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VASCULAR A10 CELL MEMBRANES CONTAIN AN ENDOTHELIN METABOLIZING NEUTRAL ENDOPEPTIDASE

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We have investigated the possible presence of endothelin-metabolizing neutral endopeptidase (NEP, EC 3.4.24.11) on A10 cell membranes using [^{125}I]-ET-1 binding and direct measurements of NEP. NEP activity of A10 cell membranes has been compared to that of solubilized rat kidney brush border membranes (KNEP). Specific [^{125}I]-ET-1 (50 pM) binding (defined with 100 nM ET-1) to A10 cell membranes was increased in a concentration dependent manner by the selective NEP inhibitors thiorphan, phosphoramidon, and SQ 28,603 {(±)-N-[2-(mercaptomethyl)-1-oxo-3-phenylpropyl]-ß-alanine} with EC_{50} values of 9.4, 28.4, and 5.7 nM respectively. At equilibrium (150 min), 70% more specific binding was apparent in the presence of these inhibitors. Phosphoramidon (2 µM) did not alter B_{max} values, but it decreased the apparent K_D for [^{125}I] ET-1 from 63 (±3) to 27 (±2) pM. Thiorphan, phosphoramidon, and SQ 28,603 inhibited A10 cell NEP activity with IC_{50} values of 5.3, 36.5, and 6.0 nM respectively, which was similar to values obtained with KNEP (3.6, 22.6, and 3.5 nM). ET-1 inhibited A10 cell NEP, and KNEP with IC_{50} values of 30 and 21.3 µM respectively. The order of inhibitory potencies: ET-3 > ET-1 = ET-2 ≥ sarafotoxin-6b was similar for both systems.These data suggest A10 cell membranes contain a NEP which has similar characteristics to NEP 24.11, and which actively metabolizes [^{125}I]-ET-1.

Endothelin (ET)-1 is a recently discovered 21-amino acid containing peptide with two intramolecular disulfide bonds which provide for significant structural rigidity. A structurally related peptide sarafotoxin is present in the venom of the Israeli burrowing asp, and this peptide exhibits most of the actions of ET-1 (1). ET-1 is a member of a family of endothelins (ET-2, ET-3) which have distinct distribution and may represent the agonists for a related family of ET receptors (2). ET-1 is the most potent vasoconstrictor peptide known, and its production and metabolism may represent important steps in the regulation of local levels of this vasoconstrictor peptide. ET-1 is synthesized from big-ET and constitutively released from vascular endothelial cells (3-

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5). The enzyme responsible for this conversion (endothelin converting enzyme, ECE) is the subject of controversy, and both aspartyl proteases (5-7), and metalloproteases (8) have been implicated. Two recent reports (9,10) have indicated that ET peptides can function as substrates for the metalloprotease neutral endopeptidase (NEP, EC 3.4.24.11), although the physiological significance of these observations remains to be established. ET-1 receptors have been demonstrated using binding techniques in vascular tissue preparations of porcine aorta (11), and rat fetal smooth muscle (A10) cells (12), and cultured human vascular smooth muscle cells (13). The present study has sought to demonstrate the presence of an ET-metabolizing enzyme activity on membrane preparations of vascular A10 cells. The observed NEP activity has been compared with the NEP (EC 3.4.24.11) found on kidney brush border membranes (14).

Materials and Methods

Materials. [^{125}I] Tyr^{13}-Endothelin-1 ([^{125}I]-ET-1) was purchased from N.E.N., Boston, MA. The peptides ET-1, ET-2, ET-3, and sarafotoxin S6 b were purchased from Peninsula Labs, Belmont, CA. Phosphoramidon was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. SQ 28,603 ({(+/-)-N-[2-(mercaptomethyl)-1-oxy-3-phenylpropyl]-ß-alanine} was synthesized as previously described (15). NEP substrate (Glutaryl-alanyl-alanly-phenylalanyl-ß-naphthylamine) was purchased from Research Plus, Inc., Bayonne, NJ. Aminopeptidase M and thiorphan were purchased from Sigma Chemicals, St. Louis, MI.

Membrane preparation. Rat fetal aortic smooth muscle cells (A10, ATCC # CRL 1476), passage numbers 30-60, were cultured at 37°C in T-75 flasks under humidified 95 % air/5 % CO_2 in HEPES-buffered Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal calf serum, 50 U/ml penicillin, 50 µg/ml streptomycin (Gibco Labs). Following the attainment of confluence (5-7 days), cells were trypsinized with 2 ml 0.25 % trypsin/1 mM EDTA and cells collected into buffer A (0.1 mM phenylmethylsulfonyl fluoride, 10 µg/ml soy bean trypsin inhibitor, 20 mM HEPES pH 7.4 , dissolved in DMEM) at a concentration of 3-4 x 10^5 cells/ml.The cell suspension was stored at -80°C for 3-24 hours, thawed using a 37°C water bath, and homogenized with a Brinkmann Polytron homogenizer (setting # 8, 2x10 sec) and centrifuged at 100,000 g for 1 hour at 4°C. The supernatant was discarded and the membranes resuspended in the above buffer A at a concentration of 0.2-0.8 mg protein/ml. The cell homogenate was stored in 5ml aliquots at -80°C until use.

Kidney neutral endopeptidase preparation and NEP assay. Neutral endopeptidase (NEP, EC 3.4.24.11) was solublized and purified from rat kidney brush border membranes (KNEP) according to the methods described previously (16). Enzyme assays for A10 cell membrane and KNEP were conducted in a total volume of 100 µl in microtiter plates containing: 20 µl of Tris buffer (62.5mM, pH 7.6), 20 µl of aminopeptidase M solution (0.2 mg protein/ml), 20 µl of inhibitor or its solvent, and 20 µl of KNEP (80 ng of protein) or A10 cell membranes (5-10 µg protein). The mixture was preincubated for 10 minutes at 25°C, and 20 µl of substrate solution (Glutaryl-alanyl-alanyl-phenylalanyl-ß-naphthylamine, 0.4 mM final concentration) was added to initiate the enzymatic reaction. The mixture was further incubated for 90 minutes at 25°C with continuous shaking after which time the reaction was terminated by the addition of 10 µl of 10% trichloroacetic acid. 150 µl of 0.05% fast garnet solution was added and the mixture was incubated for 30 minutes at 25°C with continuous shaking. Absorbance measurements of the liberated ß-naphthylamine were determined at 525 nm using a Titertek™ Multiskan Plus microtiter plater reader. Product formation, using KNEP and A10 cells, increased linearly with protein concentration (4-80 ng and 1-10 µg respectively) and with time (0-90 minutes for both preparations).

[^{125}I]-ET-1 binding. Assays were conducted in a total volume of 250 µl in tubes having microtiter plate configuration (Marsh Biomedical Products, Rochester, NY). The tubes contained: 50 µl [^{125}I]-ET-1 (40,000 cpm, equivalent to 50 pM) dissolved in assay buffer (50 mM Tris-HCl pH 7.4, 0.1 % bovine serum albumin); in the absence (total binding) or presence of 50 µl endothelin-1 (100nM final concentration, non-specific binding). The binding reaction was initiated by the addition of 100 µl of A10 membranes (10-20 µg protein). The mixture was incubated for 150 min at 37°C with continuous shaking. Membranes were filtered with a Tomtec™ cell harvester using a filtermat B pre-soaked for 1 hour in assay buffer. The filtermat was rinsed twice with 150 mM NaCl, 5 mM Tris-HCl, pH 7.4 at 4°C and the membrane preparation was filtered using a pulse wash protocol. The filtermat was impregnated with a wax based scintillant (Meltilex™, L.K.B Wallac) and counted in a Betaplate™ flatbed scintillation counter at 60 % efficiency.

Results and Discussion

The effects of the specific NEP inhibitors thiorphan, phosphoramidon, and SQ 28,603 on specific [^{125}I]-endothelin binding to A10 cell membranes is shown in Figure 1. These inhibitors produced concentration dependent increases in [^{125}I]-ET-1 binding with EC_{50} values of 9.4±1.9, (n=5), 28.4±5.9, (n=6), and 5.7±1.4 nM respectively (n=6). Concentrations at which maximal stimulation occurred were 2, 2, and 0.2 µM respectively, with increases above control of 67±7, 66±5 and 54±9% respectively. These maximum increases were not significantly different from each other (2-tailed t-test, $p >> 0.05$). A modest increase in non-specific binding was also observed by these inhibitors (data not shown), although the predominant effect was on the specific binding. These results differed from those obtained with porcine aortic and rat lung membranes by Kanse et al. who were unable to show a significant effect of phosphoramidon on [^{125}I]-ET-1 binding (11). Our finding that diverse classes of NEP inhibitors could increase specific [^{125}I]-endothelin binding to similar levels, but with different relative potencies, suggested that a membrane bound NEP which controlled

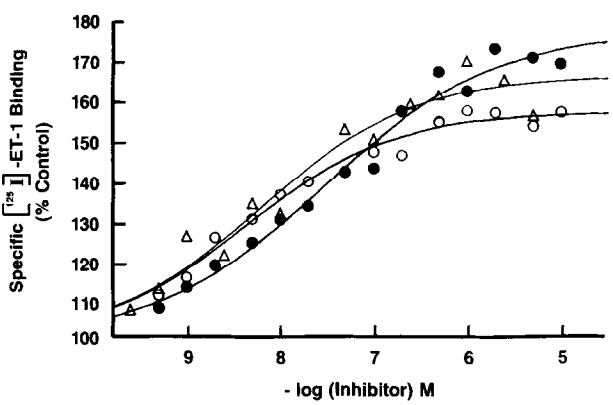
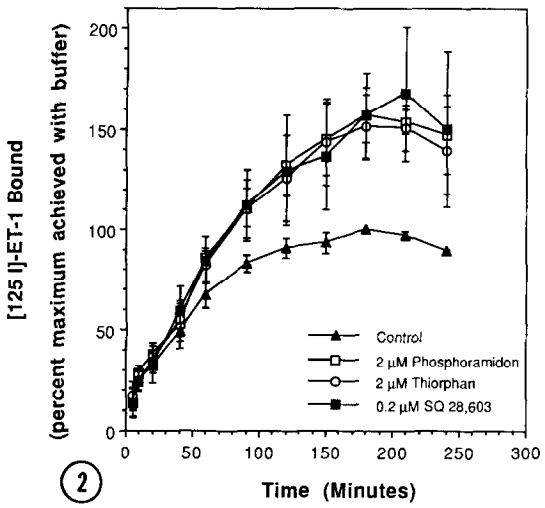


Fig 1. Stimulation of [^{125}I]-endothelin-1 binding to A10 cell membranes by NEP inhibitors. Specific binding was determined in the absence (100%) or presence of increasing concentrations of phosphoramidon (●), thiorphan (○), or SQ 28,603 (Δ). Results are representative of experiments performed 5-6 times.



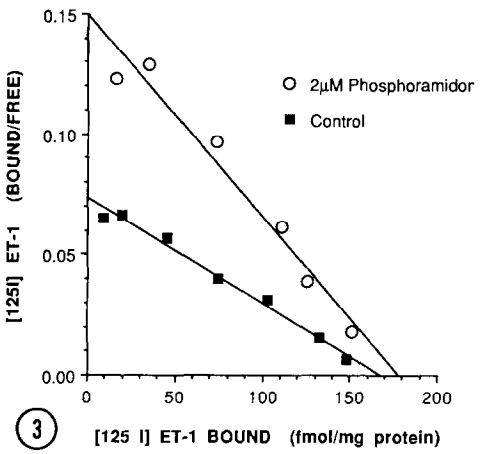


Fig 2. Effect of NEP inhibitors on the time course of specific [^{125}I]-endothelin-1 binding to A10 cell membranes. Specific binding in the absence (control) or presence of the indicated concentrations of inhibitors was determined at the stated times. Results show the mean (± SEM) of 3 experiments.

Fig 3. Scatchard transformation of specific [^{125}I]-endothelin-1 saturation binding to A10 cell membranes in the absence or presence of phosphoramidon. Results show the mean data of 3 experiments.

the amount of radioligand available for binding may be present in A10 cell membranes.

The effects of thiorphan, phosphoramidon, and SQ 28,603 on the kinetics of receptor-ligand binding were examined (Figure 2). At equilibrium (150-240 min), 70% more specific binding was observed in the presence of maximal effective concentrations of these inhibitors (determined from Figure 1). The maximum increase in binding was similar for all inhibitors, and significant augmentation of specific binding was apparent after 20 minutes. The increase in specific binding did vary considerably among A10 cell membrane preparations (130-210%), perhaps reflecting quantitative differences in enzyme levels. However the binding increases induced by these NEP inhibitors were quantitatively similar within a preparation suggesting a similar mode of action.

Figure 3 shows the saturation binding of [^{125}I]-ET-1 to A10 cell membranes in the absence and presence of a maximum effective concentration of phosphoramidon (2 μM). Specific binding data have been transformed and presented graphically as a Scatchard plot. The presence of phosphoramidon had no significant effect on the binding site maxima for [^{125}I]-ET-1 (control B_{max} = 136±41, phosphoramidon treated B_{max}=135±43 fmol / mg protein). By contrast, the apparent K_D of [^{125}I]-ET-1 decreased from control value of 63(±3) pM to 27(±2) pM (n=3) in the presence of phosphoramidon. These results are consistent with the assumption that [^{125}I]-ET-1 acts as a substrate for a membrane bound NEP, resulting in its degradation to products with no or decreased affinity for the A10 cell receptor.

TABLE 1. Effect of phosphoramidon on the pharmacological characteristics of [^{125}I]-ET-1 binding sites on A10 cell membranes

Compound	Control		2 μM phosphoramidon	
	K_i	n_H	K_i (nM)	n_H
Endothelin-1	0.08± 0.02	0.66± 0.05	0.08± 0.02	0.89± 0.03
Endothelin-2	0.30± 0.08	0.62± 0.05	0.13± 0.02	0.65± 0.03
Endothelin-3	31.7 ±9.0	0.61± 0.04	40.2 ± 6.0	0.79± 0.03
Sarafotoxin 6b	0.5 ±0.1	0.77± 0.03	1.5 ± 0.1	0.83± 0.05

Competition experiments were conducted at 50pM [^{125}I]-ET-1, and K_i values were derived from IC_{50} values using the equation of Cheng & Prusoff (17). Slope factors of the competition curves were obtained by non-linear iterative curve fitting of the binding data. The K_D values of [^{125}I]-ET-1 were 63 nM and 27 nM for control and phosphoramidon treated respectively (n=3).

In order to define the effects of putative radioligand degradation on the observed pharmacological characteristics we performed competition studies in the absence and presence of 2 μM phosphoramidon (Table 1). The specific binding of [^{125}I]-ET-1 was found to be higher at every concentration of competing compound in the presence of phosphoramidon (data not shown). However when the IC_{50} values were corrected for radioligand occupation of the receptor using the apparent K_D values generated above, there was no significant differerence in the K_i values of ET-1, ET-2, or ET-3. The K_i value of sarafotoxin 6b was three-fold higher in the presence of phosphoramidon. Slope factors of competition curves of all ET peptides were significantly less than unity, and the presence of phosphoramidon did not result in substantial changes in these parameters. These results are consistent with the notion that phosphoramidon increased the amount of both [^{125}I]-labelled ET and competing endothelin peptides available for binding equally, without affecting the pharmacological characteristics of the binding site. The slight decrease in potency seen with sarafatoxin 6b may be explained by the fact that this peptide has amino acid substitutions adjacent to each of the three proposed cleavage sites (9,10), possibly providing this peptide with increased resistance to enzymatic degradation.

Direct measurements of NEP were performed on the A10 cell membranes, and the enzyme properties were compared with those of a purified soluble NEP from rat kidney brush border membranes (NEP, EC 3.4.24.11), (16). Enzyme activity of A10 cell membranes was linearly related to time (0-90 minutes) and protein concentration (1-10 μg). Thiorphan, phosphoramidon, and SQ 28,603 inhibited product formation in a concentration dependent manner, and with IC_{50} values which were not significantly different from those obtained with the well characterized KNEP enzyme (Table 2). The sensitivity of the A10 cell membrane NEP to thiorphan indicated its difference to the

TABLE 2. Potencies of drugs as inhibitors of A10 cell, and kidney NEP, and as stimulators of [^{125}I]-ET-1 receptor binding

	IC_{50}-values (nM) NEP Inhibition				EC_{50}-values (nM): ET-binding	
Inhibitor	A10 cells	n	KNEP	n	A10 cells	n
Thiorphan	5.3 ± 1.2	5	3.6 ± 0.5	6	9.4 ± 1.9	5
Phosphoramidon	36.5 ± 2.0	4	22.6 ± 5.9	4	28.4 ± 5.9	6
SQ 28,603	6.0 ± 1.9	3	3.5 ± 4.0	4	5.7 ± 1.4	6

IC_{50} values for inhibition of NEP represent mean ±SEM of the stated number of experiments. EC_{50} values for stimulation of binding were calculated from iterative curve fitting of the stated number of curves.

recently described phosphoramidon-sensitive, thiorphan-insensitive metalloprotease which metabolized big ET to ET-1 in porcine aortic endothelial cells (8). Thus, release of immunoreactive ET from endothelial cells into the medium was markedly attenuated by 10-100 μM phosphoramidon, but was unaffected by 100 μM thiorphan. The IC_{50} values of thiorphan, phosphoramidon, and SQ 28,603 for inhibition of A10 cell NEP correlated well (r^2=0.97) with their EC_{50} values for stimulation of [^{125}I] ET-1 binding (see Table 2), which suggested that these phenomena were mediated through similar mechanisms.

ET peptides have recently been shown to be substrates for NEP 3.4.24.11 (9,10) and sarafotoxins were more resistant to the action of this enzyme (9). We therefore examined the effect of ET peptides as potential substrate inhibitors of KNEP and A10 cell NEP. Table 3 shows that ET peptides were indeed able to inhibit the NEP activity of both KNEP and A10 cell NEP with a similar rank order of inhibitory potency (ET-3 > ET-1 = ET-2 ≥ sarafotoxin 6b). ET-1 and ET-2 and sarafotoxin 6b all exerted inhibitory activity from 20-40 μM. These values are in close agreement to the K_m value of ET-1 for bovine kidney NEP (9). ET-3 was the most potent inhibitor in both systems, and it

TABLE 3. Potencies of peptides for inhibition of A10 cell and KNEP

	IC_{50} values (μM)			
Inhibitor	A10 cells	n	KNEP	n
Endothelin-1	30 ± 3.3	4	21.3 ± 1.6	3
Endothelin-2	25 ± 3.3	4	26 ± 0.1	2
Endothelin-3	3.5 ± 0.3	4	15 ± 1.4	2
Sarafotoxin 6b	40 ± 6.7	4	35 ± 2.8	2

IC_{50} values for inhibition of NEP represent means ± SEM of the stated number of experiments.

was particularly active against A10 cell NEP (IC_{50} =3.5 μM). The weaker inhibitory potency of sarafotoxin perhaps related to its slower rate of hydrolysis by NEP (9).

Recent evidence has indicated the metalloprotease NEP 24.11 can hydrolyse endothelin but the precise physiological role of this enzyme in regulating endogenous ET levels is not established. Indeed, NEP inhibitors had no effect on plasma endothelin levels following exogenous endothelin administration (18). In the present study we have demonstrated the existence of an enzyme on A10 cell membranes with similar characteristics to NEP 24.11. Inhibition of this enzyme resulted in increased [^{125}I]-ET-1 binding, which was due to a lower apparent K_D for the radioligand. These findings suggested [^{125}I]-ET-1 was hydrolysed to products which were unable to bind to the receptor with similar affinity as the parent radioligand. Hydrolysis of ET-1 by NEP has been shown to occur initially at the Ser^5-Leu^6 bond followed by cleavage at the amino side of Ile^{19} , and at the His^{16}-leu^{17} bond (9,10). The initial nick resulted in an endothelin product comparable to the parent peptide, whereas the secondary products were biologically inactive (9). Our observations are consistent with [^{125}I]-ET-1 metabolism to these secondary hydrolysis products.

Ikegawa et al. (5) have recently reported a phosphoramidon-sensitive metalloproteinase in cultured endothelial cells that was insensitive to thiorphan, and which was responsible for the conversion of big-ET to ET-1. These data suggested that the enzyme was not NEP 24.11. The A10 cell NEP was clearly thiorphan-sensitive, like KNEP, distinquishing it from the metalloproteinase of endothelial cells. It is possible that NEP 24.11 may function in vivo as an endothelin metabolizing enzyme, operating in close proximity to the ET receptor, and responsible for regulating the endogenous levels available for binding to the ET receptor. In support of this proposal, phosphoramidon has recently been shown to potentiate in vivo, and in vitro bronchoconstrictor responses to ET-1 in guinea pig (19).

It is presently uncertain whether the results obtained with fetal rat aortic smooth muscle cells exposed to cell culture conditions accurately reflects the status of smooth muscle cells in adult rats. These fetal A10 cells clearly show some differences to cultured rat aortic smooth muscle cells derived from adult rats. Thus A10 cells contain a large population of ET receptors whereas receptor levels in rat aortic smooth muscle cells in extended primary culture are very low (unpublished observations). Moreover the NEP activity in membranes of rat aortic smooth muscle cells was found to be less then 1% of that observed in A10 cell membranes at the same protein concentration. Perhaps the differences seen in NEP activities of fetal and adult cells underlies the lack of demonstrable effect of NEP inhibitors on clearance of exogenous ET-1 (18). Another possibility is that NEP 24.11 functions strictly to metabolize endothelin in its local environment thereby functioning as a house-keeping enzyme to limit receptor stimulation. Taken together with our findings, recent reports of endothelin converting

enzyme in vascular smooth muscle (20), provides evidence that the vasculature may contain all the necessary machinery for processing endothelin peptides. The expression of these regulatory enzymes, especially during pathophysiological states, may have important consequences for vasomotor function.

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References

1. Yanigisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., and Masaki, T., (1988) Nature 332, 411-415

2. Kloog, Y., and Sokolovsky, M. (1989) Trends Pharmacol Sci, 10: 212-215

3. Emori, T., Hirata, Y., Ohta, K., Shichiri, M., Shimokado, K., and Marumo, F. (1989) Biochem. Biophys. Res. Commun. 162, 217-223

4. Sawamura, T., Kimura, S., Shinmi, O., Sugita, Y., Yanagisawa, M., and Masaki, T. (1989) Biochem. Biophys. Res. Commun. 162, 1287-1294

5. Ikegawa, R., Matsumura, Y., Takaoka, M., and Morimoto, S. (1990) Biochem. Biophys. Res. Commun. 167, 860-866

6. Matsumura, Y., Ikegawa, R., Takaoka, M., and Morimoto., (1990) Biochem. Biophys. Res. Commun. 167, 203-210

7. Wu-Wong, J., Budzik, G. P., Devine, E. M., and Opgenorth, T. J. (1990) Biochem. Biophys. Res. Commun. 171, 1291-1296

8. Ikegawa, R., Matsumura, Y., Tsukahara, Y., Takaoka, M., and Morimoto, S. (1990) Biochem. Biophys. Res. Commun. 171, 669-675

9. Sokolovsky, M., Galron, R., Kloog, Y., Bdolah, A., Indig, F., Blumberg, S., and Fleminger G. (1990) Proc. Natl. Acad. Sci. USA. 87, 4702-4706

10. Vijayaraghavan, J., Scicli, A. G., Carretero, O. A., Slaughter, C., Moomaw, C., and Hersh L. B. (1990) J. Biol. Chem. 265, 14150-14155.

11. Kanse, S. M., Ghatei, M. A., and Bloom, S. R. (1989) Eur. J. Biochem. 182, 175-179

12.Takuwa, Y., Kasuya, Y., Takuwa, N., Kudo, M., Yanigisawa, M., Goto, K., Masaki, T., and Yamashita, K. (1990) J. Clin. Invest. 85, 653-658

13. Clozel, M., Fischli, W., and Guilly, C. (1989) J. Clin. Invest. 83, 1758-1761

14. George, S. G., and Kenny, A. J. (1973) Biochem J. 134, 43-57

15. Delaney J. C., Gordon E. M., and Ondetti, M. A. U. S. Patent # 4,722,810.

16. Almenoff, J., and Orlowski, M. (1983) Biochemistry 22, 590-599

17. Cheng , Y. C., and Prusoff, W. H. (1973) Biochem. Pharmacol. 22, 3099-3108

18. Asaad, M. and Dorso, C. (1991) FASEB J. in press.

19. Pons, F., Boichot, E., Lagente, V., Touvay, C., Mencia-Huerta, J. M., and Braquet, P. (1990) Pharmacologist 32, 186.

20. Hioki, Y., Okada, K., Ito, H., Matsuyama, K., and Yano, M. (1990) Biochem. Biophys. Res. Commun. 174, 446-451