Pituitary adenylate cyclase-activating peptide (PACAP) has a specific receptor PAC1 and shares two receptors VPAC1 and VPAC2 with vasoactive intestinal peptide (VIP). VPAC2 activation enhances glucose-induced insulin release while VPAC1 activation elevates glucose output. To generate a large pool of VPAC2 selective agonists for the treatment of type 2 diabetes, structure-activity relationship studies were performed on PACAP, VIP, and a VPAC2 selective VIP analog. Chemical modifications on this analog that prevent recombinant expression were sequentially removed to show that a recombinant peptide would retain VPAC2 selectivity. An efficient recombinant expression system was then developed to produce and screen hundreds of mutant peptides. The 11 mutations found on the VIP analog were systematically replaced with VIP or PACAP sequences. Three of these mutations, V19A, L27K, and N28K, were sufficient to provide most of the VPAC2 selectivity. C-terminal extension with the KRY sequence from PACAP38 led to potent VPAC2 agonists with improved selectivity (100–1000-fold). Saturation mutagenesis at positions 19, 27, 29, and 30 of VIP and charge-scanning mutagenesis of PACAP27 generated additional VPAC2 selective agonists. We have generated the first set of recombinant VPAC2 selective agonists described, which exhibit activity profiles that suggest therapeutic utility in the treatment of diabetes.
VIP or R3P3 peptide fused to a Factor Xa recognition site at its N terminus, followed by DNA extension with the large fragment of DNA polymerase I (Invitrogen). Some mutant peptides were created using mutagenesis as templates for Factor Xa. R3P2 to R3P55 were generated using R3P51 as the template, while R3P56 to R3P59 were generated using R3P41 as the template. All mutant peptides were cloned into pGEX-6p-1 (Amersham Biosciences) through XhoI and BamHI sites, or BamHI and EcoRI sites, and expressed in Escherichia coli BL21(DE3) cells (Stratagene). Cloning into pGEX-6p-1 in the above manner resulted in the fusion of the glutathione S-transferase (GST) protein N-terminal to the peptide, enabling the purification of the fusion protein by virtue of the interaction of GST with glutathione.

For saturation mutagenesis, site-directed PCR mutagenesis of the R3P3 template was performed with a mutated oligonucleotide primer containing the codons NN(NGC) to randomize amino acids of R3P65 at residues 19, 27, 29, or 30 one position at a time. The four mutant pools (one for each position) were cloned into pGEX-6p-1 as individual pools through BamHI and EcoRI sites and transformed into E. coli XL1-blue cells (Stratagene). Single clones (96 for each position) were isolated and grown up in 96-well plates for DNA purification, using the BioRobot Qiabot 9600 (Qiagen), and sequence verification. Confirmed mutations were then transformed into E. coli BL21(DE3) cells for expression. This initial random mutagenesis recovered most of the 19 possible mutations (17/19, 19/19, 13/19, and 17/19 mutations at positions 19, 27, 29, and 30, respectively) and thus greatly reduced the number of site-directed mutagenesis reactions needed. The DNA for the remaining 10 mutant peptides was generated by site-directed mutagenesis (see above).

For combinatorial mutant DNA generation (R3P174 to R3P193), PCR site-directed mutagenesis of templates R3P174 (A199G-R3P55), R3P104 (A19K-R3P56), R3P110 (A19F-R3P56), and R3P111 (A19S-R3P56) was done with oligonucleotides containing codons corresponding to the desired mutations at positions 27, 29, and 30. The mutant DNA was cloned into pGEX-6p-1 through BamHI and EcoRI sites, and single clones were isolated and expressed in E. coli BL21(DE3) cells.

Expression and Purification of Recombinant Mutant Peptides—For saturation and combinatorial mutant peptides of R3P65, only 40-mol of E. coli for expression was needed, whereas for the other mutant peptides 0.5–1 liter was necessary. Cells were grown at 37 °C. At an OD600 of 0.6–1.0, the cells were induced at a final concentration of 1 mm isopropyl-1-thio-D-galactopyranoside (Invitrogen) for 1–2 h. Cells were harvested at 7,700 × g for 15 min and frozen at −20 °C. Per 20 ml of culture, the frozen cell pellet was resuspended in 1 ml of B-PER (Pierce) with 20 μl of protease inhibitor mixture (Sigma) and 50 μg of lysozyme (Amersham Biosciences) and sonicated 3–6 times for 1 min with 15 s breaks on ice. Cellular debris was removed by centrifugation at 10,000 × g for 20 min. 20% of glutathione-Sepharose 4B (Amersham Biosciences) was thoroughly washed in phosphate-buffered saline and mixed with the lysate on a shaker overnight at 4 °C. The resin was collected from the lysate by centrifugation at 1,500 × g for 15 min, packed into a Poly-sep column (Bio-Rad), washed with 1 ml of phosphate-buffered saline followed by 0.3 ml of Factor Xa buffer (1 mg ml−1, CaCl2, 100 mM NaCl, and 50 mM Tris-HCl, pH 8.0). The peptides were cleaved off the column during an overnight incubation at 4 °C by the addition of 1 unit of Factor Xa (Amersham Biosciences) in 0.2 ml of Factor Xa buffer. For saturation and combinatorial mutant peptides of R3P65, the resulting Factor Xa eluate was used for cell assays. The other mutant peptides were further purified by C18 reverse phase-HPLC (Dynamax 300A C18 column, Rainin, Beckman System Gold HPLC) using a 2-ml loop and flow rate of 2 ml/min with the following program: 10 min of 95% Buffer A (0.1% trifluoroacetic acid/H2O), 30 min of a linear gradient to 100% Buffer B (0.1% trifluoroacetic acid/acetonitrile). Peak fractions (1 ml each) were collected. Peptide-containing fractions were lyophilized for storage and resuspended in sterile water or phosphate-buffered saline for assays. Mutant peptides so purified typically ran as a single band on 10–20% Tricine SDS-PAGE (Novex) stained in Gel Code Blue (Pierce). Typical yields of final purified peptide were 1 mg per liter of culture. Expression and purification yield was limited mainly by proteolysis at Lys-21/Lys-22 during expression of these peptides in E. coli (data not shown). Fig. 2 illustrates a typical purification of peptide R3P3.

Recombinant Mutant Peptide Purity Assessment and Concentration Determination—High-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) was used to determine the purity and concentration and to confirm the predicted mass of the saturation and combinatorial mutant peptides of R3P65. A Michrom 2002 LC was used to generate a standard curve with synthetically produced R3P66. Varying amounts of this peptide (over a range from 10 to 80 pmol) were loaded onto a 0.3 × 150 mm column with Magic C18 beads (5 μm, 300 Å). The gradient (solvent A = 5% acetonitrile, 0.4% acetic acid, 0.005% HFBA; solvent B = 100% acetonitrile, 0.4% acetic acid, 0.005% HFBA) went from 5–60% B over 20 min at a flow rate of 6 μl/min. UV detection was set at 214 nm. Using the Magic 2002 software, the goodness of fit of the linear standard curve (R2) was calculated to be 0.9971. Each recombinant mutant peptide was then loaded onto the Magic 2002 LC using the method described above and quantitation was achieved by analyzing the peptide peak against the calibration curve. To confirm the expected mass of each peptide, the eluting peptides were run directly from the HPLC column into a Finnigan LCQ Classic mass spectrometer. Using an electrospray ionization source and a method consisting of full MS over a scan range of 650–850 Da followed by data-dependent MS-MS, each peptide was analyzed. The mass for each peptide was obtained using Navigator 1.2 and Excelibur software as recommended by the manufacturer.

For all other mutant peptides, peptide molecular weight was confirmed by mass spectrometry performed on a PerSeptive Biosystems Voyager RP MALDI-TOF using the vendors suggested analysis conditions or a Hewlett Packard Model 59987A API-Electrospray LC/MS Interface with a Model 5998B MS Engine by way of a Hewlett Packard 1050 pump with a solution of 50% methyl alcohol/water and 1% acetic acid. Amino acid analysis confirmed amino acid composition of all mutant peptides (Commonwealth Biotechnologies, Inc). Analysis was performed by hydrolysis in 6 N HCl, then applied through an amino acid analyzer, typically accurate within <2%.
Competition Binding Assay and cAMP Scintillation Proximity Assay (SPA)—PACAP competition binding and cAMP accumulation assay were performed as described previously (5).

**RESULTS**

**VPAC2 Selective Agonist Can Be a Recombinant Peptide (Round 1 Mutagenesis)**—The only available VPAC2 selective agonists are two cyclized synthetic analogs of VIP (18, 20). To determine whether a recombinant VPAC2-selective peptide can be made, the four chemical modifications of one of them, Ro 25-1553 (21), were sequentially removed and tested in radiolabeled PACAP27 to the cell membrane as a result of competition from the peptide. EC_{50} is defined as the concentration of the peptide at which 50% of maximum PACAP27-induced cAMP accumulation in CHO or RIN cells transfected with the appropriate receptor is reached. Data are the mean ± S.E. of at least three experiments performed in triplicates. Ratio is a measure of selectivity, or preference for VPAC2 over VPAC1, and is derived from the ratio of IC_{50} or EC_{50} at VPAC1 versus that at VPAC2. None of these VIP-based peptides displayed any PAC1 binding or caused cAMP accumulation.

**Recombinant Expression of Peptides**—VIP mutant peptides were expressed as C-terminal fusions to glutathione S-transferase with a Factor Xa recognition site inserted immediately N-terminal to the desired peptide sequence. Factor Xa cleavage yielded recombinant peptides that were identical to their synthetic counterparts. Both synthetic and recombinant R3P3, R3P9, R3P12, R3P33, R3P55, and R3P60 were generated. In every case, recombinantly produced peptides were shown to be identical by mass spectrometry, amino acid analysis, competition binding assay (for example, see R3P3 in Fig.

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**Table I**

Mutagenesis of VIP to generate highly selective VPAC2 mutant peptides with minimal number of mutations

| Peptide | Sequence | Competition binding, IC_{50} (nM) | CAMP Accumulation, EC_{50} (nM) |
|---------|----------|----------------------------------|----------------------------------|
| VIP     | HEDAVPTDNYTLRLKGAAPVYNL SLIM* | 20 ± 2 | 1.4 ± 0.6 |
| Ro25-1553 | HEDAVPTDNYTLRLKGAAPVYNL SLIM* | 20 ± 2 | 100 ± 20 |
| R3P1    | HEDAVPTDNYTLRLKGAAPVYNL SLIM* | 100 ± 20 | 1.4 ± 0.6 |
| R3P2    | HEDAVPTDNYTLRLKGAAPVYNL SLIM* | 100 ± 20 | 1.4 ± 0.6 |
| R3P3    | HEDAVPTDNYTLRLKGAAPVYNL SLIM* | 100 ± 20 | 1.4 ± 0.6 |
| R3P4    | HEDAVPTDNYTLRLKGAAPVYNL SLIM* | 100 ± 20 | 1.4 ± 0.6 |

*Competition Binding Assay and cAMP Scintillation Proximity Assay (SPA)—PACAP competition binding and cAMP accumulation assay were performed as described previously (5).**

**Recombinant Expression of Peptides**—VIP mutant peptides were expressed as C-terminal fusions to glutathione S-transferase with a Factor Xa recognition site inserted immediately N-terminal to the desired peptide sequence. Factor Xa cleavage yielded recombinant peptides that were identical to their synthetic counterparts. Both synthetic and recombinant R3P3, R3P9, R3P12, R3P33, R3P55, and R3P60 were generated. In every case, recombinantly produced peptides were shown to be identical by mass spectrometry, amino acid analysis, competition binding assay (for example, see R3P3 in Fig.
Structure-Activity Relationship of VPAC2 Selective Agonists

Fig. 3. Competition binding assay of VIP mutant peptide R3P3. R3P3 generated by either recombinant expression (open symbols) or chemical synthesis (closed symbols) was bound to cell membrane derived from CHO cells transfected with VPAC2, VPAC1, or PAC1 at different peptide concentrations. The decrease in percent $^{125}$I-PACAP27 binding as a result of increasing concentration of R3P3 is plotted against peptide concentration. The data shown are representative of at least three similar experiments. Each data point represents the average of triplicate data with S.E. plotted.

3), and cAMP assay (data not shown) to their corresponding synthetic counterparts. Since functional screening for receptor selectivity required microgram amounts of peptides, this expression system provided an efficient and rapid method for analyzing a large number of mutant peptides and was utilized for subsequent rounds of mutagenesis.

Identification of Key VPAC2 Selective Positions within VIP and PACAP38 (Round 2 Mutagenesis)—Although R3P3 was selective for VPAC2, it contained 11 mutations from VIP, any one of which could contribute to VPAC2 selectivity. A series of 13 peptides (R3P5 to R3P17) were designed to reduce the number of candidate VPAC2 selective positions (see Tables I and II). Mutant peptides generated using PACAP as the template (R3P6, R3P15-R3P17) exhibited a significant loss in VPAC1 activity but a greater selectivity for PAC1 rather than VPAC2 (see Table II). The significant VPAC2 selectivity displayed by R3P8 but not R3P10 confirms the importance of Lys-27 and Lys-28 mutations for VPAC2 selectivity over VPAC1 (see Table I). The role of Glu-8 and Leu-26 mutations in the context of Lys-27 and Lys-28 was less clear since R3P7 did not show any VPAC2 selectivity. R3P5, R3P12, and R3P13 appeared to be the most VPAC2 selective mutant peptides out of this second round of mutagenesis, suggesting that the removal of conservative mutations, Glu-8, Lys-12, and Leu-26 found in R3P3, did not affect VPAC2 selectivity. Ending the C terminus with the GGT sequence as in the case of R3P9 and R3P12 led to slightly greater VPAC2 selectivity than C-terminal amidation as found in R3P8 and R3P13. Therefore, R3P12 was chosen as the lead peptide out of this round of mutagenesis.

Isolating Three VPAC2 Selective Positions within VIP (Round 3 Mutagenesis)—The lead peptide R3P12 from Round 2 was systematically analyzed by exchanging each mutated residue with the wild-type amino acids and by introducing these mutations into VIP individually. R3P20 to R3P30 were designed to determine the individual and combinatorial effects of the five mutations in the body of VIP by converting the R3P12 amino acids found at these positions back to those of VIP (see Table I). Changing Leu-17 and Asp-25 back to the native VIP amino acids individually as in R3P20 and R3P22 or in combination as in R3P26 do not significantly affect VPAC2 selectivity, suggesting that these two mutations do not contribute to VPAC2 selectivity. The Lys-27 mutation appears to be a major VPAC2 selectivity determinant since exchanging it for the wild-type leucine residue in R3P23, R3P27, and R3P28 greatly reduces VPAC2 selectivity in the binding assay. The Ala-19 mutation appears to play a moderate role in VPAC2 selectivity because exchanging it for the wild-type valine residue in R3P21, R3P25, R3P28, and R3P29 lowers VPAC2 selectivity to a lesser degree than the Lys-27 mutation. The importance of the Lys-28 mutation is more context-dependent because removing it alone in R3P24 has only a minor effect on VPAC2 selectivity but taking it out together with Lys-27 (R3P27), Ala-19 (R3P29), or Asp-25 (R3P30) leads to significant reduction in VPAC2 selectivity. Introducing these five mutations: Leu-17 (R3P46), Ala-19 (R3P47), Asp-25 (R3P48), Lys-27 (R3P42), Lys-28 (R3P43) into VIP individually did not result in VPAC2 selectivity, suggesting VPAC2 selectivity requires the combinatorial effect at least two mutations. Combining the three likely VPAC2 selective mutations Ala-19, Lys-27, and Lys-28 into a single R3P51 peptide resulted in a VPAC2 selective agonist that has only three mutations from VIP, yet displayed similar VPAC2 selectivity as the 11-mutation R3P3, the recomb inant version of the cyclized VIP analog Ro 25-1553. All three of these mutations are necessary since peptides containing any one of the two mutations, R3P41 (Lys-27/Lys-28), R3P49 (Ala-19/Lys-27), or R3P50 (Ala-19/Lys-28) exhibited much lower VPAC2 selectivity.

Peptides R3P37 to R3P40 were designed to generate VPAC2 selective agonists using PACAP as the template (see Table II). Although there was a general trend of preference for VPAC2 over VPAC1 displayed by R3P37 and R3P38, either no preference (R3P37) or only slight preference (R3P38) for VPAC2 over PAC1 was exhibited. When only Lys-27 and Lys-28 mutations were introduced into PACAP27 (R3P40), no significant VPAC2 selectivity was detected. Adding the Leu-17 mutation (R3P39) also did not lead to VPAC2 selectivity. Similar to the PACAP mutant peptides in Round 2, these two peptides may possess selective PAC1 agonist activity as demonstrated in the cAMP assay.

C-terminal Addition with PACAP38 Sequence to Generate More Selective VPAC2 Agonist (Round 4 Mutagenesis)—Peptides R3P31 to R3P36 were designed to decipher the role of the C-terminal GGT sequence. We sequentially removed this sequence (R3P31 to R3P33) and built the peptide back up to 31 residues with other amino acids (R3P34 to R3P36) while fixing the five other mutations (Leu-17, Ala-19, Asp-25, Lys-27, and Lys-28). Glutamine (Q) was appended to R3P33 to generate R3P34 while the NQ sequence was added to R3P33 to create R3P35 because these amino acids are favored at the C termini of helices (22). To extend the peptide to 31 residues, we chose to graft residues 29–31 of PACAP38 onto R3P33 resulting in peptide R3P36. All of these peptides displayed excellent VPAC2 selectivity (see Table I) suggesting that a 28-residue peptide without C-terminal amidation, such as R3P33, can be as VPAC2-selective as the 31-mer peptide R3P12 or R3P3. Interestingly, R3P36 displayed the greatest VPAC2 selectivity (150-fold) in the binding assay, and was an even better VPAC2 selective agonist than the VIP analog Ro 25-1553 (60-fold). These results suggest that increasing the number of positive charges at the C terminus greatly reduced VPAC1 activity without affecting VPAC2 activity.

Maximizing VPAC2 Selectivity While Minimizing Number of Mutations (Round 5 Mutagenesis)—Peptides R3P53 to R3P59 and R3P65 were designed to reduce the number of mutations found in R3P36, the lead peptide from Round 4, based on the knowledge that the lead peptide R3P51 from Round 3 did not require Leu-17 and Asp-25 mutations found on R3P36 to achieve full VPAC2 selectivity. All of these new peptides are selective for VPAC2 with the five-mutation R3P53 and R3P55 being the most selective agonists. R3P55 contains only two
(Ala-19 and Lys-27) of the original 11 mutations found in Ro 25-1553, yet it was more selective in the binding assay (180-fold versus 60-fold, respectively). Sequence comparison of R3P53 and R3P55 suggested that the Lys-28 and Tyr-31 mutations may not be crucial for the dramatic selectivity displayed since neither mutation was found in both peptides (see Table I). The four common mutations Ala-19, Lys-27, Lys-29, and Arg-30 were introduced into VIP to generate R3P65, which exhibited similar VPAC2 selectivity and potency as R3P53 and R3P55. Further reductions in the number of mutations did not result in peptides with comparable selectivity (data not shown), suggesting that all four mutations were needed to ensure maximum selectivity. Thus, we have created a highly VPAC2 selective and potent peptide that differs from VIP by only four amino acids (see Fig. 4).

High Throughput Saturation Mutagenesis of VPAC2 Selective Positions—To understand which amino acids maximally favor VPAC2 selectivity at each of the four critical VPAC2 selective positions 19, 27, 29, and 30, saturation mutagenesis at each position was performed using peptide R3P65 as a template followed by recombining the most selective mutations. This data showed that position 19 was most sensitive to substitutions in the cAMP assay (see Table III). Large, bulky, and hydrophobic amino acids at position 19 in general resulted in a loss of selectivity. In contrast, most amino acids were well tolerated for VPAC2 selectivity at position 27, with proline, arginine, and glutamine being somewhat more selective than

![Fig. 4. cAMP assay of PACAP27, VIP, and VPAC2 selective agonist R3P65. CHO cells transfected with PAC1, VPAC1, or VPAC2 were treated with increasing concentrations of PACAP27 or R3P65, followed by the measurement of accumulated cAMP. Basal and maximal-stimulated cAMP levels generally were 1 and 5 pmol per well. The data shown are representative of at least three similar experiments. Each data point represents the average of triplicate data with the S.E. plotted.](http://www.jbc.org/)

Asterisk (*) represents C-terminal amidation. Mutations from PACAP are shaded. IC50 is determined as peptide concentration at which there is a 50% decrease in binding of radiolabeled PACAP27 to the cell membrane as a result of competition by the peptide. EC50 is defined as the concentration of the peptide at which 50% of maximum PACAP27 cAMP accumulation in CHO cells transfected with the appropriate receptor is reached. Data are the mean of at least two experiments performed in triplicates. The standard errors were typically less than 30% of the mean.

| Peptide | Sequence | Competition binding, IC50 (nM) | cAMP Induction, EC50 (nM) |
|---------|----------|-------------------------------|--------------------------|
| PACAP27 | ISDGPTDSYKQAVKLYA AVL*        | VPAC | VPAC | PAC1 | VPAC | VPAC | PAC1 |
| PACAP38 | ISDGPTDSYKQAVKLYA AVL*        | 4    | 7   | 5    | 3   | 2.6  | 0.3  |
| R3P6    | ISDGPTDSYKQAVKLYA AVL*        | 80   | 250 | 90   | 5   | 3    | 0.6  |
| R3P15   | ISDGPTDSYKQAVKLYA AVL*        | 70   | 280 | 60   | 10  | 15   | 0.03 |
| R3P16   | ISDGPTDSYKQAVKLYA AVL*        | 90   | 110 | 30   | 2   | 5    | 0.14 |
| R3P17   | ISDGPTDSYKQAVKLYA AVL*        | 400  | 1500| 200  | 3   | 87   | 0.6  |
| R3P18   | ISDGPTDSYKQAVKLYA AVL*        | 100  | 300 | 200  | 2.5 | 50   | 9    |
| R3P19   | ISDGPTDSYKQAVKLYA AVL*        | 200  | 60  | 60   | 0.5 | 4    | 0.05 |
| R3P20   | ISDGPTDSYKQAVKLYA AVL*        | 120  | 70  | 80   | 0.5 | 4    | 0.05 |
| PAC1    | ISDGPTDSYKQAVKLYA AVL*        | 10   | 20  | >10000|    |
| PAC2    | ISDGPTDSYKQAVKLYA AVL*        | >10000| >10000| >10000|    |
| PAC3    | ISDGPTDSYKQAVKLYAVL*          | >10000| >10000| >10000|    |
| PAC4    | ISDGPTDSYKQAVKLYAVL*          | >10000| 1400 | >10000|    |
| PAC5    | ISDGPTDSYKQAVKLYAVL*          | 500   | 200 | >10000|    |
| PAC6    | ISDGPTDSYKQAVKLYAVL*          | >10000| >3000 | >10000|    |
| PAC7    | ISDGPTDSYKQAVKLYAVL*          | >10000| >10000| >10000|    |
| PAC8    | ISDGPTDSYKQAVKLYAVL*          | >10000| 7000 | >10000|    |
| PAC9    | ISDGPTDSYKQAVKLYAVL*          | 2600  | 40  | 2600  |    |
| PAC10   | ISDGPTDSYKQAVKLYAVL*          | >10000| >10000| >10000|    |
| PAC11   | ISDGPTDSYKQAVKLYAVL*          | >10000| 200  | 770   |    |
| PAC12   | ISDGPTDSYKQAVKLYAVL*          | 140   | 300  | >10000|    |
| PAC13   | ISDGPTDSYKQAVKLYAVL*          | 1900  | 80   | 5200  |    |
| PAC14   | ISDGPTDSYKQAVKLYAVL*          | 75000 | 7500 | 10000 |    |
| PAC15   | ISDGPTDSYKQAVKLYAVL*          | 160   | 120  | 50    |    |
| PAC16   | ISDGPTDSYKQAVKLYAVL*          | 110   | 30   | >3000  |    |
| PAC17   | ISDGPTDSYKQAVKLYAVL*          | 600   | 30   | 70    |    |
| PAC18   | ISDGPTDSYKQAVKLYAVL*          | >10000| 30   | 300   |    |
| PAC19   | ISDGPTDSYKQAVKLYAVL*          | 800   | 400  | 1600  |    |
| PAC20   | ISDGPTDSYKQAVKLYAVL*          | 360   | 200  | 800   |    |
| PAC21   | ISDGPTDSYKQAVKLYAVL*          | 40    | 10   | 60    |    |
| PAC22   | ISDGPTDSYKQAVKLYAVL*          | >10000| 50   | >10000|    |
| PAC23   | ISDGPTDSYKQAVKLYAVL*          | >10000| 4000 | >10000|    |
| PAC24   | ISDGPTDSYKQAVKLYAVL*          | 30    | 30   | 80    |    |
| PAC25   | ISDGPTDSYKQAVKLYAVL*          | 140   | 20   | 90    |    |
| PAC26   | ISDGPTDSYKQAVKLYAVL*          | 130   | 13   | 260   |    |
| PAC27   | ISDGPTDSYKQAVKLYAVL*          | 80    | 140  | 80    |    |
other amino acids. All amino acids were generally well tolerated for VPAC2 selectivity at positions 29 and 30. The most selective amino acids at positions 19 (Ala, Gly, Lys, Arg, and Ser) and 27 (Lys, Pro, Gln, and Arg) were recombined while fixing position 29 with arginine and 30 with isoleucine (see Table IV). Most of the resulting 20 mutant peptides were all highly selective for VPAC2.

**PACAP-scanning Mutagenesis**—To understand the structure-activity relationship of PACAP binding to its three receptors and identify novel VPAC2 selective mutations, we performed scanning mutagenesis of PACAP27. Because creating a more positively charged environment near the C terminus of VIP resulted in greater VPAC2 selectivity, we chose to substitute each neutral or acidic residue in PACAP27 with the basic amino acid lysine. Positions already represented by basic residues in the native PACAP27 were replaced with the acidic amino acid glutamic acid (see Table II). Adding a charge or reversing the charge at a position would have a more dramatic effect on activity than the traditional alanine-scanning mutagenesis and thus would more likely result in receptor selective agonists. As expected, these mutations were not well tolerated at most positions in PACAP27 with the exception of the C-terminal portion. Also, consistent with their ligand selectivity, the pattern of mutational effect was more conserved between PAC2 and PAC1 than PAC (Fig. 5). Charge changes at positions 1, 5, 12, and 16 affected PAC1 binding much more than VPAC1 and VPAC2. PAC13 and PAC22 displayed VPAC1 selectivity as the lysine mutations at these positions reduced VPAC2 and PAC1 binding much more than VPAC1. Lysine mutations at residues 9, 11, and 18 were much more disruptive than VPAC1 and VPAC2. PAC13 and PAC22 displayed VPAC1 selectivity as the lysine mutations at these positions reduced VPAC1 binding much more than VPAC1 and VPAC2. PAC13 and PAC22 displayed VPAC1 selectivity as the lysine mutations at these positions reduced VPAC1 binding much more than VPAC1 and VPAC2.

**Saturation mutagenesis of R3P65 to identify amino acids that will maintain VPAC2 selectivity at positions 19, 27, 29, and 30**

EC$_{50}$ is defined as the concentration of the peptide at which 50% of maximum PACAP27 cAMP accumulation in CHO cells transfected with the appropriate receptor is reached. Data are the mean of at least two experiments performed in triplicates. The standard errors were typically less than 30% of the mean. Ratio is a measure of selectivity, or preference for VPAC2 over VPAC1, and is derived from the ratio of EC$_{50}$ at VPAC1 versus that at VPAC2. NE depicts no peptide expression. Cysteine mutant peptides at all four positions also could not be expressed. None of these VIP-based peptides displayed any PAC1 binding or cAMP accumulation.

### Table III

| Amino Acids | Position 19 | | Position 27 | | Position 29 | | Position 30 |
|------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| VPAC2 EC$_{50}$ | VPAC1 EC$_{50}$ | Ratio | VPAC2 EC$_{50}$ | VPAC1 EC$_{50}$ | Ratio | VPAC2 EC$_{50}$ | VPAC1 EC$_{50}$ | Ratio |
| A           | 1000        | 1000        | 1           | 1000        | 1000        | 1           | 1000        | 1000        | 1 |
| D           | 0.51        | 15           | 30          | 0.3         | 20           | 70          | 0.4         | 40           | 100          | 0.2 |
| E           | 0.47        | 15           | 30          | 0.3         | 30           | 100         | 1           | 100          | 100          |
| F           | 0.2         | 10           | 50          | NE          | NE           | 50          | 0.37        | 20           | 50          | 0.07 |
| G           | 0.2         | 10           | 50          | NE          | NE           | 50          | 0.3         | 20           | 100         | 0.5 |
| H           | 0.2         | 10           | 50          | NE          | NE           | 50          | 0.3         | 20           | 100         | 0.5 |
| I           | 0.1         | 5            | 50          | 0.2         | 20           | 100         | 0.2         | 20           | 100         | 0.2 |
| K           | 0.2         | 100          | 500         | 0.2         | 6            | 30          | 0.11        | 20           | 200         |
| L           | 0.3         | 1            | 3           | 0.2         | 4            | 40          | 0.0         | 10           | 200         |
| M           | 0.1         | 2            | 20          | 0.19        | 3            | 15          | 0.16        | 6            | 30          | 0.18 |
| N           | 0.2         | 100          | 200         | 0.3         | 60           | 200         | 0.2         | 100          | 500         |
| O           | 0.2         | 100          | 500         | 0.2         | 100          | 500         | 0.1         | 50           | 500         |
| P           | 0.1         | 100          | 200         | 0.3         | 60           | 200         | 0.2         | 100          | 500         |
| Q           | 3           | 100          | 300         | 0.32        | 30           | 100         | 1           | 300          | 300         |
| R           | 0.1         | 4            | 40          | 0.4         | 40           | 80          | 0.17        | 60           | 300         |
| S           | 0.3         | 20           | 100         | 0.8         | 40           | 80          | 0.2         | 60           | 300         |
| T           | 0.3         | 10           | 70          | 0.8         | 40           | 80          | 0.2         | 60           | 300         |
| V           | 0.2         | 10           | 50          | 0.2         | 20           | 100         | 0.1         | 20           | 200         |
| W           | 0.6         | 300          | 500         | 0.09        | 8            | 80          | 0.4         | 80           | 200         |

### DISCUSSION

Understanding the structure-function relationship of PACAP and VIP with respect to their ability to recognize G-protein-coupled receptors PAC1, VPAC1, and VPAC2 is crucial to the study of their broad biological effects. Both peptides possess a low percentage of stable structure in aqueous solution but an increasing percentage of helicity as the ratio of organic co-solvent is increased (23-26). At ~50% organic solvent, residues 8-26 generally adopt a helical structure while the N and C termini remain highly flexible with little discernible structure. VIP analoging based on this structural information and alanine scanning (27) has been directed toward improving peptide stability and potency in BAL fluid for the treatment of asthma (21). Two of these analogs, Ro 25-1392 and Ro 25-1553, were later shown to be VPAC2 selective agonists (18, 20). As a therapeutic agent, however, the presence of N-terminal acylation, cyclization from Lys-21 to Asp-25, C-terminal amidation, and O-Me-Tyr-10 or Nle-17 on these analogs (see Fig. 1) precludes the possibility of recombinant production and may lead to manufacturability issues. In fact, several commercial vendors were requested to synthesize these analogs, but only a limited amount of Ro 25-1553 was successfully synthesized after multiple attempts, and none could make Ro 25-1392. Furthermore, these chemical modifications along with the large number of mutations may increase the probability of immune response from patients, thus preventing chronic treatment. Since none of these mutations were directed toward VPAC2 selectivity, we reasoned that only a subset of them may be required for receptor selectivity. Sequence alignment of PACAP, VIP, VPAC2-selective agonists Ro 25-1553 and Ro 25-1392, and VPAC1-selective agonist [Lys-15, Arg-16, Leu-27](VIP-(1-7)/GRF-(8–27) (28) implicates several potential VPAC2 selective determinants (see Fig. 1). Of the 11 mutations from VIP found in Ro 25-1553, Glu-8, Lys-12, and Leu-26 are highly conservative changes, and thus less likely to play a role in causing the loss of VPAC1 binding. The fact that Ro 25-1392 does not have the C-terminal GGT extension found in Ro 25-1553 suggests that the GGT sequence may not be necessary for VPAC2 selectivity. Of the remaining five mutations in Ro 25-
1553, Nle-17, Ala-19, and Asp-25 are also found in the VPAC1 selective agonist [Lys-15, Arg-16, Leu-27]VIP-(1–7)/GRF-(8–27). Because these three mutations do not prevent the VPAC1 agonist from binding tightly to VPAC1, they are probably not involved in VPAC2 selectivity. The minor modification of Y10 with a methyl group found in Ro 25-1553 is most likely not important for VPAC2 selectivity because Ro 25-1553 does not have this modification. Thus, Lys-27 and Lys-28, the remaining two mutations found in both VPAC2 selective analogs, are the most probable candidates to contribute to VPAC2 selectivity.

To understand the structure-activity relationship of VPAC2 selectivity of Ro 25-1553 and to generate novel VPAC2 selective agonists with the minimal number of mutations that can be produced in recombination, we performed several rounds of site-directed mutagenesis. All four chemical modifications found in Ro 25-1553 that would prevent recombinant expression were determined to be unnecessary for achieving high VPAC2 selectivity. A highly efficient recombinant expression system was then developed to allow a practical and rapid screen of hundreds of mutant peptides. Systematic amino acid replacement of Ro 25-1553 back to the VIP sequence revealed that only three out of the eleven mutations were needed to provide comparable VPAC2 selectivity as exhibited by Ro 25-1553. Two of the three mutations, L27K and N28K, were predicted by the sequence alignment analysis (see Fig. 1). Combining the L27K and N28K mutations with PACAP38 residues 29 and 30 greatly improved selectivity. Saturation and combinatorial mutagenesis of the resulting VIP mutant peptide containing the four key mutations, V19A, L27K, 29K, and 30R, yielded dozens of additional highly selective VPAC2 agonists.

Previous structure-activity relationship studies of the secretin/glucagon/VIP family have been limited to synthetic analogs as it has been difficult to generate fully functional peptides by recombinant means (16, 17). PACAP and VIP have been especially difficult to express because of the high number of basic amino acids, including two dibasic sites that may be particularly sensitive to proteolysis during expression. We have successfully expressed in E. coli PACAP and VIP mutant peptides using a fusion GST-Factor Xa recognition site peptide construct, which resulted in dramatically lower peptide degradation than direct expression of free PACAP and VIP (data not shown), presumably because of protection provided by the globular GST protein. The GST tag also permitted a one-step purification of the fusion protein and Factor Xa processing resulted in the release of free peptide without any extraneous sequence. The efficiency and ease of use of this expression and purification strategy permitted the generation of over 200 mutant peptides. Production and analysis of the large number of VIP and PACAP mutant peptides resulted in the identification of critical positions for conferring VPAC2 agonist selectivity as well as selection of specific amino acids within these positions that gave maximal selectivity.

A recent limited analysis of VPAC2 binding selectivity of Ro 25-1553 with several analogs of VIP and Ro 25-1553 (29) was

| Peptide | Mutant Sequences | VPAC2 EC50 | VPAC1 EC50 | Ratio |
|---------|-----------------|------------|------------|-------|
| R3P65   | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 0.6 ± 0.1  | 80 ± 9     | 130   |
| R3P174  | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 0.2 ± 0.1  | 18 ± 4     | 90    |
| R3P175  | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 2 ± 0.8    | 400 ± 100  | 200   |
| R3P176  | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 2 ± 1      | 300 ± 100  | 150   |
| R3P177  | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 1.6 ± 0.9  | 110 ± 40   | 70    |
| R3P178  | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 0.8 ± 0.4  | 80 ± 50    | 100   |
| R3P179  | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 1.5 ± 0.9  | 230 ± 80   | 150   |
| R3P180  | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 7 ± 3      | >100       | >15   |
| R3P181  | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 5 ± 1      | 300 ± 30   | 60    |
| R3P182  | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 3 ± 0.6    | 300 ± 130  | 100   |
| R3P183  | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 5 ± 0.4    | >150       | >30   |
| R3P184  | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 0.37 ± 0.1 | 10 ± 1     | 30    |
| R3P185  | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 4.5 ± 1.3  | 200 ± 20   | 40    |
| R3P186  | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 1.6 ± 0.2  | 70 ± 10    | 40    |
| R3P187  | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 1.6 ± 0.1  | >130       | >80   |
| R3P188  | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 2.2 ± 0.1  | 150 ± 50   | 70    |
| R3P189  | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 0.04 ± 0.01| 1.3 ± 0.6  | 30    |
| R3P190  | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 2 ± 0.6    | 200 ± 100  | 100   |
| R3P191  | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 3 ± 0.4    | >200       | >70   |
| R3P193  | HSDAVFTDNYTRLRKQMAKKKYNLNSLKR | 0.6 ± 0.1  | 40 ± 20    | 70    |

**TABLE IV**

Combinatorial mutagenesis of R3P65 at positions 19, 27, 29, and 30

EC50 is defined as the concentration of the peptide at which 50% of maximum PACAP27 cAMP accumulation in CHO cells transfected with the appropriate receptor is reached. Data are the mean ± S.E. of at least three experiments performed in triplicates. Ratio is a measure of selectivity, or preference for VPAC2 over VPAC1, and is derived from the ratio of EC50 at VPAC1 versus that at VPAC2. None of these VIP-based peptides displayed any PAC1 binding or activation.
Structure-Activity Relationship of VPAC2 Selective Agonists

generally consistent with our current findings, but our ability to analyze hundreds of mutant peptides using recombinant expression allowed us to fully analyze VPAC2 selectivity. For example, Moreno et al. (29) concluded that the Ala-19 mutation did not contribute to VPAC2 selectivity based on the lack of selectivity exhibited by the single V19A mutant peptide of VIP. Even though we also detected no selectivity by this mutant (R3P47), it clearly contributed to VPAC2 selectivity in the presence of the double mutant peptide Lys-27 and Lys-28 (see R3P41 versus R3P51, in Table I). Likewise, the loss of the Ala-19 mutation from R3P12 significantly reduced VPAC2 selectivity (see R3P21 versus R3P12 in Table I). Another example of the context dependence of mutational effects can be found in the conservative mutations E8D and L26I, which had little effect on VPAC2 selectivity in the context of R3P3 (see R3P3 versus R3P12 in Table I). However, in the context of R3P8, these mutations reduced VPAC2 selectivity (see R3P7 versus R3P8 in Table I). Furthermore, although VIP and PACAP share 68% of their sequence and two of three PACAP receptors, the same sets of mutations that resulted in VPAC2 selectivity in one sequence did not do the same in the other. The Lys-27 and Lys-28 mutations that contributed to VPAC2 selectivity for VIP did little for PACAP (see R3P6, R3P17, R3P37, to R3P40 in Table II). Likewise, the R12E or K15E mutation resulted in VPAC2 selectivity for PACAP but not for VIP (see PAC12 or PAC15 in Table II versus R3P44 or R3P45 in Table I).

One of our most surprising results was that grafting the KRY sequence from PACAP38 residues 29–31, a peptide with no VPAC2 selectivity, onto the VIP-based sequence greatly enhanced VPAC2 selectivity (see R3P36 versus R3P12 in Table I). Bolin et al. (21) added the GGT sequence at positions 29 to 31 to more effectively cap the helix and thus create a more stable peptide. Thus, a change in conformation induced by GGT may favor VPAC2 binding over VPAC1. Alternatively, the GGT sequence simply shifted the negatively charged C-terminal carboxylate three residues away from the original position of the 28-mer VIP, which may have removed a favorable VPAC1-specific electrostatic interaction at position 28. This increase in electrostatic potential at position 28 could also have been accomplished by C-terminal amidation, which in effect removed the C-terminal negative charge. Our data support the hypothesis of VPAC2 selectivity being favored by the elevated C-terminal electrostatic potential because grafting the basic KRY sequence from PACAP38 further reduced activity at VPAC1.

Saturation mutagenesis of VPAC2 not only identified the amino acids that favor VPAC2 selectivity at the four key positions identified in R3P65, it also supported the structural model of VIP. The dramatic loss of VPAC1 and VPAC2 activation caused by proline replacement at position 19 of R3P65 is consistent with the importance of maintaining the helical structure for its function since proline has been characterized as a "helix breaker" (30). On the other hand, proline did not significantly affect VPAC2 selectivity and potency at positions 27, 29, and 30 presumably because these residues fall outside of the predicted helical region (23–26). Most of the mutant peptides from saturation mutagenesis at positions 29 and 30 possessed at least 3-fold greater VPAC2 selectivity than R3P49 (V19A/L27K-VIP), which differs from R3P65 in not having the two amino acid extension. Basic amino acids lysine and arginine gave the greatest VPAC2 selectivity at position 29 (see R3P65 in Table I and Position 29 x R in Table III), again supporting the electrostatic potential hypothesis.

Our attempts at generating VPAC2 selective agonists by introducing mutations contained within the Ro 25-1553 peptide into the PACAP sequence did not result in significant VPAC2 selectivity, but instead yielded lead sequences for PAC1 selective agonists. Consistent with the results from VIP alanine-scanning mutagenesis (13–15), our results from the charge-scanning mutagenesis of PACAP27 indicate that the N-terminal region of the peptide is critical for binding to all three receptors. Since charge substitutions typically introduce more dramatic changes to the physical properties of peptides than alanine substitutions, most of the charge mutations within the midregion of PACAP27, which affected binding, can be explained on this basis. Only the C-terminal region appeared to tolerate these charge mutations, suggesting that it is not involved in the conformational stability of the peptide.

VPAC2 selectivity has been identified as a prerequisite for potential type 2 diabetes treatment using VIP- or PACAP-based peptides (5). VIP chemical analogs Ro 25-1553 and Ro 25-1392 have been reported as VPAC2 selective agonists (18, 20), but they were not designed to be selective for VPAC2 so that VPAC2 selective determinants were not known (21). In the current work, we have taken a systematic approach to identify the minimal VPAC2 selective determinants necessary to confer maximal VPAC2 selectivity. To accomplish this, we performed a comprehensive structure-function analysis of the interaction between VIP and PACAP with their receptors. The sequence alignment analysis (see Fig. 1) was based on the hypothesis that the VPAC2-selective peptide with a minimum number of mutations will have a sequence somewhere in between that of the native peptide VIP or PACAP and Ro 25-1553. Thus, we performed sequential mutagenesis from two directions, starting from both Ro 25-1553 and VIP. After several rounds of mutagenesis, we were able to reduce the number of potential VPAC2 selective mutations from eleven to three. In the process of deciphering the role of the C-terminal GGT sequence of Ro 25-1553, we demonstrated that further increasing the electrostatic potential at the C terminus resulted in greater VPAC2 selectivity. Manipulation of the electrostatic potential was also applied in the charge-scanning mutagenesis of PACAP27 to yield additional VPAC2 selective determinants. In addition, charge-scanning mutagenesis implicated that the C-terminal region of PACAP27 was not involved in receptor binding. Saturation mutagenesis of positions 19, 27, 29, and 30 of R3P65 supported the structure model of VIP and revealed amino acids that contribute to VPAC2 selectivity at each position. Our wide ranging structure-activity analysis of VIP and PACAP resulted in the identification of mutations that provide several hundred-fold selectivity for VPAC2 over the other two receptors recognized by VIP or PACAP. These mutant peptides exhibit biological properties and potency that fulfill therapeutic criteria necessary for the treatment of Type II Diabetes. With the increased application of robotics, fully automated high throughput mutagenesis, expression, purification, analytics, and functional assay may be possible in the near future. This powerful recombinant mutagenesis system should be adaptable to detailed structure-function analysis of other peptides.

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REFERENCES
1. Miyata, A., Arimura, A., Dahl, R. R., Minamino, N., Uehara, A., Jiang, L., Culler, M. D., and Coy, D. H. (1989) Biochem. Biophys. Res. Commun. 164, 567–574
2. Sherwood, N. M., Krueckl, S. L., and McRory, J. E. (2000) Endocr. Rev. 21, 619–670
3. Laburthe, M., and Coveyrou, A. (2002) Regul. Pept. 108, 165
4. Goeze, I., Fridkin, M., Hill, J. M., and Brenneman, D. E. (1999) Curr. Med. Chem. 6, 1019–1034
5. Tsuzuki, T., Claus, T. H., Liang, Y., Li, Y., Yang, L., Zhu, J., Dela Cruz, F., Peng, X., Chen, H., Yung, S. L., Hamren, S., Livingston, J. N., and Pan, C. Q. (2002) Diabetes 51, 1453–1460

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6. Gourlet, P., Vandermeers, A., Vandermeers-Piret, M. C., Rathe, J., De Neef, P., and Robberecht, P. (1995) *Eur. J. Pharmacol.* **287**, 7–11
7. Gourlet, P., Vandermeers, A., Vandermeers-Piret, M. C., Rathe, J., De Neef, P., and Robberecht, P. (1996) *Regul. Pept.* **62**, 125–130
8. Ando, E., Nokihara, K., Naruse, S., and Wray, V. (1996) *Biomed. Pept. Proteins Nucleic Acids* **2**, 41–46
9. Kashimoto, K., Nagano, Y., Suitani, Y., Hamanaka, K., Mizumoto, T., Tomizaki, K., Takahata, H., Nagamoto, A., Ohata, A., Yoshihara, S., and Ichimura, T. (1996) *Ann. N. Y. Acad. Sci.* **805**, 505–510
10. Gourlet, P., Vandermeers, A., Vandermeers-Piret, M. C., De Neef, P., Waelbroeck, M., and Robberecht, P. (1996) *Biochim. Biophys. Acta* **1314**, 267–273
11. Gourlet, P., Vandermeers, A., Vandermeers-Piret, M. C., De Neef, P., Waelbroeck, M., and Robberecht, P. (1997) *Peptides* **18**, 403–408
12. Gourlet, P., Vandermeers, A., Vandermeers-Piret, M. C., De Neef, P., Cnudde, J., Waelbroeck, M., and Robberecht, P. (1997) *Eur. J. Pharmacol.* **348**, 95–99
13. Nicole, P., Lins, L., Rouyer-Fessard, C., Drouot, C., Fulcrand, P., Thomas, A., Covineau, A., Martinez, J., Brasseur, R., and Laburthe, M. (2000) *J. Biol. Chem.* **275**, 24003–24012
14. Igarashi, H., Ito, T., Hou, W., Mantey, S. A., Pradhan, T. K., Ulrich, C. D., 2nd, Hecart, S. J., Coy, D. H., and Jensen, R. T. (2002) *J. Pharmacol. Exp. Ther.* **301**, 37–50
15. Igarashi, H., Ito, T., Pradhan, T. K., Mantey, S. A., Hou, W., Coy, D. H., and Jensen, R. T. (2000) *J. Pharmacol. Exp. Ther.* **303**, 445–460
16. Simonsen, A., Tjernhammar, M. L., Kalman, M., Cserpan, I., Gaselin, G., and Bartfai, T. (1988) *Eur. J. Biochem.* **178**, 343–350
17. Raingeaud, J., Lavergne, F., Lelièvre, V., Muller, J. M., Julien, R., and Cenatiempo, Y. (1996) *Biochimie (Paris)* **78**, 14–25
18. Gourlet, P., Vertongen, P., Vandermeers, A., Vandermeers-Piret, M. C., Rathe, J., De Neef, P., Waelbroeck, M., and Robberecht, P. (1997) *Peptides* **18**, 463–468
19. Picard, V., Ersdal-Badju, E., Lu, A., and Bock, S. C. (1994) *Nucleic Acids Res.* **22**, 2587–2591
20. Xia, M., Streedharan, S. P., Bolin, D. R., Gaufo, G. O., and Goetzl, E. J. (1997) *J. Pharmacol. Exp. Ther.* **281**, 629–633
21. Bolin, D. R., Michalewsky, J., Wasserman, M. A., and O'Donnell, M. (1995) *Biopolymers* **37**, 57–66
22. Richardson, J. S., and Richardson, D. C. (1988) *Science* **240**, 1648–1652
23. Fry, D. C., Madison, V. S., Bolin, D. R., Gleeley, D. N., Toome, V., and Wegryn, B. B. (1989) *Biochemistry* **28**, 2389–2409
24. Theriault, Y., Boulanger, Y., and St-Pierre, S. (1991) *Biopolymers* **31**, 459–464
25. Inooka, H., Endo, S., Kitada, C., Mizuta, E., and Fujino, M. (1992) *Int. J. Pept. Protein Res.* **40**, 456–464
26. Wray, V., Rakoschke, C., Nokihara, K., and Naruse, S. (1993) *Biochemistry* **32**, 5832–5841
27. O’Donnell, M., Garippa, R. J., O’Neill, N. C., Bolin, D. R., and Cottrell, J. M. (1991) *J. Biol. Chem.* **266**, 6389–6392
28. Gourlet, P., Vandermeers, A., Vertongen, P., Rathe, J., De Neef, P., Cnudde, J., Waelbroeck, M., and Robberecht, P. (1997) *Peptides* **18**, 1539–1545
29. Moreno, D., Gourlet, P., De Neef, P., Cnudde, J., Waelbroeck, M., and Robberecht, P. (2000) *Peptides* **21**, 1543–1549
30. O’Neil, K. T., and DeGrado, W. F. (1990) *Science* **250**, 646–651
Generation of Highly Selective VPAC2 Receptor Agonists by High Throughput Mutagenesis of Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-activating Peptide

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