Modulation of Adenylate Cyclase Activity by the Physical State of Pigeon Erythrocyte Membrane. 2. Fluidity-Controlled Coupling between the Subunits of the Adenylate Cyclase System†

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ABSTRACT: In the preceding paper, we described the parallel effect of amphipathic drugs on the fluidity and adenylate cyclase activity of pigeon erythrocyte membrane. This parallelism was found when the cyclase activity was assayed in the presence of a guanyl nucleotide and with Mg-ATP as the substrate after a preincubation of the membrane with the drugs [Salesse, R., Garnier, J., Leterrier, F., Daveloose, D., & Viret, J. (1982) *Biochemistry* (preceding paper in this issue)]. However, when the regulatory protein (N) and the catalytic unit (E) were precoupled by GppNHp or fluoride before the action of the drugs, the cyclic AMP production was never inhibited. Thus, the drug-induced fluidization appeared to interfere with the efficiency of the activating coupling between N and E. Chlorpromazine even enhanced the cyclase activity: if the catecholamine receptor (R) repressed the cyclase activity in the absence of hormone [Rodbell, M. (1980) *Nature (London) 284*, 17–22], the loss of R molecules with chlorpromazine would prevent this inhibition and lead to hyperactivity of the enzyme. On the other hand, the comparison between two states of the adenylate cyclase system, (1) N and E reversibly precoupled in the presence of GTP and (2) R, N, and E precoupled in the presence of GTP plus isoproterenol, showed no difference between the activity curves at various drug concentrations: this may be interpreted as a permanent coupling of R and N. The main control exerted by fluidity on the activity of the adenylate cyclase system would thus be at the level of the activating coupling between the N subunit and the catalytic unit in pigeon erythrocyte membrane.

Our current understanding of the functioning of the catecholamine-stimulated adenylate cyclase system involves the interaction of at least four molecular species, namely, the extracellular hormone (H) and three membrane integral proteins, the β-adrenergic receptor (R), the so-called G/F or N regulatory subunit (N), and the catalytic moiety (E) (Rodbell, 1980; Swillens & Dumont, 1980). The binding of H to R promotes its coupling with N (Limbird & Lefkowitz, 1978), which in turn allows the association of the activated N with E (Pfeuffer, 1979), resulting in the stimulation of cyclic AMP production by E. When the coupling ceases, the enzyme returns to its basal level of activity, which is very low in avian erythrocytes (Cassel & Selinger, 1977). Since these different interactions can occur through lateral diffusion of the subunits in the plane of the membrane (Orly & Schramm, 1975), it follows that the physical state of the membrane lipids must play a role in the control of these events (Hanski et al., 1979). Indeed, many attempts have been made to relate changes in fluidity or organization of membranes with changes in the adenylate cyclase activity (Hanski et al., 1979; Dipple & Houslay, 1978; René et al., 1978; Sinensky et al., 1979; Gordon et al., 1980; Houslay et al., 1980), and a possible involvement of the N subunit in this control has been suspected (Voiekov & Lefkowitz, 1980).

We have previously described the effect of amphipathic drugs on the activity of the adenylate cyclase of pigeon erythrocyte, and we correlated this activity with the fluidity of the membrane lipids: briefly, when the membrane fluidity is enhanced, the enzyme loses its activity, which is restored upon recovery of the native membrane physical state (Salesse et al., 1982).

Since the N subunit plays a central role in the stimulation of adenylate cyclase, we designed experimental conditions in which we could study the various states of coupling. It appeared that the membrane fluidity in pigeon erythrocyte membranes controlled the activating coupling between N and E, with apparently no effect on the intrinsic activity of each subunit.

Experimental Procedures

Pigeon erythrocyte ghosts were prepared as previously described (Salesse & Garnier, 1979) by hemolysis in hypotonic buffer (4 mM $MgCl_2$–10 mM Tris-HCl, pH 7.4). Chlorpromazine (CPZ)[1] was a gift from Rhône-Poulenc and 1-octanol and 1-octanoic acid were purchased from Sigma Chemical Co.

Adenylate cyclase assays were performed in two steps: a first step of preincubation of the ghosts and a second step of adenylate cyclase activity measurement. For preincubation, 1 volume of extemporaneously prepared ghosts was mixed at 37 °C with 1 volume of hypotonic buffer containing either the drug and/or different effectors according to the conditions of assay. After 20 min, the adenylate cyclase activity was assayed during 15 min at 37 °C in a final volume of 75 μL containing $\sim 10^8$ cell ghosts, 2 mM [α-^{32}P]ATP [(2–3) × 10^6 dpm], 4 mM theophylline, 7.5 mM $MgCl_2$ or $MnCl_2$, 10 mM Tris-HCl (pH 7.4), 10 mM phosphocreatine, and 0.5 mg/mL creatine phosphokinase. The different conditions of preincubation and adenylate cyclase assay are depicted in Table I.

Results

The ESR measurements of the membrane order with 5-doxylstearate are described in the preceding paper (Salesse et al., 1982), and those results at 37 °C are shown here only for comparison purpose.

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[1] Abbreviations: CPZ, chlorpromazine hydrochloride; ESR, electron spin resonance.

0006-2960/82/0421-1587$01.25/0

Table I: Conditions of Preincubation and Fluidizing Effect on Adenylate Cyclase [a]

name of assay	preincubation conditions [b]			assay conditions [c]			inhibition by fluidization
	drug	cation	effector	drug	cation	effector	
basal	yes	Mn	no	yes	Mn	none	no
F not precoupled/Mn	yes	Mn	no	yes	Mn	10 mM NaF	no
F not precoupled	yes	Mg	no	yes	Mg	10 mM NaF	yes
F precoupled	no	Mg	10 mM NaF	yes	Mg	10 mM NaF	no
Gp not precoupled	yes	Mg	no	yes	Mg	0.1 mM GppNHp	yes
Gp precoupled	no	Mg	0.1 mM GppNHp	yes	Mg	0.1 mM GppNHp	no
Gt precoupled	no	Mg	0.1 mM GTP	yes	Mg	0.1 mM GTP + 50 μM isoprot	no
H·Gt precoupled	no	Mg	0.1 mM GTP + 50 μM isoprot	yes	Mg	0.1 mM GTP + 50 μM isoprot	no

[a] Isoproterenol is abbreviated isoprot. [b] 20 min; 37 °C. [c] 15 min; 37 °C.

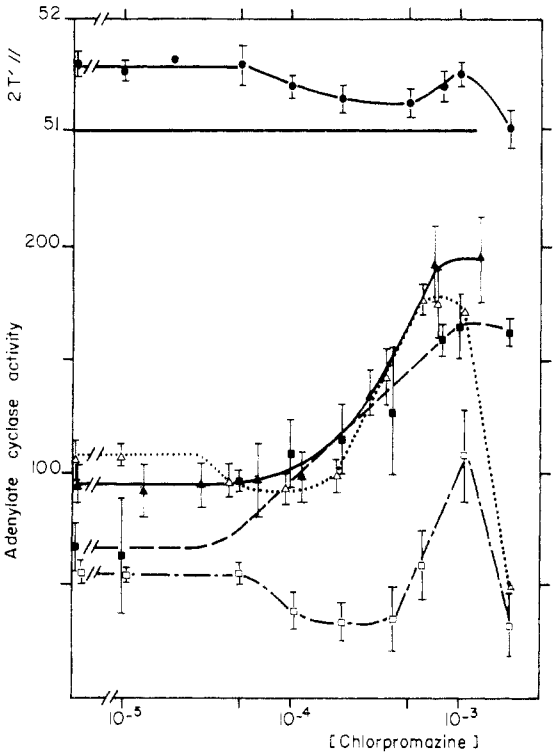

FIGURE 1: Effect of chlorpromazine at 37 °C on (top) hyperfine splitting, $2T'_{\parallel}$, of 5-doxylstearate in the membrane of pigeon erythrocyte ghosts and (bottom) adenylate cyclase activity (picomoles of cyclic AMP per minute per milligram of phospholipids) in the different cases of stimulation: (△) F not precoupled; (▲) F precoupled; (□) Gp not precoupled; (■) Gp precoupled.

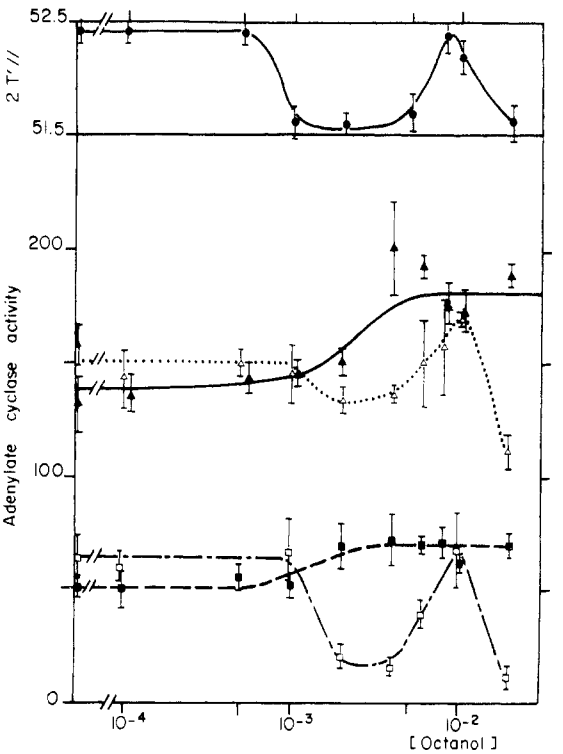

FIGURE 2: Effect of octanol at 37 °C on the membrane order of intact pigeon erythrocytes (top) and adenylate cyclase activity (bottom). Symbols are as in Figure 1.

Upon incubation with the drugs prior to addition of coupling factors, fluoride (F not precoupled), or GppNHp (G not precoupled), the enzyme stimulation followed changes of order: their shapes were polyphasic with CPZ and octanol (Figures 1 and 2) and merely decreasing with octanoic acid (Figure 3).

However, if a previous incubation with the coupling factors was performed without drug (F and Gp precoupled), a considerably modified effect of a subsequent drug addition was observed: no loss of enzymatic activity was found with the three drugs (Figures 1–3). On the contrary, CPZ above 0.1 mM (Figure 1) and, to a much lesser degree, octanol beyond 1 mM (Figure 2) allowed an hyperstimulation of the enzyme. It may be noticed that the F- and Gp-precoupled curves had the same overall shape and also the F- and Gp-not-precoupled curves.

The F-not-precoupled/Mn curves with Mn-ATP as substrate had an evolution similar to the F- and Gp-precoupled activities with Mg-ATP as the substrate (Figures 4–6) except at the highest concentrations of each drug where the F-not-precoupled/Mn activity dropped (Figures 4–6), while the two other ones reached a plateau for all the three drugs (Figures 1–3). Compared to octanol and octanoic acid, CPZ had a higher hyperstimulating effect on the F- and Gp-precoupled states (Figure 1). The low basal activity of adenylate cyclase appeared insensitive to any of the three drugs (Figures 4–6).

A possible effect of the fluidity changes on the R·N coupling was searched by comparing the precoupling with GTP and hormone for each drug (Figures 4–6). GTP was chosen as the coupling agent instead of GppNHp because, in pigeon erythrocyte membranes, GppNHp will mask a further stimulation by isoproterenol, whereas GTP will not. Figures 4–6 show no difference between the Gt- and H·Gt-precoupled activities and are similar to the Gp-precoupled curves for octanol (Figure 5) and octanoic acid (Figure 6). As in the Gp-precoupled case, CPZ (0.1 mM) began to hyperstimulate the enzyme but the cyclase activity dropped rapidly from 0.4 mM on.

Discussion

Our previous results had shown that the cationic and neutral drugs induced a polyphasic change of adenylate cyclase activity in the presence of GTP, while anionic drugs were merely inhibitory (Salesse & Garnier, 1979). In the preceding paper, these changes were related to the physical state of the mem-

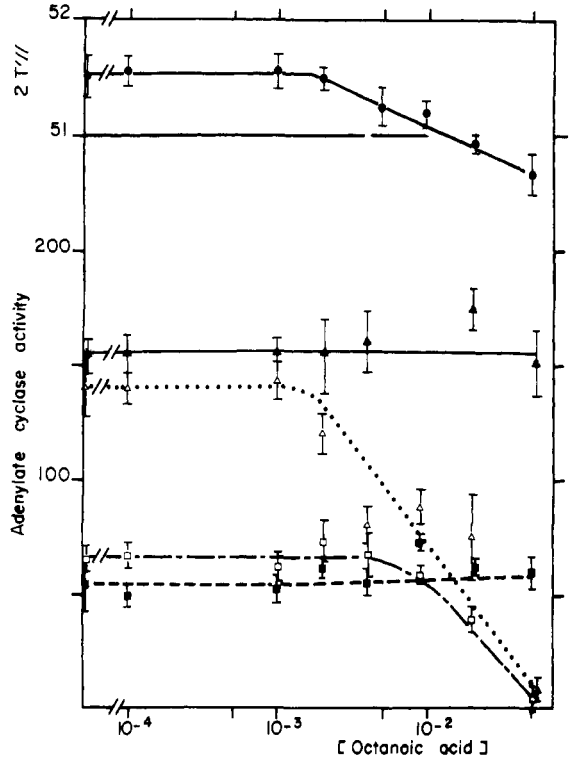

FIGURE 3: Effect of octanoic acid at 37 °C on the membrane order of intact pigeon erythrocytes (top) and adenylate cyclase activity (bottom). Symbols are as in Figure 1.

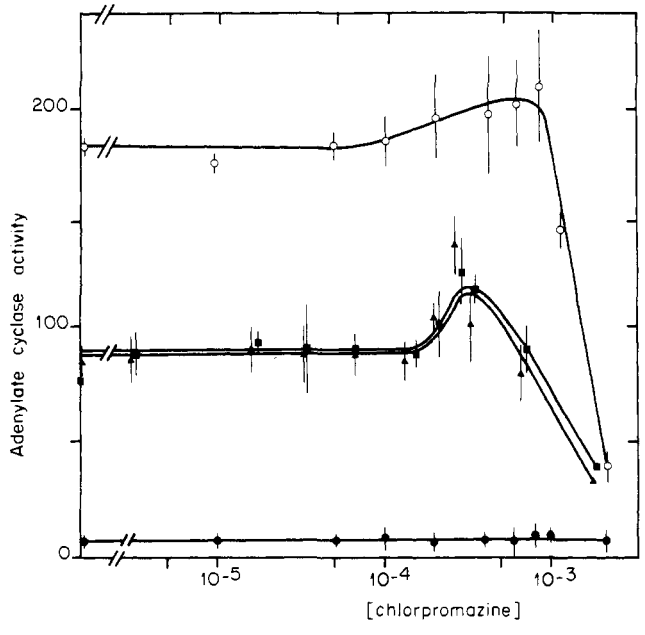

FIGURE 4: Effect of chlorpromazine at 37 °C on the activity of the adenylate cyclase in the following cases of stimulation: (●) basal with Mn-ATP as the substrate; (○) F not precoupled/Mn; (■) Gt precoupled; (▲) H·Gt precoupled.

brane (Salesse et al., 1982). Now we report that they depend on the state of stimulation of the adenylate cyclase. Table I (last column) summarizes the effect of fluidization on the different states of stimulation of the system.

Effect of the Fluidity Changes on the Coupling between N and E. The parallel changes in order and in cyclase activity are evidenced only when Mg-ATP is used as the substrate and when fluoride or GppNHp stimulation occurs after preincubation with the drugs, i.e., when the N subunit is not yet coupled to the catalytic unit E. If, however, the N and E

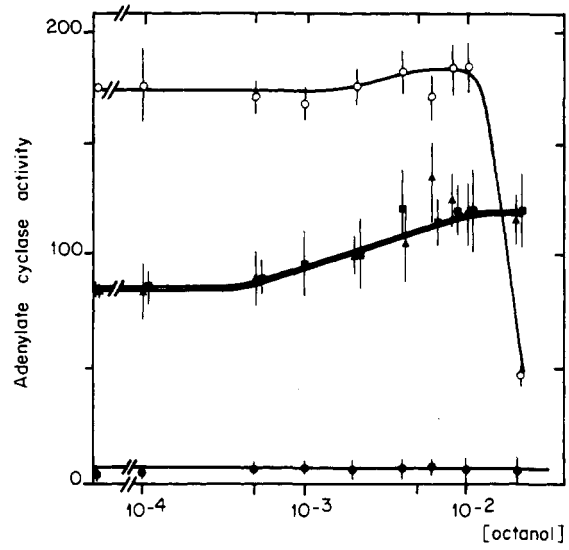

FIGURE 5: Effect of octanol at 37 °C on the activity of the adenylate cyclase. Symbols are as in Figure 4.

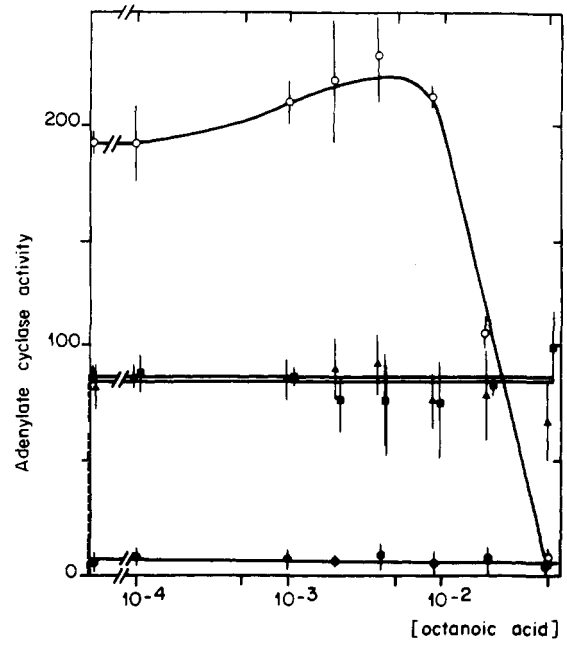

FIGURE 6: Effect of octanoic acid at 37 °C on the activity of the adenylate cyclase. Symbols are as in Figure 4.

subunits are precoupled by NaF or GppNHp prior to drug addition, the maintenance or enhancement of the cyclase activity indicates that it escapes from the control of the membrane physical state. Furthermore, in the not-precoupled states, the fact that the enzymatic system can exhibit different degrees of stimulation, even greater than the control, shows that the two protein subunits do retain their potentialities throughout the whole drug concentration range. Moreover, we have previously checked that the effect of CPZ on fluoride stimulation is reversible by washing of the drug at least up to 0.6 mM (Garnier & Singer, 1977).

Taken together, these data suggest that the coupling itself of N and E is under the dependence of the membrane fluidity which would modulate the efficiency of the activating collisions or association between N and E: disorganization of the lipid bilayer prevents the coupling while its reorganization restores this possibility.

In the presence of Mn-ATP (F not precoupled/Mn), the cyclase system behaves as in the F- or Gp-precoupled states, except at the highest concentrations of the three drugs: in some

way, it partially escapes from the control of the membrane fluidity, at the difference of the F-not-precoupled/Mg activity. Since the Mg specificity is conferred upon the catalytic unit by its coupling with the regulatory protein (Ross et al., 1978; Limbird et al., 1979), it may be concluded that in pigeon erythrocytes, the "transmission" of the fluoride stimulation itself is possible with both Mn- and Mg-ATP, but the "Mg-specificity-inducing coupling" is more dependent on the membrane fluidity. This suggests that when the N subunit is fully involved in the coupling (NaF stimulation plus Mg-ATP), the stimulation is more sensitive to the bilayer physical state at low drug concentration (below 1 mM CPZ or 10 mM octanol).

From F-not-precoupled/Mn and Gp-precoupled experiments, the catalytic activity appears hyperactivated upon fluidization of the membrane, slightly by octanol above 1 mM and much more so by CPZ beyond 0.1 mM. This effect of drugs has already been reported in other cyclase systems (Hanski et al., 1979; Houslay et al., 1980). This suggests that the fluidization brought by the drugs might release an inhibitory control exerted on the enzyme by the N subunit in the presence of resting receptors and in the absence of guanyl nucleotides (Rodbell, 1980; Lin et al., 1979). As systems devoid of receptors display high activity in the presence of guanyl nucleotides (Lin et al., 1979), this would be the origin of the high hyperactivity observed with CPZ when this drug restores the initial membrane fluidity, since at 1 mM CPZ, 90% of the β-adrenergic receptors have been irreversibly lost (Salesse & Garnier, 1979). This receptor effect occurs much later with octanol (above 10 mM) and not at all with octanoic acid.

Effect of the Fluidity Changes on the Coupling between R and N. That the absence (Gt precoupled) or presence (H·Gt precoupled) of isoproterenol during the preincubation step does not change the response of the system to the drugs means that the transmission of the hormonal stimulation to the N subunit is not under the control of the membrane physical state. This agrees with Rodbell's (1980) view that the R and N subunits would be permanently in interaction. This permanent coupling could explain that the activation of N by H·R (the receptor with the hormone bound) is not a rate-limiting step in the process of adenylate cyclase stimulation (Citri & Schramm, 1980).

Conclusion. Both fluidization (Hanski et al., 1979; Dipple & Houslay, 1978; Gordon et al., 1980; Houslay et al., 1980) and rigidification (Sinensky et al., 1979) of the bilayer have been reported to increase adenylate cyclase activity. The present study shows that, indeed, in pigeon erythrocytes, according to the state of coupling of the system, the same cause (i.e., fluidization) can have opposite effects because of the multifold regulation of the adenylate cyclase activity, which stems from the multicomponent nature of the system. Some prudence is required in the interpretation of the effect of fluidization: if a decrease in the membrane microviscosity may increase the rate of activating collisions with the catalytic subunit (Hanski et al., 1979; Dipple & Houslay, 1978; René et al., 1978; Houslay et al., 1980), it is often accompanied by a loss of membrane organization (Griffith & Jost, 1976; Schreier et al., 1978); as already noticed, this disorganization may change the degree of embedding of integral proteins into the membrane (Borochov & Shinitzky, 1976; Armond & Staehelin, 1979). It is thus conceivable that in a system requiring the mating of different subunits, this parameter is critical because it governs the transmission of the stimulation (Levitzki & Helmreich, 1979; Bakardjieva et al., 1979). In the adenylate cyclase system of pigeon erythrocyte membranes, it would mainly control the transmission between the GTP-regulatory protein and the catalytic unit.

References

Armond, P. A., & Staehelin, L. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1901–1905.

Bakardjieva, A., Galla, H. J., & Helmreich, E. J. M. (1979) *Biochemistry 18*, 3016–3023.

Borochov, H., & Shinitzky, M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4526–4530.

Cassel, D., & Selinger, Z. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3307–3311.

Citri, Y., & Schramm, M. (1980) *Nature (London) 287*, 297–300.

Dipple, I., & Houslay, M. D. (1978) *Biochem. J. 174*, 179–190.

Garnier, J., & Singer, S. J. S. (1977) in *Electrical Phenomena at the Biological Membrane Level* (Roux, E., Ed.) pp 35–41, Elsevier, Amsterdam.

Gordon, L. M., Sauerheber, R. D., Esgate, J. A., Dipple, I., Marchmont, R. J., & Houslay, M. D. (1980) *J. Biol. Chem. 255*, 4519–4527.

Griffith, O. H., & Jost, P. C. (1976) in *Spin Labeling* (Berliner, L. J., Ed.) pp 453–523, Academic Press, New York.

Hanski, E., Rimon, G., & Levitzki, A. (1979) *Biochemistry 18*, 846–853.

Houslay, M. D., Dipple, I., Rawal, S., Sauerheber, R. D., Esgate, J. A., & Gordon, L. M. (1980) *Biochem. J. 190*, 131–137.

Levitzki, A., & Helmreich, E. J. M. (1979) *FEBS Lett. 101*, 213–219.

Limbird, L. E., & Lefkowitz, R. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 228–232.

Limbird, L. E., Hickey, A. R., & Lefkowitz, R. J. (1979) *J. Biol. Chem. 254*, 2677–2683.

Lin, M. C., Lin, C. S., & Whitlock, J. P., Jr. (1979) *J. Biol. Chem. 254*, 4684–4688.

Orly, J., & Schramm, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3433–3437.

Pfeuffer, T. (1979) *FEBS Lett. 101*, 85–89.

René, E., Pecker, F., Stengel, D., & Hanoune, J. (1978) *J. Biol. Chem. 253*, 838–841.

Rodbell, M. (1980) *Nature (London) 284*, 17–22.

Ross, E. M., Howlett, A. C., Fergusson, K. M., & Gilman, A. G. (1978) *J. Biol. Chem. 253*, 6401–6412.

Salesse, R., & Garnier, J. (1979) *Biochim. Biophys. Acta 554*, 102–113.

Salesse, R., Garnier, J., Leterrier, F., Daveloose, D., & Viret, J. (1982) *Biochemistry* (preceding paper in this issue).

Schreier, S., Polnaszek, C. F., & Smith, I. C. P. (1978) *Biochim. Biophys. Acta 515*, 395–436.

Sinensky, M., Minneman, K. P., & Molinoff, P. B. (1979) *J. Biol. Chem. 254*, 9135–9141.

Swillens, S., & Dumont, J. E. (1980) *Life Sci. 27*, 1013–1028.

Voiekov, V. L., & Lefkowitz, R. J. (1980) *Biochim. Biophys. Acta 629*, 266–281.