Biochemical Pharmacology, Vol. 38, No. 9, pp. 1481–1489, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

^{125}I-IODOPINDOLOL BINDING TO FROG ESOPHAGEAL PEPTIC CELLS

DETECTION OF AMINE UPTAKE AND β-ADRENERGIC RECEPTORS COUPLED TO PEPSINOGEN SECRETION*

KENNETH E. J. DICKINSON, WAYNE ANDERSON, HARUKI MATSUMOTO, NAOMI UEMURA and BASIL I. HIRSCHOWITZ†

Division of Gastroenterology, University of Alabama at Birmingham, Birmingham, AL 35294, U.S.A.

(*Received* 13 *June* 1988; *accepted* 16 *September* 1988)

Abstract—The β-adrenergic receptors (β-ARs) coupled to pepsinogen secretion on frog esophageal peptic cells have been compared to frog erythrocyte β-ARs using the radioligand ^{125}I-iodopindolol (^{125}I-PIN). ^{125}I-PIN binding to intact peptic cells was time and temperature dependent. Saturation and competition experiments established that a large component of this binding represented radioligand uptake, which was energy dependent, pH sensitive, Na^+ independent, and inhibited by agents that depress cellular ATP or disrupt proton gradients. This uptake system, which was absent from frog erythrocytes, appeared similar to that recently described for a number of mammalian cells. ^{125}I-PIN bound to a single class of sites on peptic cell homogenates with a K_D = 64 (±5) pM. Binding to cell homogenates and a proportion of the binding to intact cells was inhibited by β-agonists and antagonists with pharmacological characteristics similar to typical β_2-ARs of frog erythrocytes. The number of β-ARs in these peptic cell preparations was 1300 (± 240) sites/cell. Isolated peptic cells were poorly responsive to isoproterenol stimulation even in the presence of the phosphodiesterase inhibitor IBMX (3-isobutyl-1-methylxanthine). Pretreatment of cells with the phorbol ester TPA (12-*O*-tetradecanoylphorbol-13-acetate) (100 nM) promoted isoproterenol stimulation of pepsinogen secretion. Catecholamine agonists stimulated pepsinogen secretion with an order of potency: isoproterenol > epinephrine ≫ norepinephrine, which was identical to that determined for inhibition of ^{125}I-PIN binding. These findings indicate that frog peptic cells contain β_2-ARs functionally coupled to pepsinogen secretion.

Catecholamines activate target cell metabolism following binding to specific receptors. β-Adrenergic receptors (β-ARs) represent a class of receptors which are coupled through guanine nucleotide regulatory proteins (G_s) to activation of adenylate cyclase, the product of which is the intracellular messenger cyclic AMP (cAMP). Production of this messenger, with activation of protein kinase A, is thought to be the major activation pathway for β-ARs.

We have been studying pepsinogen secretion and, although control of peptic cell activity by the cholinergic nervous system is well established, the contribution of the sympathetic nervous system is somewhat controversial. Thus, catecholamines have been shown to have stimulatory [1] and inhibitory effects upon histamine- or pentagastrin-stimulated pepsinogen secretion *in vivo* [2]. However, studies of pepsinogen secretion using intact mammalian gastric tissue or dispersed peptic glands are complicated by the presence of closely associated heterogeneous cell types including parietal, endocrine and mast cells. Effects of catecholamines on these cells may modulate histamine release from mast cells [3] or somatostatin release from somatostatin (D) cells. Inhibition of canine gastric acid and pepsin release by isoproterenol has been thought to be mediated by the latter mechanism [4].

The presence of β-ARs on peptic cells has been suggested, however, by studies showing isoproterenol-stimulated pepsinogen secretion in rabbit gastric glands [5–7] and intact frog esophageal mucosa [8]. Pepsinogen secretion can be stimulated in cultured canine chief cells by high concentrations of epinephrine [9]. Moreover, agents that increase the cellular concentration of cAMP (cAMP analogues, cholera toxin, cAMP phosphodiesterase inhibitors, forskolin) also stimulate pepsinogen secretion in preparations of gastric glands and peptic cells [10–13]. These findings indicate a functioning cAMP messenger system in peptic cells, and we were interested in establishing directly whether β-ARs were present on isolated peptic cells and whether they were coupled to pepsinogen secretion.

The esophagus of the American bullfrog *Rana catesbeiana* contains mucosal peptic glands which do not contain acid secreting cells. We have used isolated peptic cells from this organ, and studied β-adrenergic receptor binding sites on intact cells and membrane preparations using the radioligand ^{125}I-

* This work was supported by funds from the Gastroenterology Education and Development Fund (GEDF). Contributions to this fund by Glaxo, Inc. and Boehringer Ingelheim are gratefully acknowledged.

† Address for reprints: Basil I. Hirschowitz, M.D., Division of Gastroenterology, University of Alabama at Birmingham, University Station, Birmingham, AL 35294.

iodopindolol (^{125}I-PIN). Binding site characteristics have been compared to the well characterized β-ARs on frog erythrocytes [14], and related to stimulation of pepsinogen secretion by adrenergic agonists.

MATERIALS AND METHODS

Drugs

^{125}I-Iodopindolol (2200 Ci/mmol) was purchased from New England Nuclear, Boston, MA. Imipramine HCl, (±)-pindolol, (±)-timolol maleate, 5-hydroxytryptamine HCl, reserpine, monensin, (+)- and (−)-isoproterenol bitartrate, (−)-epinephrine bitartrate, (−)-norepinephrine bitartrate, sodium azide, EDTA and bovine serum albumin (BSA) were purchased from the Sigma Chemical Co., St. Louis, MO. The following drugs were gifts of the indicated pharmaceutical companies: (−)- and (+)-propranolol HCl, Ayerst Laboratories, New York, NY; (±)-alprenolol HCl, Hassle, Sweden; and desmethylimipramine, Smith Kline & French, Philadelphia, PA.

Preparation of frog peptic cells and erythrocytes

Peptic cells were isolated as described previously [12] and used after a 3- to 4-hr equilibration period. Frog blood was collected into heparinized tubes and washed three times in amphibian Ringer. The buffy coat was removed, and the washed erythrocytes were suspended in oxygenated amphibian Ringer at 10^6 cells/ml and used immediately for binding studies.

Preparation of peptic cell membranes

Peptic cells were homogenized in 10 ml buffer A (50 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM EDTA) using a Polytron (Brinkmann) homogenizer (3 × 5 sec bursts) and centrifuged at 40,000 *g* for 20 min at 4°. The pellet was washed twice in 20 ml of this buffer, and the washed membranes were resuspended in buffer at approximately 350 μg protein/ml and used immediately for binding studies.

^{125}*I-PIN binding*

Intact cells. ^{125}I-PIN (50–75 pM) was incubated for 2 hr at 25° with 3–5 × 10^5 erythrocytes or 1–3 × 10^5 peptic cells under an atmosphere of 95% O_2/5% CO_2 in a total volume of 0.5 ml of amphibian Ringer. The reaction was terminated by filtration through Whatman GF/C filters, and the filters were washed three times with 5 ml of cold isotonic phosphate-buffered saline, pH 7.4. Radioactivity associated with the filters was determined with an Isoflex γ counter (Micromedic Systems Inc., Horsham, PA).

Membranes. Membranes (100–200 μg protein) were incubated with ^{125}I-PIN (50–75 pM) for 2 hr at 25° in buffer A. Bound and free radioligand were separated by vacuum filtration using a cell harvester (Scatron Inc., Sterling, VA). Expriments conducted in amphibian Ringer or buffer A gave similar results (data not shown).

Conditions were chosen such that <10% of radioligand was bound at equilibrium. "Specific" binding to β-ARs of intact peptic cells was defined as the binding displaceable by 0.1 μM timolol. Specific binding to β-ARs of other preparations was calculated as described in the text. Inhibition constants were calculated for drugs using the equation of Cheng and Prusoff [15].

Pepsinogen secretion

Secretion of pepsinogen from isolated peptic cells was determined using 3 × 10^5 cells/tube as previously described [12]. Incubation proceeded for 30 min at 25° in 1 ml total volume of amphibian Ringer and secretagogues. Ascorbic acid (0.1 mM) was included to prevent oxidation of catecholamines. Some cells were pretreated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 10^{-7} M, for 10 min and then washed three times before incubation. After incubation, the cells were centrifuged for 10 sec at 13,000 *g*, and aliquots of supernatant fluid (30 μl) were assayed for pepsinogen using a modified assay based on the acid hemoglobin substrate method [16]. This highly sensitive assay allowed significant dilution of supernatant fluid such that there was no interference by added drugs in the subsequent spectrophotometric assay of peptic products. Such problems commonly arise with isoproterenol which absorbs strongly at 280 nm and reacts with Folin–Ciocalteau reagent to produce interfering products. Pepsinogen secretion was expressed as percentage of total cellular pepsinogen.

RESULTS

^{125}*I-PIN binding to frog erythrocytes and peptic cells*

Figures 1 and 2 show the characteristics of ^{125}I-PIN binding to intact frog erythrocytes and peptic cells. Binding to erythrocytes displayed saturability, and Scatchard analysis of the specific binding data (defined using 1 μM alprenolol) yielded a K_D = 32 (± 3) pM, N = 3, with binding site maxima = 6.6 (± 0.37) fmol/10^6 cells equivalent to 4000 (± 220) sites/cell (Fig. 1). By contrast, ^{125}I-PIN binding to peptic cells approached linearity over a similar radioligand concentration range. Scatchard analysis of ^{125}I-PIN binding which was displaceable by 0.1 μM timolol indicated a single high affinity site (K_D = 60 (± 5) pM, B_{max} = 1300 (± 240) sites/cell, N = 5). By contrast, Scatchard analysis of binding displaceable by 1 μM alprenolol revealed the presence of a large-capacity low-affinity site (K_D > 2 nM). Binding of I-PIN to this "site" was further inhibited by 1 mM alprenolol.

Figure 2 shows the pharmacological characteristics of ^{125}I-PIN binding to erythrocytes and intact peptic cells using 40 pM ^{125}I-PIN. Erythrocytes showed the classical characteristics of β-adrenergic receptors with the antagonist timolol exhibiting high affinity (K_i = 0.5 nM). (−)-Propranolol was approximately 100-fold more potent than (+)-propranolol. The agonist (−)-isoproterenol had a K_i value of 1 μM [14]. By contrast, displacement curves of ^{125}I-PIN binding to peptic cells were markedly biphasic with overall IC_{50} values which were not consistent with β-AR binding sites. ^{125}I-PIN binding to a proportion (17%) of sites was similar to that found for binding to erythrocytes, which indicated the existence of β-ARs on peptic cells. The major proportion of ^{125}I-PIN binding, however, was characterized by non-stereoselectivity for the isomers of propranolol and

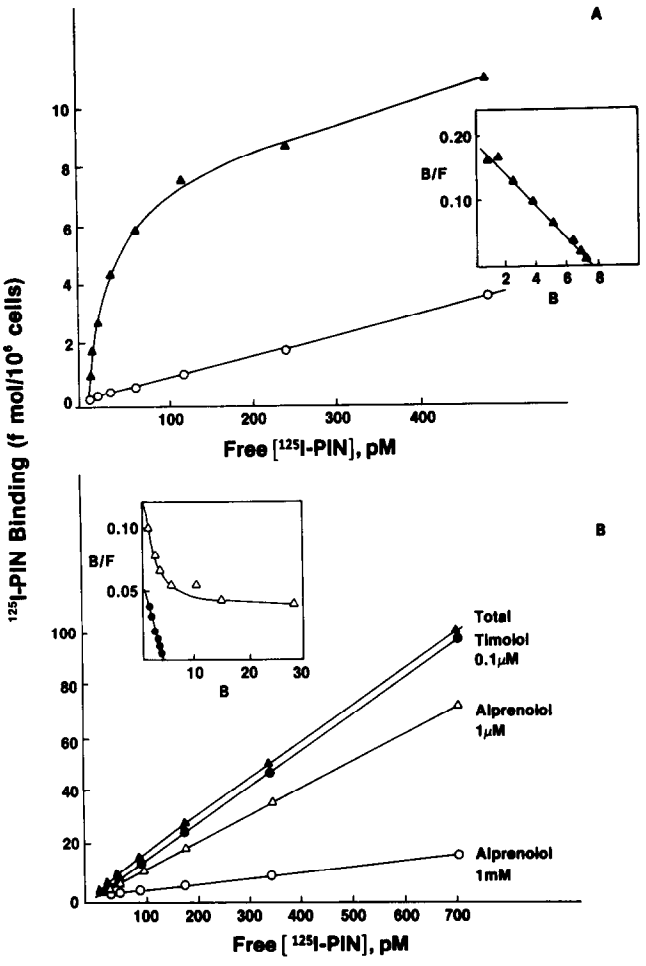

Fig. 1. Saturation isotherms of ^{125}I-PIN binding to frog erythrocytes and peptic cells. (A) Binding of ^{125}I-PIN to erythrocytes in the absence (▲) and presence (○) of 1 μM alprenolol was assessed as described in Materials and Methods. The inset shows a Scatchard plot of the specific binding data, and the calculated K_D and B_{max} values were 40 pM and 4600 sites/cell. Identical data was obtained with 0.1 μM timolol to define specific binding. (B) Binding to intact peptic cells in the absence (▲) and presence of the indicated concentrations of β-AR antagonists. The inset shows Scatchard plots of the specific binding defined with 0.1 μM timolol (●) or 1 μM alprenolol (△). The calculated K_D and B_{max} values of ^{125}I-PIN for the high-affinity sites were 65 pM and 2100 sites/cell. Results are representative of experiments performed three times.

markedly diminished affinity for timolol and isoproterenol.

Washing labeled cells three times with 5 ml hypotonic 5 mM phosphate (pH 7.4) buffer resulted in a 53% decrease in the radioactivity associated with peptic cells (data not shown). These data suggested that the low-affinity "site" represented uptake of radioligand into the peptic cells. To examine the characteristics of the β-ARs, binding experiments were conducted with peptic cell homogenates. ^{125}I-PIN binding to these preparations did not show the presence of the low-affinity site, and Scatchard analysis of specific binding data was linear with a K_D value for ^{125}I-PIN = 64 (± 5) pM, and a B_{max} value of 2.5 (± 0.6) fmol/mg protein, N = 6. Figure 3 shows inhibition curves of the catecholamines isoproterenol, epinephrine and norepinephrine in competition with ^{125}I-PIN binding to frog peptic cell membranes. The relative potencies of these agonists were identical to those observed for frog erythrocyte membranes [14, 17] and were indicative of a β_2-AR classification. Inhibition constants calculated for isoproterenol, epinephrine and norepinephrine were 0.38 (± 0.14), 1.1 (± 0.08), and 24.4 (± 9) μM (N = 3–5) respectively. Hill slope factors of isoproterenol and epinephrine competition curves were 0.57 and 0.58, respectively, whereas the norepinephrine curve exhibited a slope factor of 0.8. Similar findings have been made with frog erythrocyte membranes (data not shown) which may relate to a reduced ability of norepinephrine to induce the GTP-sensitive high-affinity ternary complex in these systems [18].

Characteristics of ^{125}I-PIN uptake in peptic cells

We were interested in further defining the characteristics of ^{125}I-PIN uptake by peptic cells. Binding experiments were therefore conducted in the presence of 0.1 μM timolol to occupy peptic cell β-ARs.

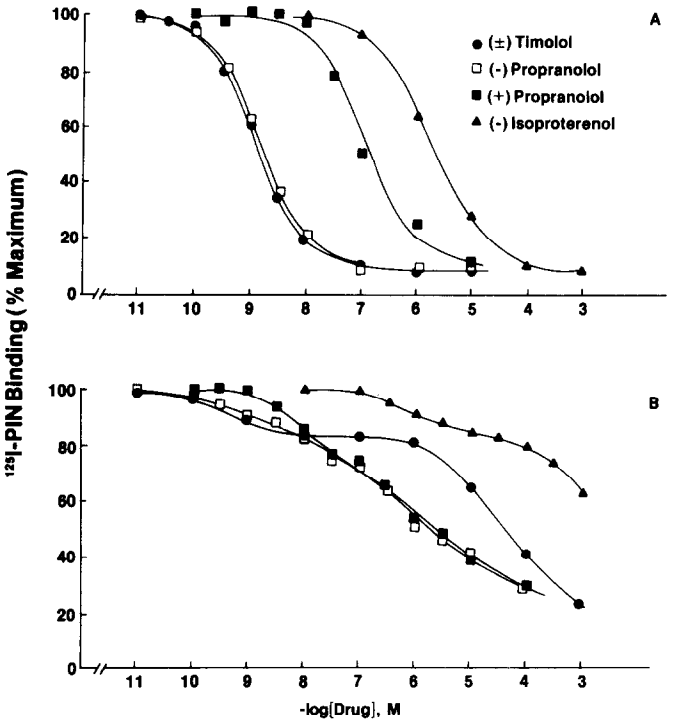

Fig. 2. Pharmacological characteristics of ^{125}I-PIN binding to erythrocytes (A) and peptic cells (B). Binding of ^{125}I-PIN (40 pM) to cells was determined in the presence of increasing concentrations of the indicated competing agents. Binding data were calculated as percent maximal binding and plotted against log drug concentration. Results show representative curves of experiments peformed three to five times.

Figure 4 compares the time and temperature dependence of ^{125}I-PIN uptake into peptic cells with ^{125}I-PIN binding to erythrocytes. ^{125}I-PIN binding to β-ARs on erythrocytes was clearly temperature dependent, reaching steady state at 25° and 37° after 2 and 1 hr, respectively, and was depressed markedly at 4°. Uptake of ^{125}I-PIN into peptic cells also showed significant temperature dependence with little uptake observed at 4°. Maximum uptake at 25° was achieved after 20 min and maintained for a further 40 min. ^{125}I-PIN uptake at 37° was more rapid reaching a significantly higher maximum after 5 min, followed by radioligand efflux over the next 50 min.

The pH dependence of ^{125}I-PIN binding to β-ARs

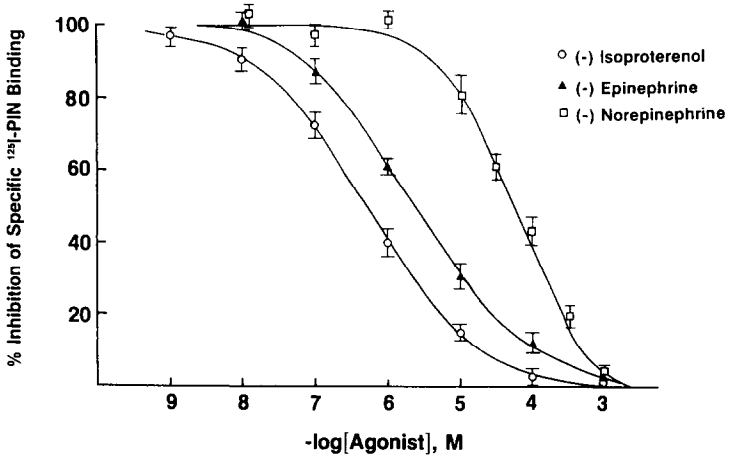

Fig. 3. Inhibition of ^{125}I-PIN binding to peptic cell homogenates by adrenergic agonists. Membranes were incubated with ^{125}I-PIN (60–70 pM) in the presence of increasing concentrations of (−)-isoproterenol, (−)-epinephrine, or (−)-norepinephrine. Specific binding was measured and is plotted against log agonist concentration. Results show the mean (± SEM) of experiments conducted three to five times.

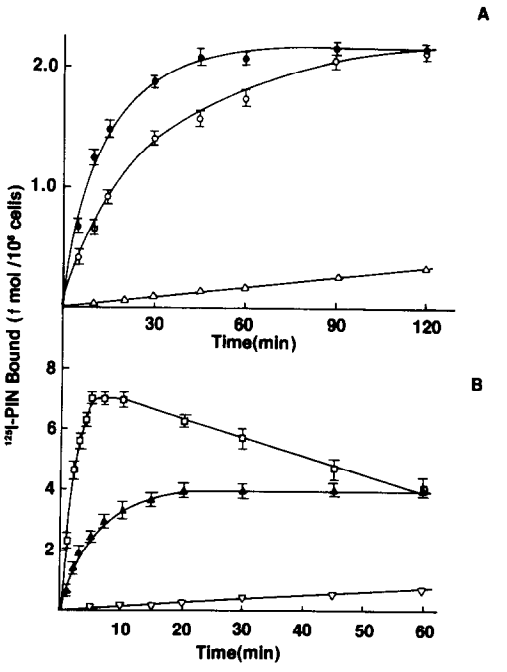

Fig. 4. Time and temperature dependence of ^{125}I-PIN binding to frog erythrocytes (A) and ^{125}I-PIN uptake by peptic cells (B). (A) Frog erythrocytes were incubated with ^{125}I-PIN (40 pM) for the indicated times at 4° (△), 25° (○) or 37° (●). Binding in the absence and presence of 0.1 μM timolol was measured and specific β-AR binding is plotted against time. (B) Peptic cells were incubated with ^{125}I-PIN (40 pM) at 4° (▽), 25° (▲) and 37° (□), in the presence of timolol (0.1 μM) to block ^{125}I-PIN binding to β-ARs. At the indicated times, ^{125}I-PIN associated with peptic cells was determined following filtration and washing. Results show the mean (± SEM) of experiments conducted three times.

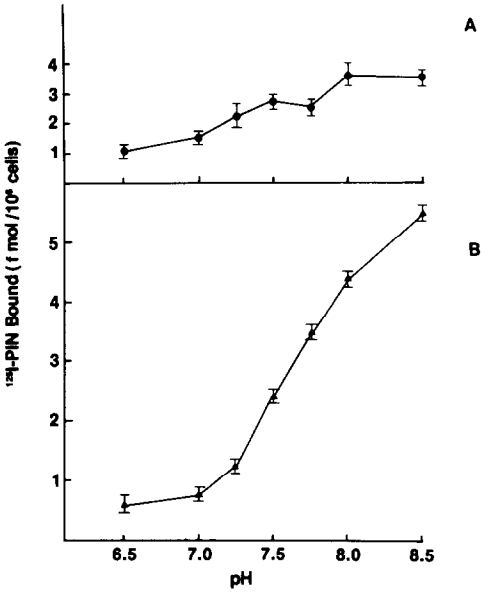

Fig. 5. The pH dependence of ^{125}I-PIN binding to frog erythrocytes (A) and ^{125}I-PIN uptake into peptic cells (B). Erythrocytes were incubated with ^{125}I-PIN (60 pM) for 2 hr at 25° in amphibian Ringer buffered with 16 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethansulfonic acid (HEPES), under an atmosphere of 100% O_2. Specific binding to β-ARs was calculated as described in Materials and Methods and is plotted against the medium pH. (B) Peptic cells were incubated with ^{125}I-PIN at the indicated pH values for 45 min at 25° in the presence of 0.1 μM timolol. ^{125}I-PIN associated with cells was determined as described in the legend of Fig. 4. Results show the mean (± SEM) of experiments performed three times.

on frog erythrocytes and uptake into peptic cells are shown in Fig. 5. Binding to β-ARs was increased slightly between pH 7 and 8.5 (by 130%) with no significant difference seen between pH 7.25 and 7.75. By contrast, ^{125}I-PIN uptake was markedly dependent on pH, increasing 700% between pH 7 and 8.5.

Replacement of medium sodium ions by choline did not affect ^{125}I-PIN uptake, suggesting that the uptake was independent of external Na^+ (data not shown).

The abilities of drugs to inhibit ^{125}I-PIN uptake into peptic cells have been compared with their abilities to inhibit binding to β-ARs on frog erythrocytes (Table 1). Uptake was inhibited by the β-adrenergic antagonists alprenolol, pindolol and propranolol with IC_{50} values in the range of 4–25 μM, with the hydrophilic antagonist timolol having diminished activity (IC_{50} = 100 μM). The marked difference between the ability of timolol to occupy β-ARs and to inhibit uptake made the agent the most suitable for discriminating between receptor binding and uptake.

^{125}I-PIN uptake showed no evidence of stereoselectivity since the optical isomers of propranolol and isoproterenol were equally effective inhibitors. A lack of stereoselectivity was also seen with (L) and (D) amino acids (data not shown). Catecholamines were poor inhibitors of ^{125}I-PIN uptake, and no difference was seen between the agonists tested. By contrast, the established β_2-AR receptor classification for frog erythrocytes was confirmed by the use of these agonists [14]. Carbachol, serotonin and histamine were found to be inactive at the ^{125}I-PIN uptake site, with only the cholinergic agonist oxotremorine showing weak activity.

Reserpine, imipramine and DMI, agents which exert potent effects at amine transport systems, were effective inhibitors of ^{125}I-PIN uptake into peptic cells, with EC_{50} values similar to that of the β-adrenergic antagonist propranolol. Monensin, a Na/H^+ ionophore which dissipates proton gradients, was the most potent inhibitor of ^{125}I-PIN uptake having an IC_{50} of 1.4 μM. The metabolic inhibitor sodium azide was also able to inhibit uptake at reduced molar concentrations. These agents which interact with amine carriers or deplete cellular ATP were generally inactive at β-ARs of frog erythrocytes.

To determine whether ^{125}I-PIN uptake by peptic cells was a saturable process, ^{125}I-PIN was diluted with (−)-pindolol and uptake into peptic cells was measured. Under these conditions ^{125}I-PIN uptake was saturable (data not shown), and we estimate an "apparent" K_m value for I-PIN uptake of 16 μM.

β-AR coupled pepsinogen secretion

Having established the presence of β-ARs on frog

Table 1. Comparison of IC_{50} values of drugs for inhibition of ^{125}I-PIN uptake into peptic cells and their inhibition constants for β-ARs on frog erythrocytes

Drug	IC_{50} for uptake (M)	K_i for β-ARs (M)
β-AR Antagonists		
(±)-Alprenolol	$7.3 (\pm 2.4) \times 10^{-6}$	$8 (\pm 1.5) \times 10^{-10}$
(±)-Pindolol	$2.5 (\pm 0.6) \times 10^{-5}$	$5 (\pm 0.8) \times 10^{-10}$
(±)-Timolol	$1.0 (\pm 0.2) \times 10^{-4}$	$5 (\pm 1.6) \times 10^{-10}$
(−)-Propranolol	$3.9 (\pm 1.6) \times 10^{-6}$	$7 (\pm 0.7) \times 10^{-10}$
(+)-Propranolol	$4.2 (\pm 1.7) \times 10^{-6}$	$5.3 (\pm 1.1) \times 10^{-8}$
β-Agonists		
(−)-Isoproterenol	$2.4 (\pm 0.6) \times 10^{-3}$	$9 (\pm 2.1) \times 10^{-7}$
(+)-Isoproterenol	$1.7 (\pm 0.5) \times 10^{-3}$	$3.8 (\pm 1.2) \times 10^{-5}$
(−)-Epinephrine	$3 (\pm 0.6) \times 10^{-3}$	$3.8 (\pm 0.9) \times 10^{-5}$
(−)-Norepinephrine	$3 (\pm 0.4) \times 10^{-3}$	$4.5 (\pm 1.1) \times 10^{-4}$
Other drugs		
Monensin	$1.4 (\pm 0.5) \times 10^{-6}$	$>10^{-5}$
Reserpine	$4.3 (\pm 1.5) \times 10^{-6}$	$>10^{-4}$
Imipramine	$3.9 (\pm 1.9) \times 10^{-6}$	$1.1 (\pm 0.4) \times 10^{-5}$
Desmethylimipramine (DMI)	$3.5 (\pm 2.2) \times 10^{-6}$	NT
Serotonin	$>10^{-3}$	$2.1 (\pm 0.3) \times 10^{-5}$
Oxotremorine	$2.3 (\pm 0.6) \times 10^{-4}$	NT
Sodium azide	$5.2 (\pm 4.0) \times 10^{-3}$	$>10^{-3}$

Inhibition by drugs of ^{125}I-PIN uptake into peptic cells was determined and IC_{50} values were calculated. ^{125}I-PIN was used at 50–60 pM, and β-AR binding sites were occupied by 0.1 μM timolol. K_i values of drugs for inhibition of ^{125}I-PIN binding to β-ARs on frog erythrocytes were calculated from competition curves using the equation of Cheng and Prusoff [15]. Results are the means (± SEM) of experiments performed three to five times. NT = not tested.

esophageal peptic cells by direct binding studies, we were interested in determining whether they were functionally coupled to pepsinogen secretion. We were disappointed to observe, however, that peptic cells were somewhat unresponsive to isoproterenol activation. Thus, maximum secretory responses to isoproterenol were generally 0.5 to 1.5% of total pepsinogen/30 min (see Fig. 6) which represented approximately 20% of maximal secretion elicited by cholinergic agonists. This reponse was unchanged in the presence of the cAMP phosphodiesterase inhibitor IBMX, although basal secretion was enhanced by this agent (data not shown). One possibility for the poor isoproterenol response was the release of somatostatin from D cells in the cell preparation. Experiments were therefore conducted in the presence of somatostatin antibody (courtesy of Dr. T. Yamada, Ann Arbor, MI). Isoproterenol-stimulated pepsinogen secretion was similar in the absence and presence of this antibody (data not shown). These data suggested that somatostatin release did not contribute to the poor isoproterenol stimulation.

Isoproterenol responsiveness could be enhanced, however, by preincubation of peptic cells with the protein kinase C activator TPA. Figure 6 shows the dose–response curve for isoproterenol-stimulated pepsinogen secretion in TPA (0.1 μM) pretreated cells. Maximum isoproterenol-stimulated secretion was 3.4% of total pepsinogen/30 min, which represented a 200% increase in secretion compared with control cells. TPA pretreatment had no significant effect on the EC_{50} for pepsinogen secretion stimulated by isoproterenol, epinephrine or norepinephrine, nor on the K_i values of agonists for β-ARs of peptic cell membranes. The inactive protein kinase C activator 4-α phorbol was unable to augment isoproterenol-stimulated pepsinogen secretion. The β-AR antagonist timolol (10 μM) totally inhibited iso-

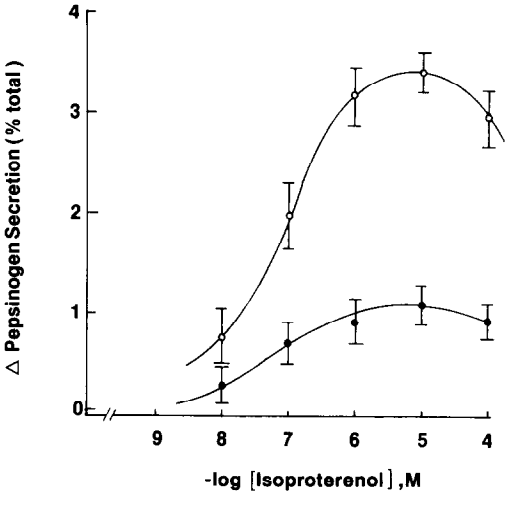

Fig. 6. Effect of TPA preincubation upon isoproterenol-stimulated pepsinogen secretion of frog peptic cells. Peptic cells were preincubated with (○) or without (●) 10^{-7} M TPA for 10 min at 25° and washed three times in amphibian Ringer containing 0.2% BSA. Treated and non-treated cells were stimulated with increasing concentrations of isoproterenol for 30 min at 25°, and pepsinogen secretion was assessed as described in Materials and Methods. Δ Pepsinogen (increase above unstimulated control) is plotted against log isoproterenol concentration. Results show the mean (± SEM) of experiments performed five to eight times.

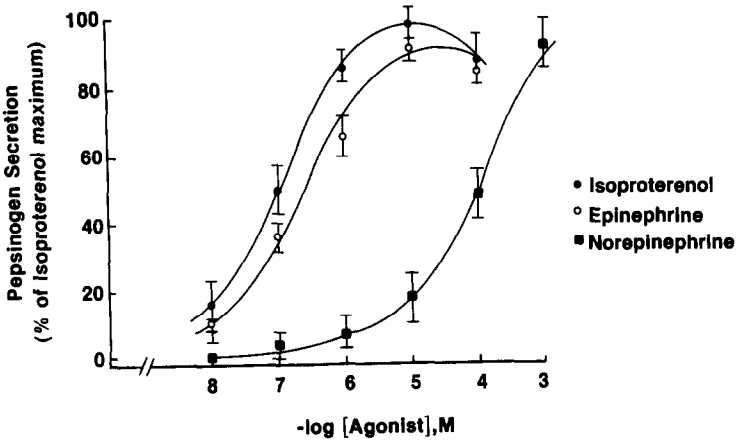

Fig. 7. Dose–response curves of catecholamine-stimulated pepsinogen secretion from frog peptic cells. Peptic cells were pretreated with TPA as described in the legend of Fig. 6. The washed cells were exposed to increasing concentrations of the indicated catecholamines, and the pepsinogen secreted for 30 min at 25° was measured. Secretion expressed as percent isoproterenol maximal is plotted against log agonist concentration. Results show the mean (± SEM) of experiments performed five to eight times.

proterenol-stimulated pepsinogen secretion (data not shown).

Figure 7 shows the dose–response curves of catecholamine-stimulated pepsinogen secretion in TPA-pretreated cells. Isoproterenol and epinephrine were potent secretagogues with EC_{50} values of 0.1 and 0.22 μM respectively. Norepinephrine was some 1000-fold weaker (EC_{50} = 100 μM) although it did appear to be a full agonist, evoking a maximal secretion equivalent to that of isoproterenol. The order of potencies of catecholamines for stimulating secretion was similar to that for inhibition of ^{125}I-PIN binding to peptic cell β-ARs. These data indicated that β_2-ARs were functionally coupled to pepsinogen secretion.

DISCUSSION

This study has sought to examine directly the presence of β-ARs coupled to pepsinogen secretion on frog esophageal peptic cells. We have used the established radioligand ^{125}I-iodopindolol to characterize the β-AR binding sites on intact cells and membrane preparations. However, binding of ^{125}I-PIN to intact cells was complicated by significant radioligand uptake into peptic cells. Thus, although a small proportion of binding sites showed characteristics of β-ARs, i.e. they exhibited high affinity for timolol, stereoselectivity, and appropriate affinity for isoproterenol, a larger proportion did not demonstrate these characteristics. Since washing cells in hypotonic buffer resulted in cell lysis and decreased "binding", it became clear that the non-β-AR sites represented radioligand uptake into peptic cells. Temperature reduction has normally been used to inhibit uptake processes and allow cell surface sites to be determined. However, in view of the slow rates of association of ^{125}I-PIN with β-ARs, this approach was not possible. To overcome the problem of radioligand uptake, we prepared peptic cell homogenates and established that ^{125}I-PIN bound to a single high-affinity population of sites having the same pharmacological characteristics as the β_2-ARs of frog erythrocytes [14].

We examined the characteristics of the ^{125}I-PIN uptake in some detail since this seems to represent a ubiquitous and metabolically important amine transport system which is common to a number of mammalian cells [19, 20]. Uptake of ^{125}I-PIN into peptic cells was saturable, temperature and pH dependent, Na^+ independent, non-stereoselective and inhibited by agents that depress cellular ATP, disrupt pH gradients, or interact with amine transport systems. This uptake system has many characteristics similar to the postulated carrier-mediated amine transporter described for a number of cultured mammalian cells [19, 20]. Interestingly, ^{125}I-PIN uptake was not observed with frog erythrocytes which are nucleated cells. These findings argue against the concept that accumulation of radioligand represents non-specific diffusion of radioligand into acidic compartments of the cell [21]. Moreover, uptake of amines into HeLa cells has been reported to be independent of pK_a or hydrophobicity of the drug [19]. Instead, uptake has been suggested to occur by a carrier-mediated transport system which utilizes a proton gradient as the energy source. Our studies demonstrated that uptake was markedly dependent on temperature and extracellular pH. Elevated temperature resulted in ^{125}I-PIN uptake followed by radioligand efflux, and similar results have been reported for amine uptake into HeLa cells [19] and catecholamine transport in synaptic vesicles [22]. The sensitivity of ^{125}I-PIN uptake to monensin, an agent that dissipates proton gradients, suggests that the driving force for uptake in our system may be a proton gradient. The Na^+ independence and lack of affinity of catecholamines, serotonin and histamine clearly distinguish the amine uptake system of peptic cells from synaptic vesicle or platelet amine transport [23, 24] or histamine uptake [25].

Our studies did not discriminate the localization

of the putative amine tranporter to either the plasma membrane or lysosomal compartments, although the former localization has been reported for HeLa cells [19]. The physiological significance of such a system and its normal substrates are presently unknown. We have examined a number of amino acids and polyamines (e.g. spermine) as potential substrates and observed poor affinity for the transport system. In view of the fact that it was absent on frog erythrocytes, it may not exhibit a critically important metabolic function for all cells.

The functional experiments clearly demonstrated the presence of β-ARs on isolated peptic cells which were coupled to pepsinogen secretion. These functional β-ARs had an identical pharmacological specificity to the sites labeled by ^{125}I-PIN and they could be classified as β_2-ARs. Frog peptic cells therefore differ from rabbit glands which have been reported to have β_1-ARs which are coupled to pepsinogen secretion [6]. These variations may relate to the different sympathetic neutrotransmitters utilized by Amphibia (epinephrine) and Mammalia (norepinephrine). Thus, β_2-ARs may function in Amphibia as the "innervated" receptor, whereas this function is served in mammals by β_1-ARs [26]. Alternatively, the β_1-ARs of rabbit peptic glands may be a further example of the unusual distribution of β_1-ARs in that species. Thus, rabbit lung, unlike most other mammals also contains a predominance of β_1-ARs [27].

The maximum stimulation to isoproterenol that we observed in isolated peptic cells represented approximately 10–20% of the maximal response to cholinergic agonists. One possibility for the poor isoproterenol response of cells was β-AR-mediated release of somatostatin from D cells in our cell preparation. Significant amounts of somatostatin have been observed in frog esophageal mucosa [28], and somatostatin has been reported to inhibit agonist and messenger-stimulated pepsinogen secretion in peptic cells [29]. However, the presence of a significant number of D cells was unlikely to be important in view of experiments showing that isoproterenol responsiveness was similar in the absence or presence of somatostatin antibody.

Since these peptic cells responded to forskolin and the cAMP phosphodiesterase inhibitor IBMX [12], we could assume a functioning cAMP messenger system coupled to secretion. The available evidence points to a relatively inefficient coupling of β-AR with effector (adenylate cyclase) in these cells. The efficiency of coupling could be increased by treatment of cells with phorbol ester which activates protein kinase C. Thus, the state of phosphorylation of components of the effector system may modulate the activity of the β-ARs coupled to pepsinogen secretion. Recently phorbol esters have been shown to augment isoproterenol-stimulated β-AR responsiveness in frog erythrocytes, by phosphorylation of the catalytic subunit of adenylate cyclase [30, 31]. These observations suggest that the activity of the β-AR system may be modulated by activators of protein kinase C pathway. Thus, activation of peptic cells by agonists that stimulate protein kinase C, e.g. muscarinic agonists or bombesin, would be expected to augment β-AR stimulation. In support of this contention, synergistic responses to combinations of isoproterenol and bombesin or bethanechol have been observed in these cells [11].

In conclusion, we have established the presence of β-ARs on frog esophageal peptic cells by direct binding studies and measurements of catecholamine-stimulated secretory responses. In addition, peptic cells contained an uptake system for amines which is distinct from the β-AR binding sites, and which is absent from frog erythrocytes. The characteristics of this uptake system suggests that it serves to transport amines into the peptic cell, although its exact physiological significance is unknown. The pharmacological characteristics of peptic cell β_2-ARs were similar to those present on frog erythrocytes. The number of β-AR sites was less than those on erythrocytes, but similar to the number of peptic cell muscarinic cholinergic receptors (1200 sites/cell). However, in contrast to the marked secretory response to muscarinic agonists, the responses to isoproterenol were relatively modest. The efficiency of β-AR coupling could be enhanced by phorbol esters which activate protein kinase C, and may represent a possible molecular mechanism by which muscarinic agonists and catecholamines produce synergistic secretory responses in peptic cells.

Acknowledgement—The authors would like to thank Debbie Beam for manuscript preparation.

REFERENCES

1. Anichkov SV and Grechishkin LL, Influence of catecholamines on pepsinogen production. *Arch Int Pharmacodyn Ther* **180**: 281–288, 1969.
2. Daly MJ and Stables R, The effect of (−) isoprenaline and (±) salbutamol on pepsinogen and acid secretion in the dog. *Br J Pharmacol* **59**: 323–325, 1977.
3. Sanders MJ and Soll AH, Characterization of receptors regulating secretory function in the fundic mucosa. *Annu Rev Physiol* **48**: 89–101, 1986.
4. Bech K and Moberg KU, β-Adrenergic agonists inhibit gastric acid and pepsin secretion through somatostatin release in dogs. *Scand J Gastroenterol* **21**: 531–536, 1985.
5. Koelez HR, Hersey SJ, Sachs G and Chew CS, Pepsinogen release from isolated gastric glands. *Am J Physiol* **243**: G218–G225, 1982.
6. Skoubo-Kristensen E and Frykland J, Adrenergic stimulation of pepsinogen release from rabbit isolated gastric glands. *Naunyn Schmiedebergs Arch Pharmacol* **330**: 37–41, 1985.
7. Ballantyne GH, Schafer DE, Tyshkov M, Zdon MJ, Fratesi GR, Modlin IM and Roberts JR. Evidence for dual modulation of pepsinogen secretion using isoproterenol, carbachol, CCK-8, forskolin, 8-bromo-cAMP and A23187 probes. *Ann Surg* **204**: 559–565, 1986.
8. Shirakawa T and Hirschowitz BI, Interaction between stimuli and their antagonists on frog esophageal peptic glands. *Am J Physiol* **249**: G668–G673, 1985.
9. Sanders MJ, Amirian DA, Ayalon A and Soll AH, Regulation of pepsinogen release from canine chief cells in primary monolayer culture. *Am J Physiol* **245**: G641–G646, 1983.
10. Raufman JP, Kasbekar DK, Jensen RT and Gardner JD, Potentiation of pepsinogen secretion from dispersed glands from rat stomach. *Am J Physiol* **245**: G525–G530, 1983.

11. Raufman JP and Cosowsky L, Actions of cholera toxin on dispersed chief cells from guinea pig stomach. *Biochim Biophys Acta* **928**: 199–207, 1987.
12. Matsumoto H, Dickinson KEJ, Shirakawa T, Komiyama K and Hirschowitz BI, Synergism between cellular messengers and agonist combinations in pepsinogen secretion. *Am J Physiol* **253**: G557–G565, 1987.
13. Hersey SJ, Owirodu A and Miller M, Forskolin stimulation of acid and pepsinogen secretion by gastric glands. *Biochim Biophys Acta* **755**: 293–299, 1983.
14. Mukherjee C and Lefkowitz RJ, Direct studies of β-adrenergic receptors in intact frog erythrocytes. *Life Sci* **19**: 1897–1906, 1976.
15. Cheng Y and Prusoff W, Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymic reaction. *Biochem Pharmacol* **22**: 3099–3108, 1973.
16. Matsumoto H, Dickinson KEJ, Anderson W and Hirschowitz BI, Pepsinogen secretion from perifused frog peptic glands: Rapid transients detected with a modified pepsin assay. *Life Sci* **42**: 1237–1244, 1987.
17. Dickinson KEJ and Nahorski SR. Atypical characteristics of frog and chick erythrocyte β-adrenoceptors. *Eur J Pharmacol* **74**: 43–52, 1981.
18. Kent RS, DeLean A and Lefkowitz RJ, A quantitative analysis of beta-adrenergic receptor interactions: Resolution of high and low affinity states of the receptor by computer modelling of ligand binding data. *Mol Pharmacol* **17**: 14–23, 1980.
19. Lysko PG and Henneberry RC, Differentiation between amine transport and β-adrenergic receptor-mediated binding in cultured mammalian cells. *Mol Pharmacol* **28**: 338–347, 1985.
20. Henneberry RC, Jahangeer S and Lysko PG, Transport of beta-adrenergic antagonists in the absence of beta-adrenergic receptors in rat pituitary tumor cells. *Biochem Pharmacol* **35**: 2687–2692, 1986.
21. Meier KE and Ruoho AE, Uptake of L-[propyl-2,3-^{3}H]dihydroalprenolol by intact HeLa cells. *Biochim Biophys Acta* **804**: 331–340, 1984.
22. Toll L and Howard BD, Role of Mg^{2+}-ATPase and a pH gradient in the storage of catecholamines in synaptic vesicles. *Biochemistry* **17**: 2517–2523, 1978.
23. Sneddon JM, Blood platelets as a model for monoamine-containing neurones. *Prog Neurobiol* **1**: 151–198, 1971.
24. Njus D, Knoth J and Zaalakiam M, Proton-linked transport in chromaffin granules. *Curr Top Bioenerg* **11**: 107–147, 1981.
25. Berglindh T and Sachs G, Histamine uptake and release from isolated gastric glands. In: *Hormone Receptors in Digestion and Nutrition* (Eds Roselin G, Fromageot P and Bonfils S), pp. 373–381. Amsterdam, Elsevier/North Holland Biomedical Press, 1979.
26. Stene-Larsen G, Comparative aspects of cardiac adrenoceptors: Characterization of the β_2-adrenoceptor as a common "adrenaline" receptor in vertebrate hearts. *Comp Biochem Physiol* **70C**: 1–12, 1981.
27. Rugg EL, Barnett DB and Nahorski SR, Coexistence of $beta_1$ and $beta_2$ adrenoceptors in mammalian lung: Evidence from direct binding studies. *Mol Pharmacol* **14**: 996–1005, 1978.
28. Fong JC, Ko LW, Hong SC and Ho LM Somatostatin modulation of neurally controlled pepsinogen from frog esophageal mucosa. *Fed Proc* **46**: 1233, 1987.
29. Heldman AW, Matsumoto H and Hirschowitz BI, Somatostatin-14 pretreatment inhibits pepsinogen secretion from protein kinase dependent pathways. *Gastroenterology* **92**: 1800, 1987.
30. Sibley DR, Jeffs RA, Kiefer D, Nambi P and Lefkowitz RJ, Phorbol diester treatment promotes enhanced adenylate cyclase activity in frog erythrocytes. *Arch Biochem Biophys* **244**: 373–381, 1986.
31. Yoshimasa T, Sibley DR, Bouvier M, Lefkowitz RJ and Caron MG, Cross-talk between cellular signalling pathways suggested by phorbol-ester-induced adenylate cyclase phosphorylation. *Nature* **327**: 67–70, 1987.