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Mutant Constructs of the β-Adrenergic Receptor that Are Uncoupled from Adenylyl Cyclase Retain Functional Activation of Na-H Exchange

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SUMMARY

β-Adrenergic receptor (βAR) agonists modulate a number of intracellular effectors; for example, they stimulate adenylyl cyclase and Ca^{2+} channels, inhibit Na^+ channels and Mg^{2+} efflux, and activate Na-H exchange. Regulation of adenylyl cyclase, Ca^{2+}, Na^+, and Mg^{2+} by the βAR is mediated through receptor coupling to the GTP-binding protein G_s. We have previously determined, however, that βAR stimulation of Na-H exchange occurs independently of receptor coupling to G_s. In the present study, we analyzed mutant βARs containing deletions of amino acid residues within the third cytoplasmic domain, to determine whether there is a structural basis for the ability of the βAR to couple divergently to the G_s-dependent stimulation of adenylyl cyclase and the G_s-independent activation of Na-H exchange. Receptor constructs with deletions of residues 222–229 and 258–270, which were previously shown to be defective in coupling to G_s and adenylyl cyclase, retained an isoproterenol-induced activation of Na-H exchange that was similar in time course and magnitude to that observed with the wild-type βAR. These results confirm our previous findings that the βAR activates Na-H exchange independently of G_s, and they further suggest that distinct molecular determinants of the receptor divergently stimulate adenylyl cyclase and Na-H exchange.

βARs are members of a family of receptor proteins that mediate their cellular actions through activation of G proteins. The most widely studied effector of βAR action is the enzyme adenylyl cyclase, which is stimulated by the interaction of the βAR with the G protein G_s. Besides stimulating adenylyl cyclase activity and increasing intracellular cAMP, the βAR also modulates the levels of other intracellular second messengers. Activation of the βAR stimulates the opening of voltage-dependent Ca^{2+} channels (1, 2), inhibits the activity of voltage-dependent Na^+ channels (3), and inhibits Mg^{2+} efflux (4). Although the modulation of Ca^{2+}, Na^+, and Mg^{2+} fluxes by the βAR may occur independently of changes in intracellular cAMP, regulation of these ion fluxes requires receptor coupling to G_s.

We have recently determined that the βAR also stimulates activity of the NaH-1 exchanger, resulting in a cytoplasmic alkalinization in a HEPES buffer (5). Receptor coupling to Na-H exchange is observed in multiple cell types that endogenously express the βAR (5, 6) and in mouse L cells that have been transfected with either the wild-type hamster β_2AR (6), the human β_1AR,[1] or the turkey erythrocyte βAR (6). Like βAR-mediated regulation of ion channels, βAR activation of Na-H exchange occurs independently of changes in cAMP production. Unlike ion channel regulation, however, activation of Na-H exchange by βAR agonists is not mediated by G_s. Stimulation of Na-H exchange by the βAR is insensitive to cholera toxin (5, 6) and is observed in *cyc*$^-$ variants of S49 lymphoma cells (5), a clonal line that does not express the α subunit of G_s (7, 8). Together, these data indicate that the βAR activates the NaH-1 exchanger through a G_s-independent pathway.

In the present study, we used a molecular genetic approach to characterize further the ability of the βAR to stimulate adenylyl cyclase and Na-H exchange divergently. We found that mutant receptors containing deletions within the third cytoplasmic domain, which rendered them defective in activating the G_s-adenylyl cyclase system, nevertheless retained functional activation of Na-H exchange. These results confirm our previous findings that the βAR activates Na-H exchange in-

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[1] D. L. Barber and C. D. Strader, unpublished observations.

ABBREVIATIONS: βAR, β-adrenergic receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BCECF, 2,7-biscarboxyethyl-5(6)-carboxyfluorescein; CYP, cyanopindolol; G protein, guanine nucleotide-binding protein; EIPA, ethylisopropylamiloride; pH_i, intracellular pH; PGE_1, prostaglandin E_1; AM, acetoxymethyl ester.

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dependently of receptor coupling to G_s, and they identify that there is a molecular basis for divergent signaling by the receptor, in that distinct structural determinants of the receptor independently activate adenylyl cyclase and the NaH-1 exchanger.

Materials and Methods

Mutagenesis and expression. The cloning, expression, and oligonucleotide-directed mutagenesis of the hamster βAR have been described (9, 10). The ligand-binding characteristics and activation of adenylyl cyclase by the mutant receptors used in this study were reported previously (11). Mutant receptors were expressed in L cells as previously described, using the expression vector pSVL, and cell lines expressing the receptors were selected by resistance to G-418 (10). Cells were grown in Dulbecco's modified Eagle's medium containing 1 mg/ml G-418 and 10% fetal calf serum, in 5% CO_2. For pH_i determinations, cells were subcultured on glass coverslips and maintained in culture for 3–4 days before the experiment.

pH_imeasurements. Na-H exchange activity was determined by monitoring pH_i, using the fluorescent pH-sensitive dye BCECF. Cells plated on coverslips were loaded with 5 μM BCECF-AM for 10 min at 37°, washed, and placed in a cuvette maintained in a thermostatically controlled cuvette holder within a Shimadzu RF5000 spectrofluorometer. Fluorescence measurements were made by exciting the dye alternately at 440 and at 500 nm, with a constant emission at 530 nm. The BCECF excitation ratio was calibrated by the high-K^+/nigericin technique (12), and pH_i was determined with a conversion program developed by G. Boyarsky (University of Texas, Galveston). The rate of alkalinization (dpH_i/dt) was determined by taking the first derivative of the slope of the pH_i tracing between 30 and 60 sec after the onset of alkalinization. Data represent the mean ± standard error of the indicated number of determinations. Statistical significance was analyzed by use of the unpaired Student's t test. pH_i determinations were made in a nominally HCO_3^--free, HEPES-buffered solution containing (in mM) 145 sodium, 5 potassium, 150 chloride, 1.0 magnesium, 1.8 calcium, 1.0 SO_4, 1.0 PO_4, 10 glucose, and 32.2 HEPES, titrated to a pH of 7.4. *N*-Methyl-D-glucamine was used to replace Na^+ in determinations made in the absence of Na^+.

cAMP determination. Intracellular cAMP was measured in cells maintained for 3–4 days in 16-mm wells, as previously described (5, 13). Cells were incubated in either the absence or the presence of isoproterenol or isoproterenol plus propranolol. All incubations contained 10 μM 3-isobutyl-1-methylxanthine. cAMP was quantitated by radioimmunoassay (Biomedical Technologies, Cambridge, MA), and results were expressed as pmol of cAMP/16-mm well.

Radioligand binding assays. Receptor density was determined by whole-cell radioligand binding. Cells maintained for 4 days in 16-mm wells were washed with Hanks' balanced salt solution, supplemented with 0.1% bovine serum albumin, and 2 mM glutamine. Cells were then incubated in Hanks' containing 100 pM ^{125}I-CYP, in the absence (total binding) and the presence of 1 μM propranolol (nonspecific binding). Incubations were for 120 min at 37°. The reactions were stopped by washing cells with Hanks' containing 1 μM propranolol. Cells were lysed in 1 N NaOH, and the bound ^{125}I-CYP was quantified using a γ counter. Results are expressed as fmol of CYP binding/10^6 cells and represent the mean ± standard error of the indicated number of separate cell passages.

Materials. BCECF-AM was obtained from Molecular Probes (Eugene, OR), and ^{125}I-CYP was purchased from New England Nuclear (Boston, MA). EIPA was a gift of Dr. Burchard (Frankfurt, Germany).

Results

Regions of the βAR that are involved in activating G_s and adenylyl cyclase have been identified by genetic and biochemical analysis. Secondary structural prediction suggests that the βAR, like other members of the G protein-coupled receptor family, consists of seven transmembrane helices, connected by alternating extracellular and cytoplasmic hydrophilic domains (Fig. 1). Deletion mutagenesis has implicated the regions at the ends of the third intracellular domain of the receptor, adjacent to the fifth and sixth transmembrane domains, in the interaction with G_s (11). The deletion of eight amino acid residues from the amino terminus of this domain results in a mutant βAR [(D222–229)βAR] that is uncoupled from G_s and does not activate adenylyl cyclase, whereas the deletion of 12 amino acids from the carboxyl terminus of this domain [(D258–270)βAR] markedly attenuates G_s-dependent activation. In contrast, deletions of similar size from the central portion of this region of the receptor do not affect the ability of the receptor to couple to G_s and activate adenylyl cyclase. Molecular replacement studies of other G protein-coupled receptors have also demonstrated the involvement of regions within the third intracellular domain in defining the selectivity of the interaction between receptors and G proteins (14–16).

To determine whether similar molecular determinants of the βAR are required for functional coupling to Na-H exchange, we studied transfected L cells expressing mutant receptors having a series of deletions within the third intracellular domain of the protein. The coupling of these mutant receptors to G_s and to the activation of adenylyl cyclase has been characterized previously (11). Wild-type L cells do not express detectable levels of the βAR and do not activate adenylyl cyclase in response to the βAR agonist isoproterenol (10). These wild-type L cells had a resting pH_i of 7.10 ± 0.05 and showed no change in pH_i in response to 1 μM isoproterenol (five experiments) (Fig. 2). L cells stably transfected with the wild-type hamster lung β_2AR expressed 12.6 ± 2.1 fmol of CYP binding sites/10^6 cells (four experiments). Addition of isoproterenol (1 μM) to $L_1(\beta AR)_{WT}$ resulted in an alkalinization of resting pH_i from 7.08 ± 0.04 to 7.19 ± 0.03. Maximal alkalinization occurred within 12.81 ± 0.83 min, and the initial rate of alkalinization (dpH_i/dt) was 2.67 ± 0.18 × 10^{-4} pH units/sec (six

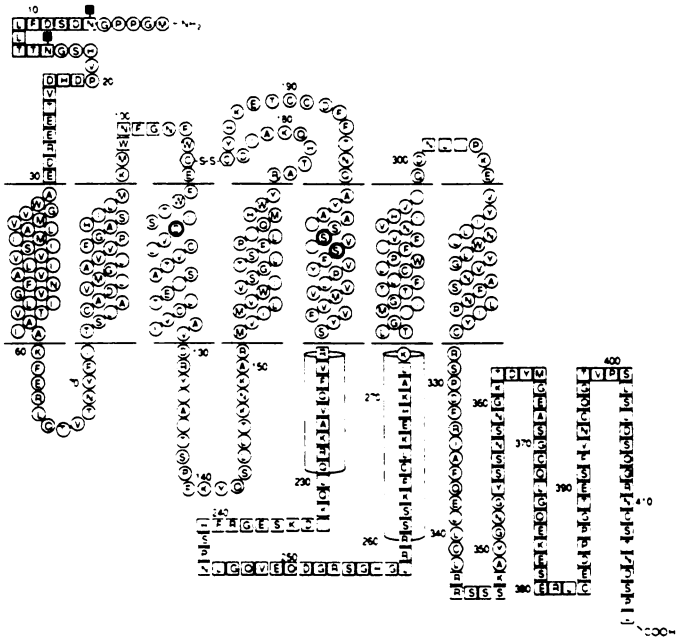

Fig. 1. Schematic diagram of the hamster lung β_2AR.

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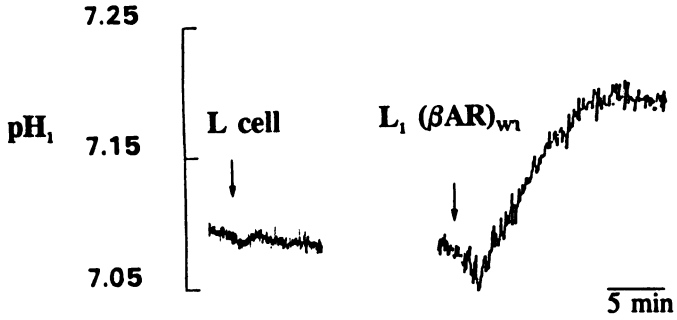

Fig. 2. Effect of isoproterenol on pH_i in cells expressing βAR. Cells were loaded with BCECF-AM in a HEPES buffer and analyzed as described in Materials and Methods. Isoproterenol (1 μM) was added, at the *arrows*, to control L cells (*left*) and L cells expressing the wild-type βAR (L_1) (*right*).

experiments) (Fig. 2; Table 1). The alkalinization in response to isoproterenol was inhibited by propranolol (10 μM) and was not observed in the absence of extracellular Na^+ or the presence of EIPA (50 μM), a relatively selective inhibitor of Na-H exchange (data not shown). These results indicate that the isoproterenol-induced alkalinization was mediated by activation of Na-H exchange.

To identify potential molecular determinants of the βAR that may be required for activating Na-H exchange, we first studied a receptor construct containing a relatively large deletion (residues 239–273) in the third cytoplasmic domain. L cells expressing the receptor construct [D(239–272)βAR] had 18.2 ± 2.4 fmol of CYP binding sites/10^6 cells. The resting pH_i in these cells was 7.04 ± 0.05. After the addition of isoproterenol, however, no change in steady state pH_i was observed (12 experiments) (Fig. 3; Table 1). We previously determined that L cells have an endogenously expressed PGE_1 receptor that is divergently coupled to the activation of adenylyl cyclase and Na-H exchange (6). In L cells expressing D(239–272)βAR, although we were unable to observe a pH_i change in response to isoproterenol, in the presence of PGE_1 (100 nM) resting pH_i increased from 7.07 ± 0.06 to 7.20 ± 0.09 (nine experiments) (Fig. 3). The PGE_1-induced alkalinization was dependent on extracellular Na^+ and was not observed after pretreatment with EIPA (data not shown). Hence, activation of a receptor coupled to adenylyl cyclase in these cells was still able to stimulate Na-H exchange.

Because D(239–272)βAR appeared to be uncoupled from Na-H exchange, we studied receptor constructs having smaller deletions (8–14 amino acids in length) that completely spanned the 239–272 segment, to identify more precisely the amino acid residues critical for activating the exchanger. A total of five additional constructs were tested for functional coupling to Na-H exchange. In contrast to the D(239–272)βAR, however, each of these additional mutant receptors retained an isoproterenol-induced (1 μM) alkalinization (Fig. 4; Table 1). For each of these mutants, the alkalinization in response to isoproterenol was not observed in the absence of extracellular Na^+ or the presence of EIPA (50 μM) (data not shown) and was reversed after the addition of propranolol (Fig. 4). In several pH_i determinations using receptor constructs containing deletions of 8–14 amino acids, the onset of alkalinization after the addition of isoproterenol was 30–60 sec slower than that observed with the wild-type receptor. We are currently uncertain whether this delayed alkalinization has biological significance or was due to a time lag in agonist mixing within the cuvette. Our findings, however, indicate that the delay is not restricted to either mutant receptors or receptors uncoupled from G_s. Similar delays in alkalinization have been observed after activation of PGE_1 and parathyroid hormone receptors (6), and we observed delays with βAR constructs that were both coupled to G_s [D(250–259)βAR] and uncoupled from G_s [D(222–229)βAR]. When the means of 5–12 determinations for each construct were evaluated, however, the maximal change in pH_i (0.10–0.12 units), the time required to attain maximal alkalinization (11.95–13.14 min), and the initial rate of alkalinization (2.62–2.88 × 10^{-4} pH units/sec) for the five mutant constructs were not significantly different from those observed with the wild-type βAR (Table 1). Of particular interest was the finding that cells expressing mutant receptors that were previously identified as being poorly coupled to or uncoupled from G_s and adenylyl cyclase [D(258–270)βAR and D(222–229)βAR, respectively] had a resting pH_i, maximal pH_i change, and initial alkalinization rate (dpH_i/dt) that were not significantly different from those of receptor constructs functionally coupled to adenylyl cyclase, including the wild-type βAR, D(229–236)βAR, D(238–251)βAR, and D(250–259)βAR ($p > 0.2$) (Table 1).

To confirm that D(222–229)βAR was, in fact, uncoupled from adenylyl cyclase under the conditions in which activation of Na-H exchange was observed, we determined the effect of isoproterenol on intracellular cAMP in three independent cell passages (Fig. 5). In cells expressing D(250–259)βAR, used as a positive control, isoproterenol induced a dose-dependent increase in cAMP accumulation, with an apparent EC_{50} of 42 ±

TABLE 1

Effect of isoproterenol on pH_i

Cells expressing the indicated constructs of the hamster lung β_2AR were loaded with BCECF-AM, and pH_i was determined as described in Materials and Methods. Rates of alkalinization (dpH_i/dt) were determined between the initial 30 and 60 sec after the onset of alkalinization. t_{max} represents the time required to reach maximal alkalinization after the addition of isoproterenol. Receptor density (fmol of ^{125}I-CYP binding sites) was determined in intact cells.

Receptor construct	Resting pH_i	ΔpH_i	Alkalinization rate, dpH_i/dt (× 10^{-4})	t_{max}	^{125}I-CYP binding sites	Adenylyl cyclase stimulation[a]
			pH units/Sec	*min*	*fmol/10^6 cells*	*fold*
βAR_{WT} (6)[b]	7.08 ± 0.04	0.11 ± 0.01	2.68 ± 0.18	12.81 ± 0.83	12.6 ± 2.1 (4)	3.2
D(239-272) (12)	7.04 ± 0.05	<0.02			18.2 ± 2.4 (4)	1.0
D(222-229) (12)	7.05 ± 0.04	0.11 ± 0.01	2.73 ± 0.38	12.49 ± 0.86	25.6 ± 1.8 (6)	1.0
D(229-236) (5)	7.04 ± 0.02	0.12 ± 0.02	2.68 ± 0.17	12.02 ± 1.26	31.6 ± 2.26 (3)	3.1
D(238-251) (5)	7.10 ± 0.06	0.12 ± 0.02	2.88 ± 0.37	12.96 ± 0.91	28.7 ± 3.83 (3)	6.3
D(250-259) (7)	7.04 ± 0.04	0.10 ± 0.01	2.76 ± 0.27	11.95 ± 1.25	22.9 ± 1.67 (4)	3.0
D(258-270) (6)	7.08 ± 0.02	0.10 ± 0.01	2.62 ± 0.30	13.14 ± 1.06	7.8 ± 1.02 (3)	1.3

[a] Results from Ref. 11 on the ability of isoproterenol (1 μM) to activate adenylyl cyclase in cells expressing each of the receptor constructs.
[b] Numbers in parentheses are numbers of experiments.

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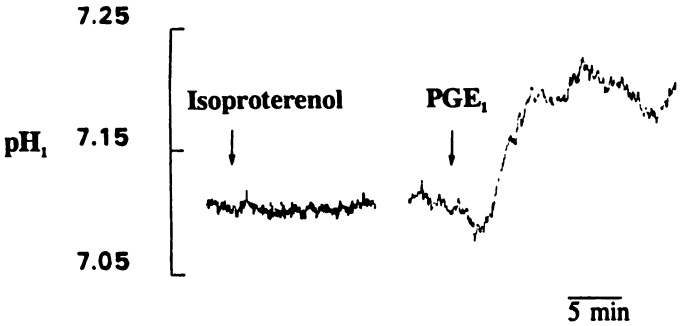

Fig. 3. Effect of isoproterenol and PGE_1 on pH_i in cells expressing D(239–272)βAR. Time course of pH_i after the addition of isoproterenol (1 μM) (*left*) or PGE_1 (100 nM) (*right*), at the *arrows*, to L cells expressing a mutant βAR containing a deletion of amino acid residues 239–272.

3 nM, similar to the value previously reported for isoproterenol activation of adenylyl cyclase (11). The response to isoproterenol in these cells was competitively inhibited by propranolol (10 μM). In contrast, isoproterenol (10^{-9} to 10^{-5} M) had no effect on cAMP accumulation in cells expressing D(222–229)βAR.

Discussion

Previous work from this laboratory has demonstrated that agonist stimulation of the βAR activates Na-H exchange, resulting in an intracellular alkalinization in a HEPES buffer. Two lines of evidence suggest that this signaling event proceeds through a pathway that does not involve stimulation of the G protein G_s. First is the observation that treatment of cells with cholera toxin, which activates G_s, decreases the EC_{50} for βAR agonist-induced cAMP accumulation, without altering the dose-response curve for βAR stimulation of Na-H exchange (5, 6). Second, βAR activation of Na-H exchange is observed in *cyc*$^-$ variants of S49 lymphoma cells (5), a mutant cell line lacking a functional G_s (7, 8).

In the present study, we analyzed mutant βARs containing deletions of amino acid residues within the third cytoplasmic domain, to determine whether the receptor molecule itself divergently couples to adenylyl cyclase and Na-H exchange. We observed that two mutant constructs of the β_2AR that are unable to stimulate G_s and increase intracellular cAMP levels retained full functional coupling to Na-H exchange. D(222–229)βAR was previously shown not to couple to the G_s-adenylyl cyclase system when expressed in L cells, as determined both by the absence of a GTP-mediated decrease in agonist affinity and by the absence of any stimulation of cAMP accumulation upon exposure to adrenergic agonists (11). This absence of an effect on cAMP accumulation was confirmed in the present study. D(258–270)βAR has been determined to be poorly coupled to the G_s-adenylyl cyclase pathway; cAMP levels stimulated by this mutant receptor are markedly attenuated, and GTP has only a small effect on the affinity of adrenergic agonists for the receptor (11). Neither of these mutations in the βAR had any effect on its ability to activate Na-H exchange, as assessed by dpH_i/dt and maximal pH_i increase. These results confirm our previous findings that the βAR activates Na-H exchange independently of receptor coupling to G_s. More importantly, the current results indicate that there is a structural basis for the divergent signaling of the βAR and that distinct molecular determinants may activate adenylyl cyclase and Na-H exchange.

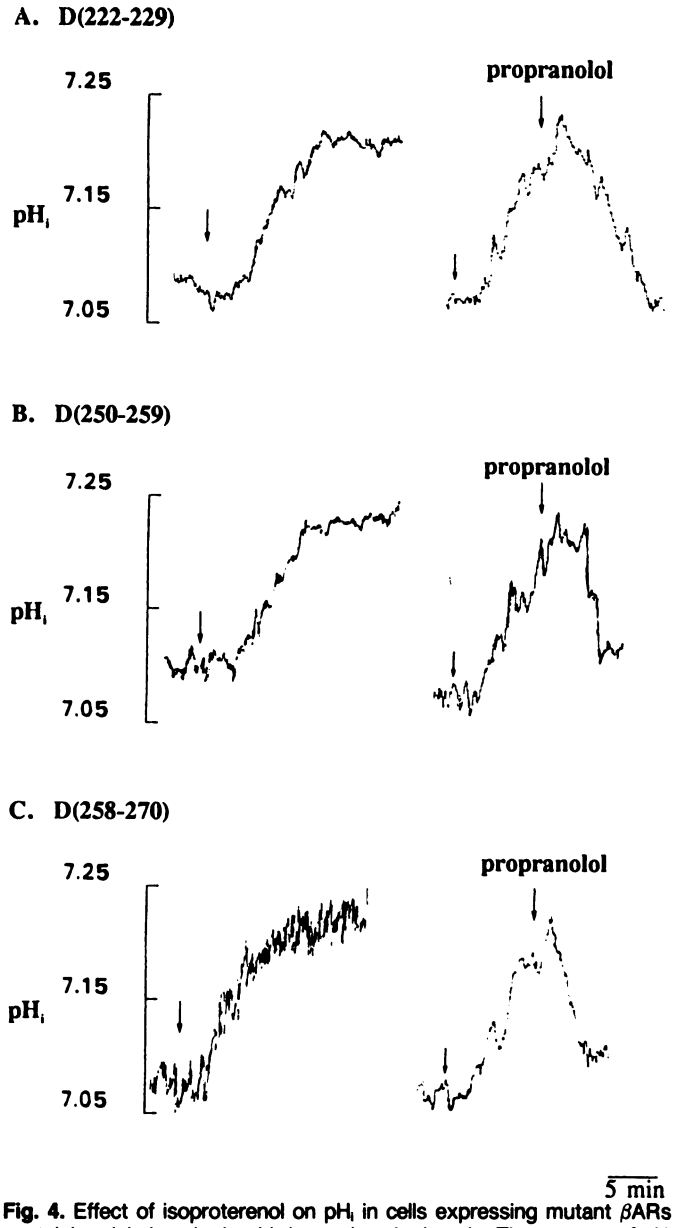

Fig. 4. Effect of isoproterenol on pH_i in cells expressing mutant βARs containing deletions in the third cytoplasmic domain. Time course of pH_i after the addition of isoproterenol (1 μM), at the *arrows*, to cells expressing D(222–229)βAR (A), D(250–259)βAR (B), or D(258–270)βAR (C). *Left*, effect of isoproterenol alone. *Right*, ability of propranolol (10 μM) to reverse the isoproterenol-induced alkalinization. Tracings are representative of 5–12 determinations.

An exception to this separation in signaling is the concurrent loss of coupling to Na-H exchange and adenylyl cyclase with receptor construct D(239–272)βAR. For adenylyl cyclase stimulation, the structural determinants within this region could be further mapped by the construction of smaller deletion mutations, as described above. In marked contrast, this nestled deletion approach failed to reveal a smaller portion of the 239–272 region that is important for activation of the Na-H exchange pathway. Mutants D(238–251)βAR, D(250–259)βAR, and D(258–270)βAR completely span the region removed from the βAR with the larger deletion, D(239–272)βAR. The ability of all three of the mutant receptors with smaller deletions within this region to activate Na-H exchange indicates that none of the individual amino acids within the sequence 238–

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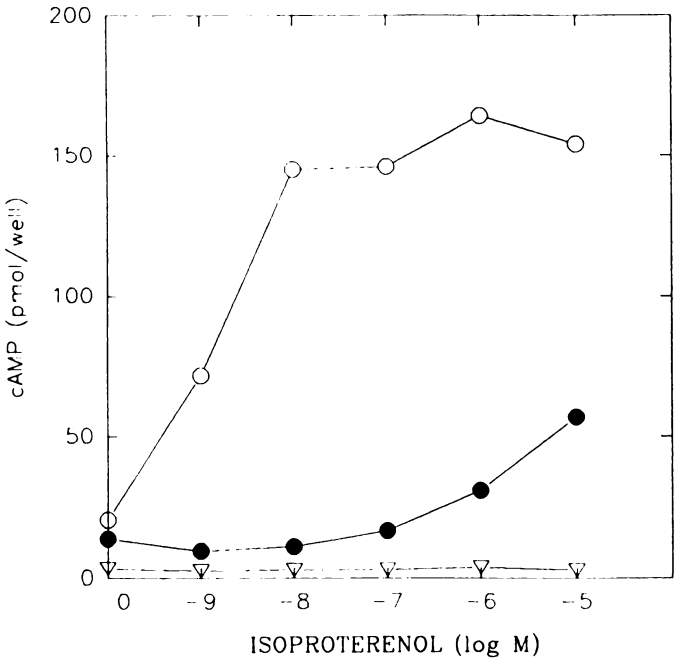

Fig. 5. Effect of isoproterenol on intracellular cAMP in cells expressing mutant βARs. Isoproterenol dose-response curve for cAMP in L cells expressing D(250–259)βAR in the absence (○) and presence (●) of 10 μM propranolol and in L cells expressing D(222–229)βAR (▽). Results are expressed as the mean of duplicate determinations from three separate cell passages.

270 of the βAR is directly involved in coupling to the exchanger. It seems likely that the inability of isoproterenol to stimulate Na-H exchange in cells expressing D(239–272)βAR arises from some conformational effect of this large deletion on adjacent regions of the receptor protein.

Although the particular residues of the βAR that are required for activation of Na-H exchange remain undetermined, these residues are clearly distinct from those in the third cytoplasmic domain that are required to activate adenylyl cyclase. Additional hydrophobic segments of the receptor, however, may be critical for coupling to the exchanger. Although the amino- and carboxyl-terminal regions of the third intracellular domains of G protein-coupled receptors have been shown by deletion and replacement mutagenesis to be important determinants of the activation of several different G protein-linked signaling pathways, including adenylyl cyclase stimulation (11), phospholipase C activation (14, 15, 17, 18), and K^+ channel activation (15), other intracellular domains of these receptors have also been implicated in activating effector pathways. The second intracellular domain of hybrid M_1 muscarinic/β-adrenergic receptors was found to play a role in determining the signaling specificity of the chimeric receptor (14). Similar studies using hybrid receptors or single-residue replacements within other intracellular regions of α- and βARs also suggest that residues in the carboxyl-terminal tail of the receptor that are adjacent to the seventh hydrophobic domain play a role in mediating receptor activation of G proteins (18–20). Peptide mapping analysis of rhodopsin and of the βAR confirms the importance of these other intracellular regions of the receptor for G protein activation (21, 22). Identification of the structural determinants of the βAR, other than the third cytoplasmic domain, that are involved in signaling activation of Na-H exchange will require further genetic and pharmacological analysis.

Acknowledgments

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