc, myocardial cells. (d) An autoradiogram of the lung. Arrows indicate the large tube-or vessel-like structures. (e) A bright-field photomicrograph of the lung. ar, pulmonary artery. br, bronchi. Bar, 1 mm for (a) and (d), and 75 μm for (b), (c) and (e).

Preparation of tissue sections

Adult male ddY mice were anaesthetized with ether and killed by decapitation. Organs were removed and immediately frozen in isopentane at −50°C without any preparative measures. Dorsal root ganglia (DRG) were removed from L4–L6 of the spinal cord and processed similarly. Sections of 8 μm thickness were cut on a cryostat and thaw-mounted onto poly-L-lysine (Sigma) coated slides. They were briefly air-dried and kept at −80°C until use.

In situ *hybridization with radiolabelled probes*

In situ hybridization was carried out essentially as described previously (Shigemoto *et al.*, 1992). The frozen sections were warmed to room temperature, fixed with 4% formaldehyde in phosphate-buffered saline for 10 min, rinsed in phosphate-buffered saline, and then acetylated with 0.25% acetic anhydride/0.1 M triethanolamine/0.9% NaCl/pH 8, for 10 min at room temperature. After dehydration in an ascending ethanol series, the sections were air-dried and stored at −80°C until use. Hybridization was carried out in a buffer containing 50% formamide/2 × SSC/10 mM Tris-HCl, pH 7.5/1 × Denhardt's solution/10% dextran sulphate/0.2% sodium dodecyl sulphate/100 mM dithiothreitol (DTT)/500 μg ml^{-1} sheared single-stranded salmon sperm DNA/250 μg ml^{-1} yeast tRNA. The riboprobe, preheated at 80°C for 10 min in 1 M DTT, was added to the hybridization buffer at 7×10^4 d.p.m. μl^{-1}. The hybridization solution was applied to the sections, which were then covered with a coverslip which was sealed in place with rubber cement. After incubation at 57°C for 6 h, the slides were immersed at room temperature in 2 × SSC to remove the coverslips. They were then washed in 2 × SSC/10 mM β-mercaptoethanol at 60°C for 1 h. The sections were then treated with 20 μg ml^{-1} ribonuclease A in 0.5 M NaCl/10 mM Tris-HCl, pH 7.5/1 mM ethylenediamine tetraacetic acid (EDTA), for 30 min at 37°C, followed by an additional wash in 0.1 × SSC at 60°C for 1 h. After dehydration in an ascending ethanol series, the slides were air-dried and exposed to β-Max film (Amersham) for 7 days at room temperature, or were

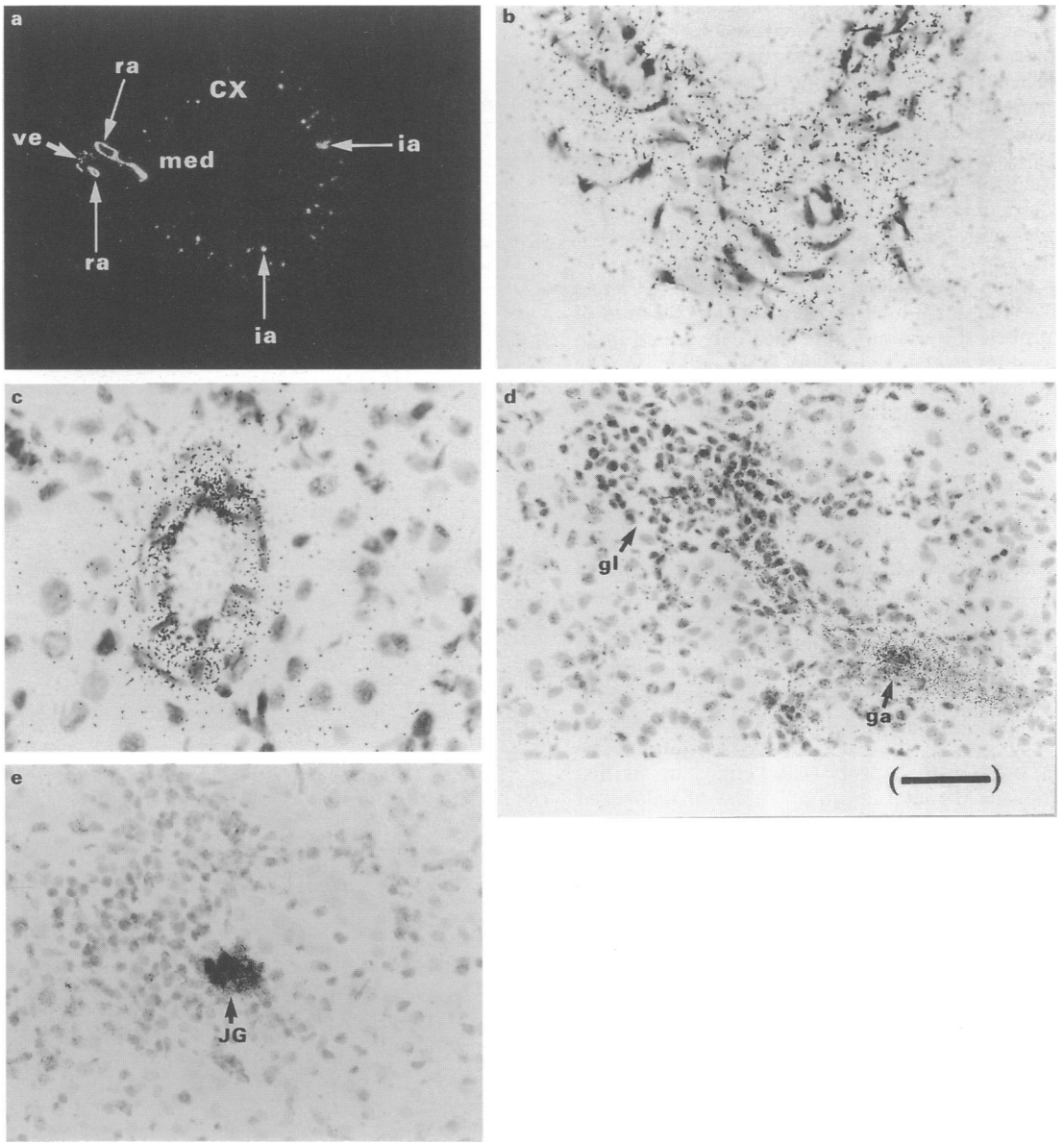

Figure 3 Regional and cellular localization of IP receptor (a–d) and renin (e) mRNAs in the kidney. (a) An autoradiogram of the kidney; cx, cortex; med, medulla; ra, renal artery; ve, renal vein; ia, interlobular artery. Apparent labelling in the renal vein is due to the deposition of ^{35}S-probe at the section edge. (b and c) Bright-field photomicrographs of the renal artery and interlobular artery, respectively. (d) A bright-field photomicrograph of the glomerular region; gl, glomerulus; ga, glomerular arteriole. (e) A bright-field photomicrograph showing renin mRNA expression, using the Dig-labelled probe, in the glomerular region. JG, juxtaglomerular cells. Bar, 1.8 mm for (a) and 75 μm for (b), (c), (d) and (e).

dipped in NTB2 (Kodak), a nuclear track emulsion which is diluted 1 : 1 with distilled water. After exposure for 30 days at 4°C, the dipped slides were developed in D-19 developer (Kodak), fixed, and counterstained with haematoxylin-eosin.

In situ *hybridization with Dig-labelled probes*

The *in situ* hybridization with Dig-labelled probes, and double *in situ* hybridization with both Dig-labelled and radiolabelled probes, were carried out as follows. Slides were pretreated as described and subjected to prehybridization at 55°C for 2 h in 50% formamide/5 × SSC/5 × Denhardt's solution/10 mM EDTA/250 $\mu g\ ml^{-1}$ yeast tRNA/20 mM DTT/500 $\mu g\ ml^{-1}$ sheared single-stranded salmon sperm DNA. Hybridization was carried out with a Dig-labelled probe, or with a mixture of a radiolabelled probe and Dig-labelled probe, at 55°C for 16 h. After hybridization, sections were washed four times in 2 × SSC/10 mM DTT (each wash was for 10 min at 55°C), incubated with 50 $\mu g\ ml^{-1}$ ribonuclease A as described, and then washed twice in 50% formamide/2 × SSC/10 mM DTT, for 30 min at 55°C. Sections were dehydrated in an ethanol series, and dried. Sections were rehydrated in 0.1 M Tris HCl, pH 7.4/0.9% NaCl, blocked with 1% unimmunized sheep serum (DaKo) for 30 min, and then incubated with 0.1% alkaline phosphatase-conjugated anti-digoxigenin antibody in the solution described above containing 1% sheep serum, for 2 h at room temperature. After washing in the above solution, the sections were immersed in 0.1 M Tris HCl/0.15 M NaCl/50 mM $MgCl_2$/pH 9.5 (the colouring buffer) twice, for 5 min each time. Visualization of the antibody was carried out in colouring buffer containing 337.5 $\mu g\ ml^{-1}$ nitroblue tetrazolium chloride and 1.75 $\mu g\ ml^{-1}$ 5-bromo-4-chloro-3-indolylphosphate for 15 h. The colouring reaction was terminated by washing the slides in 10 mM Tris HCl/1 mM EDTA/pH 8.0, twice, for 2 h each time. For the detection of radiolabelled probes, the sections were then dehydrated in an ascending ethanol series and coated with 2.5% collodion. The dipping of slides in a nuclear track emulsion and the subsequent developing procedure were conducted as described above.

Results

Northern blot analysis in various mouse organs has shown that IP receptor mRNA is most abundantly expressed in the thymus, followed by the spleen heart and lung (Namba *et al.*, 1994). To identify the exact tissue and cellular localization of the IP receptor mRNA, we performed *in situ* hybridization using a ^{35}S-labelled riboprobe. We also examined co-localization of the IP receptor mRNA with mRNAs of other receptors and peptide precursors, by double *in situ* hybridization. The specificity of a signal with a probe was verified by its disappearance following the addition of an excess of unlabelled probe, or by using a sense probe with the same construct.

The regional and cellular localization of IP receptor mRNA in the thymus and spleen, was examined first. Autoradiography of the thymus tissue showed that hybridization signals were visible only in the medulla and not in the cortex, and that these signals were abolished by the addition of an excess of unlabelled probe (Figure 1a, b). In a microscopic examination of the thymic medulla, autoradiographic grains were detected over a number of small-sized mononuclear cells scattered in the medulla but not in those cells with a large cytoplasm, indicating that IP receptor mRNA was expressed predominantly in thymocytes and not in stromal cells such as the epithelial cells (Figure 1c). In the spleen, positive signals were observed in regions corresponding to the white pulp and as punctate signals scattered in the red pulp (Figure 1d). The hybridization signals in the white pulp were diffusely distributed in lymphocytes (Figure 1e). On the other hand, as shown in Figure 1f, autoradiographic grains in the red pulp were specifically concentrated in megakaryocytes.

IP receptor mRNA expression was subsequently examined in the heart and the lungs. Figure 2a represents an autoradiogram of heart tissue. The hybridization signals were observed in regions corresponding to the aorta and in vessel-like structures of small-calibre in the parenchyma (Figure 2a). In the bright-field examination, hybridization signals in the aorta were detected in the smooth muscle cells, and those in the parenchyma were located in the smooth muscle cells of the coronary arteries (Figure 2b and c). No significant signals were detected either in veins, including the vena cava, or in myocardial cells (Figure 2c and data not shown). The autoradiogram of the lung shown in Figure 2d, revealed strong hybridization signals in the large tube or vessel-like structures and similar structures of various sizes which were widely distributed in the lung parenchyma (Figure 2d). The emulsion-dipped section showed that the silver grains were confined to arteries of large to small calibre and were located over the smooth muscle cells (Figure 2e). In contrast, significant hy-

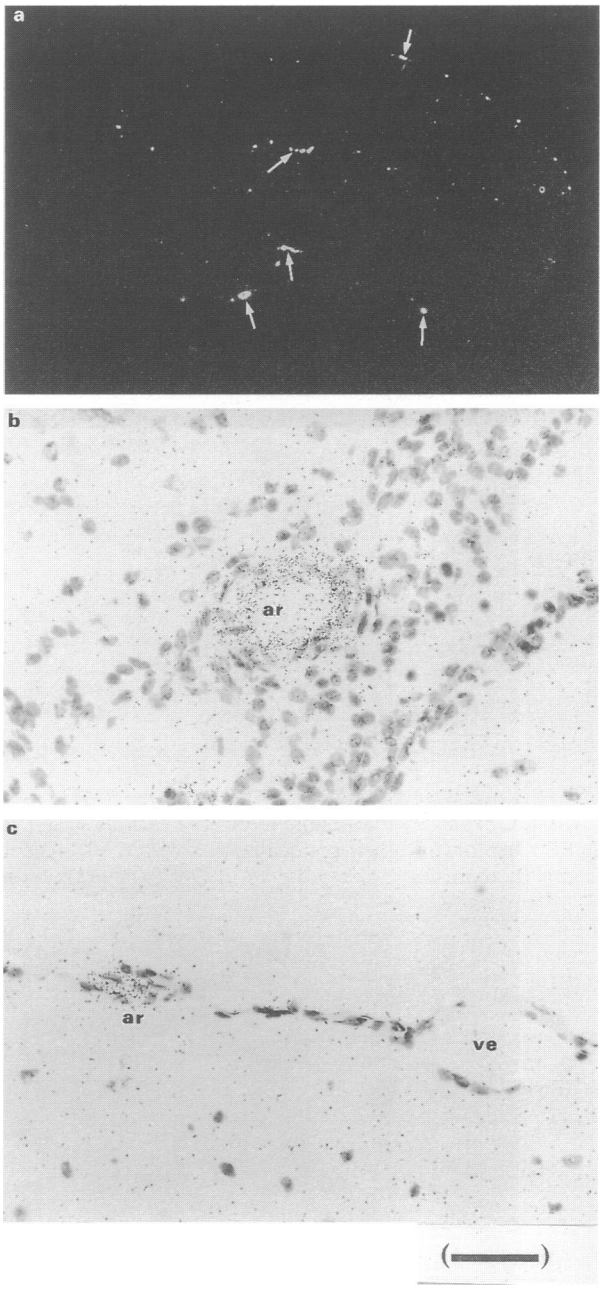

Figure 4 Regional and cellular localization of IP receptor mRNA in the brain. (a) An autoradiogram of a sagittal section of the brain; (b and c) photomicrographs; ar, artery. ve, vein. Bar, 1.5 mm for (a) and 100 μm for (b) and (c).

bridization signals were not seen in the bronchi (Figure 2e), the pulmonary veins, the alveolar epithelium, or the interalveolar septum (data not shown).

Figure 3 shows our *in situ* hybridization study of the kidney. Although this organ as a whole did not give strong signals with Northern blot analysis (Namba *et al.*, 1994), the signals detected in this analysis were distinct. These signals were seen in a vessel-like structure at the hilus, and in dot-like structures in the cortex (Figure 3a). In a bright-field photomicrograph of the former, the grains were observed in the renal artery and its branch, where smooth muscle cells were labelled extensively (Figure 3b). A similar analysis of the latter structures revealed the labelling to be confined to the interlobular arteries, as shown in Figure 3c. Examination of the renal cortex also disclosed significant labelling in the wall of the arterioles connected to the glomeruli (Figure 3d). The size of these arterioles indicated that they were the afferent arterioles of the glomeruli. Interestingly, little signal was seen in those portions of the arterioles which were most proximal to the glomerulus. Because prostacyclin is known to stimulate renin release, IP receptor mRNA expression was examined in the juxta- glomerular (JG) cells. As shown in Figure 3e, the JG cells labelled with the probe for mouse renin mRNA were located in the proximal portion of the afferent arteriole, where little hybridization signal for the IP receptor mRNA was found.

IP receptor mRNA expression was also examined in the nervous system, including the brain, spinal cord and sensory ganglia. Figure 4a shows a macroscopic autoradiogram of a sagittal section of the brain, revealing the distribution of hybridization signals in this tissue. Analysis using the emulsion-dipped sections revealed that the autoradiographic grains were concentrated in the smooth muscle cells of arteries but not in veins (Figure 4b and c). No significant labelling for IP receptor mRNA was seen in the neurones or glia of this section (Figure 4b). Similarly, there was an absence of labelling in the neurones and glia in transverse sections of the spinal cord (data not shown). Contrary to findings in the brain and spinal cord, a high density of signals was found in the DRG and these signals were found exclusively on neurones (Figure 5a–c). About 40% of the neurones in the ganglia were labelled intensively by the IP mRNA probe. Both large (25–60 μm) and small (15–25 μm) neurones were labelled, and 60% of the labelled cells were small in size. In order to characterize those neurones positive for IP receptor mRNA, we carried out double *in situ* hybridization with a probe for the PPTA mRNA. As shown in Figure 6a, a population of DRG neurones which were PPTA mRNA-positive was detected. A parallel experiment with nuclear staining indicated that about 30% of the DRG neurones expressed the PPTA mRNA, about 70% of which were positive for IP receptor mRNA.

We next examined the expression of the various subtypes of the EP receptors and their co-localization with the IP receptor. As already reported (Sugimoto *et al.*, 1994a), the expression of EP_3 receptor mRNA was clearly observed in DRG neurones. In addition, weaker but significant labelling was also found with both the EP_1 and EP_4 receptor mRNA probes. Labelling for EP_1, EP_3 and EP_4 receptor mRNAs was found in about 30%, 50% and 20% of the total neurones, respectively. In some neurones these mRNAs were co-localized with IP receptor mRNA (Figure 6b–d). About 25%, 41% and 24% of the IP receptor-positive neurones expressed the EP_1, EP_3 and EP_4 receptors, respectively.

In situ hybridization screening for IP receptor mRNA expression was also performed in trachea, thyroid gland, parathyroid gland, oesophagus, stomach, jejunum, ileum, colon, adrenal gland, liver and pancreas. However, in the organs of the gastrointestinal tract, high nonspecific hybridization signals, due mainly to an edging effect, precluded the definitive identification of expression sites. In other organs, we did not detect specific hybridization signals, suggesting that the expression of the IP receptor mRNA, if present, was considerably lower in these organs.

Discussion

The cells expressing IP receptor mRNA in various organs, and the relative density of this expression, are summarized in Table 1. IP receptor mRNA is expressed by various types of cells, including neurones in the sensory ganglia, thymocytes and splenic lymphocytes, megakaryocytes and arterial smooth muscle cells in various organs. The highest density of expression was seen in DRG neurones, and high expression was also detected in megakaryocytes. Moderate labelling was detected in the arterial smooth muscle cells of a variety of tissues. Low levels of labelling were observed in mature thymocytes and lymphocytes. Significant labelling was not detectable in either the trachea or the spinal cord.

Our study demonstrated that IP receptor was expressed ubiquitously by the smooth muscle cells of arteries of various sizes in a variety of organs. This finding is consistent with

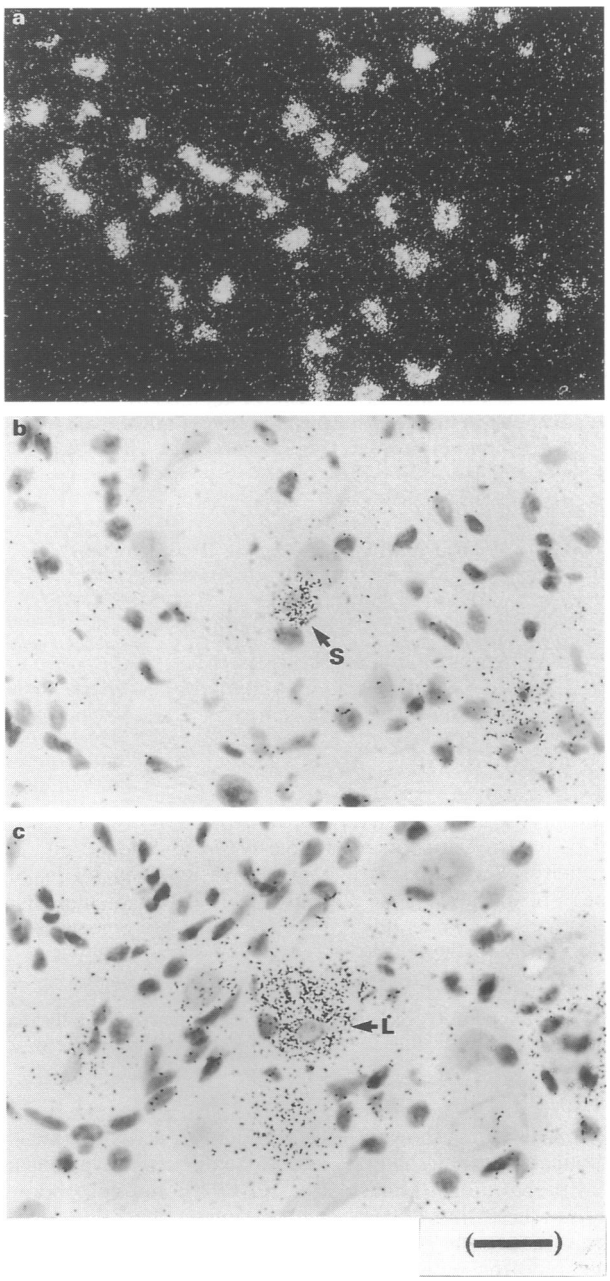

Figure 5 Regional and cellular localization of IP receptor mRNA in the dorsal root ganglia. Dark- (a) and bright-field (b and c) photomicrographs of dorsal root ganglia are shown. S, small-sized neurones. L, large-sized neurone. Bar, 500 μm for (a) 75 μm for (b) and (c).

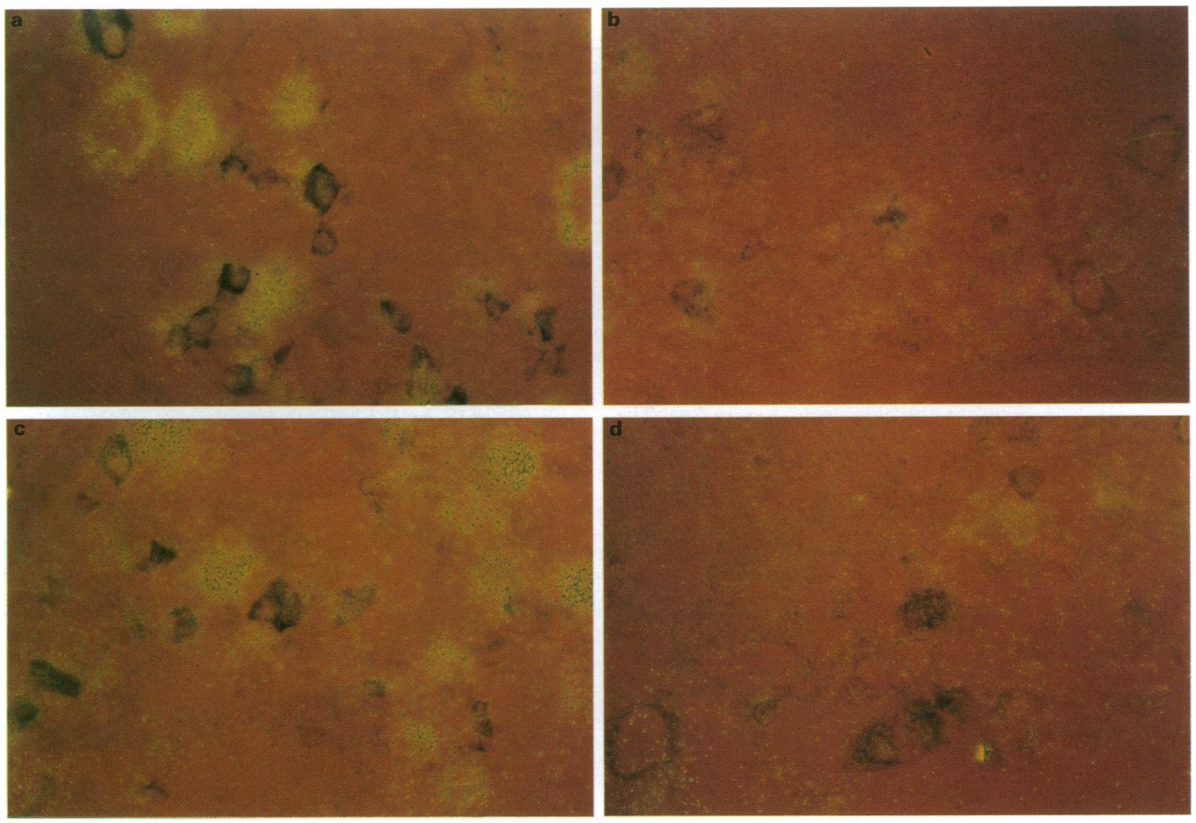

Figure 6 Co-localization of IP receptor mRNA with preprotachykinin A (PPTA) mRNA and mRNA for the EP-receptor subtype. (a) Co-localization of IP receptor mRNA and PPTA mRNA. The IP receptor and PPTA mRNAs were hybridized with ^{35}S-labelled and Dig-labelled probes, respectively. Signals obtained with the radiolabelled probe are shown in green, and those with the Dig-labelled probe are dark brown. (b), (c) and (d), co-localization of IP receptor mRNA and EP_1 (b), EP_3 (c) and EP_4 (d) receptor mRNAs. In these experiments, IP receptor mRNA was hybridized with Dig-labelled probe and mRNA for the EP receptor subtypes was hybridized with the radiolabelled probes. Bar, 50 μm.

Table 1 Cellular localization of mouse IP receptor mRNA

Organ	*Labelled cells*	*Relative grain densities on cell bodies*
Thymus	Mature thymocyte	+
Spleen	Lymphocyte	+
	Megakaryocyte	+ + +
Heart	Smooth muscle cells of aorta	+ +
	Smooth muscle cells of coronary artery	+ +
Lung	Smooth muscle cells of pulmonary artery	+ +
Trachea		–
Kidney	Smooth muscle cells of renal artery	+ +
	Smooth muscle cells of interlobular artery	+ +
	Smooth muscle cells of afferent arteriole	+ +
Brain	Smooth muscle cells of cerebral artery	+ +
Dorsal root ganglion	Neurone	+ + + +
Spinal cord		–

Distribution of IP receptor mRNA is summarized on the basis of the results presented in Figures 1–5. Relative grain densities: + + + +, very high; + + +, high; + +, moderate; +, low; –, background level.

previous studies showing the vasodilator activity and the radioligand binding of PGI_2 and its analogues, in various arterial preparations (Halushka *et al.*, 1989), and corroborates the hypothesis that PGI_2 plays a key role in controlling blood supply in various organs. Our results also showed that the same receptor molecule was expressed by arterial smooth muscle cells and by the platelet precursor cells, the megakaryocytes. Previous pharmacological studies have noted the different responsiveness of platelets and vascular smooth muscle cells to PGI_2 and to several PGI_2 analogues. For example, Corsini *et al.* (1987) found that (5Z)-carbacyclin was as effective as PGI_2 in stimulating adenylate cyclase in human platelets, while it was a partial agonist in the rabbit mesenteric artery where it antagonized PGI_2 activity. On the basis of these results, they indicated that the IP receptor in platelets may be different from that in arteries. Our results do not appear to support this hypothesis, and suggest that the observed difference may be species-dependent. Our results showed that the IP receptor was expressed only in arteries and not in veins, and that this expression was limited to the smooth muscle cells and was not seen in endothelial cells. Previous studies, however, have shown that PGI_2 causes the contraction of veins. For

example, Schrör *et al.* (1981) reported that contraction of the bovine coronary vein was induced by PGI_2. PGI_2 has also been reported to cause the endothelium-dependent relaxation of coronary arteries in the pig (Shimokawa *et al.*, 1988). Because both PGI_2 and iloprost used as IP receptor agonists in many studies are known to cross-react with the EP_1 receptor (Dong *et al.*, 1986; Armstrong *et al.*, 1989), the above findings may be due to activation of the EP_1 receptor by these compounds. We did not observe significant expression of IP receptor mRNA in mouse myocardium in this study. There are controversial reports of PGI_2 action on myocardial cells. Alloatti *et al.* (1991) reported that PGI_2 increased the calcium current in a concentration-dependent manner in guinea-pig myocardial cells, whereas Couttenye *et al.* (1985) reported that PGI_2 had no direct influence on rat myocardial cells.

PGI_2 is well known as an important regulator of renal blood flow in the dog (Bolger *et al.*, 1978; Lifschitz, 1981) and of the glomerular filtration rate in the rat kidney (Schor *et al.*, 1981). The major resistance vessels in the kidney are the afferent and efferent arterioles and possibly the interlobular arteries (Källskog *et al.*, 1976). IP receptor mRNA was expressed in the interlobular arteries as well as the glomerular arterioles, which were presumed to be afferent arterioles from their diameter. PGI_2, therefore, appears to act on these arteries to increase blood supply to this organ. PGI_2 is also known to stimulate the release of renin both *in vivo* and *in vitro* (Bolger *et al.*, 1978; Oates *et al.*, 1979). Two mechanisms of renin release by PGI_2 have been proposed: (1) PGI_2 acts directly on the JG cells to stimulate the release of renin; (2) PGI_2 induces vasodilatation in the afferent arterioles, causing a decrease in blood pressure, which indirectly stimulates the release of renin. The present study is consistent with the idea that PGI_2 causes the release of renin by the latter mechanism. IP receptor mRNA was detected in the afferent arterioles but not in the JG cells, although we cannot completely exclude the possibility that a small amount of IP receptor was also present in the latter.

Our results suggest that PGI_2 may be an important regulator in the immune and nervous systems. The IP receptor transcripts were abundant exclusively in the thymic medulla where CD4+8− and CD4−8+ single positive thymocytes are harboured. In a preliminary experiment, we observed positive *in situ* hybridization signals in both the CD4+8− and the CD4−8+ thymocyte populations isolated by a cell sorter, and the generation of cyclic AMP in response to cicaprost was detected in both populations (H. Oida & S. Narumiya, unpublished observation), suggesting that the IP receptor was expressed in single positive thymocytes. Several groups have reported that PGI_2 is produced in thymic nurse cells and phagocytic cells (Homo-Delarche *et al.*, 1985; McCormack *et al.*, 1991). We also detected the production of 6-keto-$PGF_{1\alpha}$, a major metabolite of PGI_2, in two thymic stromal cell lines, TSt-4 and TEC 1-4 (H. Oida & S. Narumiya, unpublished observation). PGI_2, therefore, seems to be produced by stromal cells and to act on medullary thymocytes. Thus, PGI_2 may have novel immuno-modulatory actions. IP receptor mRNA was also detected in the white pulp of spleen tissue where peripheral lymphocytes are harboured. This finding indicates that PGI_2 also acts on peripheral lymphocytes. Whereas IP receptor mRNA expression is high in mouse thymus and spleen, Northern blot analysis detected very little signal in human thymus and only weak expression in rat thymus (Nakagawa *et al.*, 1994; Sasaki *et al.*, 1994). These results suggest that the immuno-modulatory role of PGI_2 may differ among species.

IP receptor mRNA was highly expressed in both large and small neurones to nearly the same degree in the DRG. The expression of IP receptor mRNA in small DRG neurones is in good agreement with the hypothesis that PGI_2 causes hyperalgesia (Ferreira *et al.*, 1974; Juan, 1979). Substance P (SP) is present in primary afferent neurones and works as a neurotransmitter of nociceptive neurones in the spinal cord (Otsuka & Yoshioka, 1993). About 70% PPTA (SP precursor), mRNA-positive neurones co-expressed IP receptor mRNA. It is, therefore, likely that the IP receptor has an important role in hyperalgesia in the SP-containing neurones. On the other hand, about half of the IP receptor mRNA-positive neurones did not express PPTA mRNA, indicating that the IP receptor has some function in neurotransmission of other sensory signals. The present study also demonstrated that three subtypes of the PGE receptor, EP_1, EP_3 and EP_4, in addition to the IP receptor, are expressed in DRG neurones. Each of these receptors have different signal transduction pathways. The EP_1 receptor activates calcium channels, the IP and EP_4 receptors stimulate adenylate cyclase, while the EP_3 receptor inhibits adenylate cyclase (Ushikubi *et al.*, 1995). Approximately, 20–40% of IP receptor mRNA-positive neurones co-express EP_1, EP_3 and EP_4. These findings have raised several questions, including whether all of these prostanoid receptors are expressed in the peripheral endings of the primary afferent neurones, or whether some of them are expressed in the peripheral endings and others in the central endings in the spinal cord. Furthermore, if they are expressed in the same endings, how they interact with each other also remains to be determined. Actions in the periphery have been accepted as the primary mechanism of the hyperalgesia induced by PGs. However, Malmberg & Yaksh (1992) recently showed that cyclo-oxygenase inhibitors administered into the intrathecal space blocked the hyperalgesia induced by the activation of spinal glutamate and SP receptors, indicating the presence of action sites for prostanoids in the spinal cord. We did not detect mRNA for either the IP or the EP_3 receptor in the spinal cord (this study and Sugimoto *et al.*, 1994c). These observations suggest that some of the prostanoid receptors expressed by DRG neurones are localized in the central endings in the spinal cord and act to facilitate the release of SP from the afferent neurone terminals. Immunohistochemistry using antibodies specific for each receptor will clarify the identification and localization of these receptors in the sensory neurotransmission pathway.

In summary, the present study showed that the IP receptor was distributed not only in tissues known to be sensitive to PGI_2, but also in other systems such as the immune and nervous systems, suggesting that PGI_2 has novel functions in these various systems. It also indicated that the actions of PGI_2 in both blood vessels and in platelets are mediated via the same type of receptor.

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