Steroidogenic Action of Calcium Ions in Isolated Adrenocortical Cells

Ernesto J. PODESTA, Alfred MILANI, Hans STEFFEN and Robert NEHER
Friedrich Miescher-Institut, P.O. Box 273, CH-4002 Basel, Switzerland

(Received 25 June 1979)

The corticotropin-induced increase of total intracellular and receptor-bound cyclic AMP in isolated rat adrenocortical cells was strictly dependent on extracellular Ca^{2+} . A rise in bound cyclic AMP with rising Ca^{2+} concentrations was accompanied by a decrease in free cyclic AMP-receptor sites. A Ca^{2+} -transport inhibitor abolished the rise in bound cyclic AMP induced by corticotropin. These data suggested that during stimulation by corticotropin some Ca^{2+} has to be taken up in order to promote the rise of the relevant cyclic AMP pool. In agreement with this view, adenylate cyclase activity from isolated cells proved also to be dependent on a sub-millimolar Ca^{2+} concentration in the presence of corticotropin and GTP. When cells were treated under specific conditions, corticosterone production could be activated by Ca^{2+} in the absence of corticotropin (cells primed for Ca^{2+}). Ca^{2+} -induced steroidogenesis of these cells, in the absence of corticotropin, was also accompanied by an increase in total intracellular and receptor-bound cyclic AMP, as was found previously with corticotropin-induced steroidogenesis in non-primed cells. Calcium ionophores increasing the cell uptake of Ca^{2+} were not able, however, to increase the cyclic AMP pools in non-primed cells, unlike corticotropin in non-primed cells or Ca^{2+} in cells primed for Ca^{2+} . It was concluded that during stimulation by either corticotropin or Ca^{2+} a possible cellular uptake of Ca^{2+} must be very limited and directed to a specific site which may affect the coupling of the hormone-receptor–adenylate cyclase complex.

The requirement for extracellular Ca^{2+} in the adrenal steroidogenic response to corticotropin (ACTH) was described many years ago (Birmingham *et al.*, 1953). There is good evidence that Ca^{2+} is not needed for the corticotropin–receptor interaction in either intact cells or membrane fractions, but is required for the stimulation of adenylate cyclase by corticotropin in subcellular particles from adrenal tissue (Bär & Hechter, 1969; Lefkowitz *et al.*, 1970; Kelly & Koritz, 1971; Sayers *et al.*, 1972; Haksar & Peron, 1973; Kowal *et al.*, 1974; Yanagibashi *et al.*, 1978). Nevertheless, the mechanism of action of Ca^{2+} remains controversial (Dazord *et al.*, 1975; Lymangrover & Martin, 1978). Other possible sites of action of this cation in the stimulation of adrenal steroidogenesis by corticotropin have been discussed (Birmingham *et al.*, 1960; Peron *et al.*, 1965; Whysner *et al.*, 1966; Farese, 1971; Jaanus & Rubin, 1971; Jaanus *et al.*, 1972; Bower & Kitabchi, 1974; Jefcoate & Orme-Johnson, 1975; Pfeiffer & Tchen, 1975; Arthur *et al.*, 1976; Farese & Prudente, 1978; Warner & Carchman, 1978). Our finding that acute steroidogenesis in isolated adreno-

cortical cells could be triggered in the absence of corticotropin by extracellular Ca^{2+} alone presented under specific conditions (cells primed for Ca^{2+}) (Neher & Milani, 1976, 1978*a,b*) offered a good opportunity to study the mechanism of action of Ca^{2+} in these cells.

There is now strong evidence that cyclic AMP and cyclic AMP-dependent protein kinase are obligatory mediators in steroidogenesis (Podesta *et al.*, 1979; Rae *et al.*, 1979; Sala *et al.*, 1979). In the present paper we have investigated whether steroidogenesis in isolated rat adrenocortical cells, triggered by Ca^{2+} in the absence of corticotropin, follows the same mechanism of action as steroidogenesis triggered by corticotropin. We also studied the interrelation between extracellular Ca^{2+} and the total intracellular and receptor-bound cyclic AMP pools.

Materials and Methods

Materials

Chemicals and rats were obtained as described previously (Podesta *et al.*, 1979) with the following

additions: calcium ionophore A23187 was a gift from Dr. R. L. Hamill of Lilly Research Laboratories, Indianapolis, IN, U.S.A.; X-14547A was a gift from Dr. U. Solms, F. Hoffmann-La Roche and Co., A.G., Basel, Switzerland; ionomycin was a gift from Mr. S. J. Lucania of E. R. Squibb and Sons, Princeton, NJ, U.S.A.: [α - 32 P]-ATP (12.4 Ci/mmol) and 45 CaCl₂ (20 mCi/mg of Ca) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. L-[Gly-2- 3 H]glutathione (2.48 Ci/mmol) was from New England Nuclear, Boston, MA, U.S.A.; L-glutathione, reduced and oxidized form, and EGTA were from Fluka A.G., Buchs, Switzerland; verapamil was from Knoll A.G., Liestal, Switzerland; nucleotides, phosphocreatine and creatine kinase were from Boehringer, Mannheim, Germany; silicone oils AR20 and AR200 were from Wacker-Chemie G.m.b.H., München, Germany; ion-exchange resins Dowex AG50W-X4 (200–400 mesh) and AG50W-X8 (50–100 mesh) were from Bio-Rad Laboratories, Richmond, CA, U.S.A.; alumina (neutral, grade 1) was from Camag, Muttenz, Switzerland.

Cell preparation and incubation of adrenocortical cells of zona fasciculata-reticularis type

These were carried out as described by Podesta *et al.* (1979).

Preparation of Ca²⁺-responsive cells (cells primed for Ca²⁺)

The trypsin treatment of 20 decapsulated adrenals followed published procedures (Neher & Milani, 1978a). The cell pellet obtained from the last wash with 20 ml of Krebs–Ringer bicarbonate solution, pH 7.5–7.7 (KRB; Krebs & Henseleit, 1932), supplemented with 0.01 M-glucose (G), 0.5% (w/v) bovine serum albumin (A) and 0.1% (w/v) lima-bean trypsin inhibitor was resuspended in 60 ml of KRBGA medium, pH 7.5–7.7, containing only 0.25 mM-Ca²⁺ instead of 2.5 mM-Ca²⁺, at 20°C, pre-incubated for 60 min at 37°C under O₂/CO₂ (19:1) without shaking, passed through a nylon filter, centrifuged for 10 min at 100g and separated from the supernatant. This final pellet was resuspended in 30 ml of freshly prepared Ca²⁺-free KRBG medium containing 0.25% gelatin (KRBG Gel–Ca) as described earlier (Neher & Milani, 1978a) at 5–10°C. The cell count was usually in the range of 250 000 cells per adrenal.

Preparation of incubation mixtures for Ca²⁺-induced steroidogenesis

Incubations were done in duplicate or triplicate in 12 mm × 75 mm Falcon plastic tubes with shaking (100 cycles/min) for different periods of time. Each tube was loaded with 0.5 ml of KRBG Gel–Ca medium, pH 7.5–7.7, including additions. Additions

were made in the same medium at a concentration which was 10 times that required in the final incubation mixture of 1 ml. Obligatory additions (final concns.) were 0.3 mM-3-isobutyl-1-methylxanthine (a phosphodiesterase inhibitor) and 6 mM-glutathione (either reduced form, GSH, or oxidized form, GSSG). Optional additions were CaCl₂, corticotropin, or others in concentrations as indicated in the Figures and Tables. Final media used other than KRBGA (for stimulation by corticotropin or KRBG Gel–Ca (for stimulation by Ca²⁺) are also indicated in the legends of the Tables.

The loaded tubes were then cooled to 0–5°C immediately before the addition of 0.5 ml of cell suspension per tube. The complete mixture was gassed with O₂/CO₂ (19:1) for 1 min at 0–5°C, and the tubes were capped and kept for 15–20 min at 0–5°C before incubation at 37°C in a water bath. The incubation was stopped by cooling the tubes in ice/water and all further processing was done at 0–4°C.

Assays

The steroid assay, analysis of total intracellular and intracellular protein-bound cyclic AMP, the radioimmunoassay of cyclic AMP, the measurement of free cyclic AMP-receptor sites and the cyclic nucleotide phosphodiesterase assay were done as described earlier (Podesta *et al.*, 1979).

Preparation of crude adrenal-cell adenylate cyclase

The cell pellet from 28 adrenals was resuspended in 10 ml of cold 2 mM-Tris/HCl buffer, pH 7.5, shell-frozen directly in the homogenizer vessel and homogenized during thawing with a loose-fitting Teflon pestle (10–20 strokes) until no more unbroken cells could be detected by microscopy. The homogenate was centrifuged at 0°C for 20 min at 48 000g and the pellet resuspended in 2 mM-Tris/HCl, pH 7.5. The pellet from half an adrenal was used in a final reaction volume of 100 μ l for the adenylate cyclase assay described by Salomon *et al.* (1974).

Protein assay

This was done by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

⁴⁵Ca-uptake experiments

These were carried out by using Dowex AG1-X8 (50–100 mesh) (Gasko *et al.*, 1976), and by the silicone-oil technique of Gerisch & Wick (1975), with a mixture of 45% (v/v) silicone oil AR 20 and 55% (v/v) AR 200 for the rapid separation of adrenocortical cells from Krebs–Ringer media.

Results and Discussion

Cells stimulated by corticotropin

The good correlation of the acute steroidogenic

response with total intracellular and receptor-bound cyclic AMP pools (Podesta *et al.*, 1979) prompted us to investigate the dependence of these two cyclic AMP pools on the extracellular Ca^{2+} concentration, which determines the degree of steroidogenesis by a given corticotropin concentration. Fig. 1 shows that the production of both total intracellular and bound cyclic AMP in corticotropin-stimulated adrenocortical cells is highly dependent on the extracellular Ca^{2+} concentration and that both pools increase sharply from 0.1 to 2.5 mM- Ca^{2+} . No increase in these two parameters was seen over the whole range of Ca^{2+} concentrations in the absence of corticotropin.

In the presence of corticotropin the Ca^{2+} -dependent increase in occupied cyclic AMP-receptor sites (bound cyclic AMP) was accompanied by a Ca^{2+} -dependent decrease in free receptor sites of about 25%. No change in free receptor sites was induced by corticotropin in the absence of Ca^{2+} [control,

Fig. 1. Dependence of the production of total intracellular (circles) and bound (triangles) cyclic AMP on the extracellular Ca^{2+} concentration in the presence (open symbols) or absence (closed symbols) of 28 pM-corticotropin

The incubations were carried out for 10 min at 37°C, in the presence of 0.3 mM-3-isobutyl-1-methylxanthine. Results are mean values $\pm$ S.D., $n = 4$.

100.00 ± 1.02 ; + corticotropin (10 pM) 99.6 ± 1.06 (means $\pm$ S.D., $n = 3$).

Inhibitors of Ca^{2+} transport such as verapamil are known also to inhibit corticotropin-induced steroidogenesis (Neher & Milani, 1978b; Warner & Carchman, 1978). We decided to see whether this inhibition could be explained by an effect on the intracellular cyclic AMP pools. Table 1 shows that verapamil decreased both steroid formation and the production of total intracellular and bound cyclic AMP in corticotropin-stimulated cells. The effect of verapamil is due to a shift in the corticotropin dose-response curve. The decrease in the response was less, but still evident, in cells stimulated by cyclic AMP (results not shown). This could be explained by a side-effect of verapamil or by the fact that Ca^{2+} may be important in some step beyond cyclic AMP. The effect on the dose-response curve is in agreement with the fact that supra-physiological concentrations of corticotropin can overcome the lack of Ca^{2+} to a large degree.

It was concluded that the corticotropin-induced production of cyclic AMP depends on some cellular uptake of Ca^{2+} . However, we were not able to measure directly a significant difference in ^{45}Ca uptake between corticotropin-stimulated and unstimulated cells, using two different procedures under various conditions (results not shown). The accuracy of these methods is insufficient to record a possible corticotropin-dependent uptake of a few c.p.m. of $^{45}\text{Ca}^{2+}$ necessary to raise the concentration in the small cytosolic compartment, e.g. from 0.1 to 1 μM , when a few million c.p.m. of $^{45}\text{Ca}^{2+}$ is present in the extracellular volume in the absence or presence of 2.5 mM- Ca^{2+} . On the other hand, calcium ionophores, such as A23187, X-14547A or ionomycin, promoted a marked Ca^{2+} uptake in isolated cells without stimulating steroidogenesis or increasing intracellular or bound cyclic AMP pools at all (results not shown).

Moreover, these calcium ionophores inhibited both corticotropin- and cyclic AMP-stimulated steroidogenesis in isolated cells (Neher & Milani, 1978a; Warner & Carchman, 1978), owing to either non-specific Ca^{2+} uptake or other side reactions.

Table 1. Effect of corticotropin and verapamil on receptor-bound cyclic AMP, total intracellular cyclic AMP and corticosterone production

Rat adrenocortical cells were incubated in KRBGA medium, pH 7.4, for 15 min at 37°C in the presence of 2.5 mM- Ca^{2+} and 0.3 mM-3-isobutyl-1-methylxanthine, and in the absence or presence of corticotropin and verapamil. Results are mean values of close duplicates.

Corticotropin (pM)	Verapamil (μM)	Cyclic AMP (fmol/ 10^5 cells)		Corticosterone (ng/ 10^5 cells)
		Receptor-bound	Total intracellular	
0	0	50	300	1.6
17	0	250	2700	45.2
17	60	75	600	16.2

Taking all these data together, we tentatively concluded that the effect of extracellular Ca^{2+} must be due to a very specific Ca^{2+} uptake in respect of both quantity and localization. This is in agreement with the findings of Matthews & Saffran (1973) that the transmembrane potential of corticotropin-treated adrenal tissue did not change in the presence or absence of Ca^{2+} .

One obvious target enzyme for Ca^{2+} is the adenylate cyclase in the plasma membrane, as the activity of this enzyme from bovine adrenal tissue and cultured adrenal tumour cells was reported to be dependent on sub-millimolar Ca^{2+} concentrations (Bär & Hechter, 1969; Lefkowitz *et al.*, 1970; Kelly & Koritz, 1971; Kowal *et al.*, 1974). We also found that corticotropin activation of adenylate cyclase from isolated rat adrenocortical cells was dependent on sub-millimolar calcium concentrations in the presence of both GTP and Gpp[NH]p (guanosine 5'-[β -imidol]triphosphate) (Fig. 2). With GTP as

Fig. 2. Dependence of adenylate cyclase activity in the 48000g pellet from isolated adrenocortical cells on Ca^{2+} concentration in the presence of 1 mM-EGTA and 0–2 mM- CaCl_2 .

The free Ca^{2+} concentration was calculated by using the K_{CaEGTA} value of Schwarzenbach *et al.* (1957), taking into consideration the presence of 0.5 mM-ATP and 2.5 mM- Mg^{2+} at pH 7.5 and 30°C. Incubation in the presence of 10 μM -GTP (●—●) or of 10 μM -Gpp[NH]p (○—○), incubation in the presence of 27 μM -corticotropin and 10 μM -GTP (●—●) or 27 μM -corticotropin and 10 μM -Gpp[NH]p (○—○). Results are mean values of triplicates; s.d. for all means was less than $\pm 10\%$.

cofactor the corticotropin-stimulated activity was maximal between 10 and 100 μM free Ca^{2+} ($P < 0.001$ versus zero Ca^{2+}), whereas with Gpp[NH]p as cofactor the dependence on Ca^{2+} was less marked, the optimal free Ca^{2+} concentration being at about 0.1 μM ($P < 0.005$ versus zero Ca^{2+}). In contrast, the activation of adenylate cyclase by either cofactor in the absence of corticotropin was always highest at 1 nM- Ca^{2+} or less. Therefore it is unlikely that Ca^{2+} supports the binding of GTP or Gpp[NH]p to the cyclase complex in the absence of corticotropin. It is also unlikely that Ca^{2+} affects the hydrolysis of GTP, because the cation also supports the corticotropin-dependent activation of the cyclase by the hydrolysis-resistant Gpp[NH]p. The results mentioned above suggest that in the presence of corticotropin and cofactors Ca^{2+} may be involved in the coupling of the hormone-receptor-cyclase complex.

Cells stimulated by Ca^{2+} in the absence of corticotropin

When the preparation of rat adrenocortical cells and the presentation of Ca^{2+} was modified, as reported earlier (Neher & Milani, 1976, 1978a), the cells were found to become responsive to Ca^{2+} in the absence of corticotropin, producing a high output of corticosterone. We may call this type of cell 'cells primed for Ca^{2+} ', in contrast with those that need in addition corticotropin for induction of steroidogenesis. Yanagibashi *et al.* (1978) briefly described bovine adrenocortical cells behaving like these primed cells.

We have further improved the procedure to obtain cells primed for Ca^{2+} from decapsulated rat adrenals as reported in the Materials and Methods section. The first step comprises a preincubation in a Ca^{2+} -poor medium, which may affect some physical properties of the cell membrane and thus facilitate the subsequent access of Ca^{2+} to a specific site, as discussed above. In the final step, Ca^{2+} is added in the presence of gelatin, 3-isobutyl-1-methylxanthine and glutathione, probably forming a colloid-like species in transition from the ionized to a precipitated form. 3-Isobutyl-1-methylxanthine was added in order to counteract the high phosphodiesterase activity (Podesta *et al.*, 1979). Glutathione, in either the reduced (GSH) or the oxidized form (GSSG), was found to support the stimulation by Ca^{2+} . It is not clear whether this particular effect of extracellular glutathione has anything to do with the high adrenal glutathione reductase activity (Harding & Nelson, 1964) or with regulation of adrenal mitochondrial hydroxylation (Wickramasinghe, 1974; Sulimovici & Boyd, 1967). This effect does not seem to be due to the redox properties of glutathione, since the response is sometimes more pronounced in the presence of GSSG than of GSH, and vice versa.

Glutathione did not complex Ca²⁺, and [³H]GSH did not interact with cell-membrane proteins under the incubation conditions. Glutathione had no direct effect on adenylate cyclase nor on phosphodiesterase activity (results not shown).

With cells primed for Ca²⁺ we investigated whether the acute steroidogenesis in these cells could also be correlated with the total intracellular and bound cyclic AMP pool, as reported earlier for corticotropin-responsive cells (Podesta *et al.*, 1979), or whether a different mechanism of action is involved.

Table 2 shows clearly that the two cyclic AMP pools increased with increasing extracellular Ca²⁺ concentration and with time, in parallel to corticosterone production. As shown previously in non-primed cells (Podesta *et al.*, 1979), bound cyclic AMP correlates better than total intracellular cyclic AMP with steroidogenesis. This also suggests a mediating role of cyclic AMP in the steroidogenesis of cells primed for Ca²⁺, as was the case in corticotropin-responsive cells.

We further investigated whether the preparation of cells primed for Ca²⁺ selected a particular cell population or whether they still contained corticotropin receptors on their surface and were responsive to corticotropin. This was made possible by the availability of conditions for the dissociation of the two triggers. A good stimulation by the Ca²⁺ trigger

is dependent on the presence of extracellular phosphate, which is not required for stimulation by supra-threshold doses of corticotropin. On the other hand, stimulation by more than 30pM-corticotropin can be obtained at Ca²⁺ concentrations too low to activate the Ca²⁺ trigger. Table 3 shows that cells primed for Ca²⁺ still possess corticotropin receptors. Again, bound cyclic AMP concentrations reflect the changes in corticosterone, the high concentration of 2700pM-corticotropin leading to an excessive production of cyclic AMP (Podesta *et al.*, 1979). In this respect, a difference between the Ca²⁺ trigger and corticotropin trigger seemed to become apparent. By comparing the production of total intracellular and bound cyclic AMP after 15 min stimulation of unprimed cells by corticotropin and of primed cells by Ca²⁺ to a comparable degree of steroidogenesis, and at maximal total intracellular cyclic AMP production, it was found that the ratio of bound to intracellular cyclic AMP seemed to differ in the two sets of stimulated cells (Table 4). Since *in vitro* calcium had no effect on the binding of cyclic AMP to its receptor protein (results not shown), the higher ratio in cells primed for Ca²⁺ may point to a Ca²⁺-dependent shift of cyclic AMP from the total intracellular pool to the receptor-protein-bound pool. In some experiments during the preparation of primed cells (preincubation in 0.25 mM-

Table 2. Production of receptor-bound cyclic AMP, total intracellular cyclic AMP and corticosterone in cells primed for Ca²⁺

Rat adrenocortical cells were incubated in KRBG Gel—Ca medium, pH 7.5–7.8, containing 0.3 mM-3-isobutyl-1-methylxanthine and 6 mM-glutathione after addition of 0.25 or 2.5 mM-CaCl₂ at 37°C. Results are mean values of close duplicates.

Extracellular Ca ²⁺ (mM)	Time (min)	Cyclic AMP (fmol/10 ⁵ cells)		Corticosterone (ng/10 ⁵ cells)
		Receptor-bound	Total intracellular	
0.25	0	31.2	327	2.1
	15	46.0	434	34.8
	30	58.5	282	71.3
2.50	0	42.2	307	2.2
	15	257.4	995	56.9
	30	312.0	421	119.6

Table 3. Responsiveness of cells primed for Ca²⁺ to extracellular Ca²⁺ or corticotropin
The final suspension of cells primed for Ca²⁺ was in phosphate-free KRBG Gel—Ca medium and incubated in the presence of 0.1 or 3 mM-CaCl₂, 0 or 1 mM-KH₂PO₄ and 0, 82 or 2700 pM-corticotropin, for 15 min at 37°C. Results are mean values of close duplicates.

Ca ²⁺ (mM)	P _i (mM)	Corticotropin (pM)	Corticosterone (ng/10 ⁵ cells)	Receptor-bound cyclic AMP (fmol/10 ⁵ cells)
3	0	0	2.4	39
3	1	0	36.0	78
3	0	82	46.5	56
0.1	1	0	2.4	43
0.1	1	2700	39.0	300

Table 4. Comparison of receptor-bound cyclic AMP and total intracellular cyclic AMP in cells primed for Ca^{2+} and in unprimed cells, ratio of bound/intracellular cyclic AMP and corticosterone production

The cells primed for Ca^{2+} were prepared as described in the Materials and Methods section and incubated in the presence of 0.3 mM-3-isobutyl-1-methylxanthine and 6 mM-glutathione with or without 2.5 mM- CaCl_2 for 15 min at 37°C . The unprimed cells were prepared without preincubation and suspended finally in KRBGA medium and incubated in the presence of 0.3 mM-3-isobutyl-1-methylxanthine for 15 min at 37°C in the absence or presence of corticotropin. Results are mean values of close duplicates.

	Cyclic AMP (fmol/ 10^5 cells)		Ratio bound/ intracellular cyclic AMP	Corticosterone (ng/ 10^5 cells)
	Receptor-bound	Total intracellular		
Cells primed for Ca^{2+}				
Ca^{2+} (mM)				
0	85.1	332.5	0.26	2.9
2.5	210.0	725.2	0.29	41.4
Unprimed cells				
Corticotropin (pM)				
0	54.1	335.6	0.16	4.1
10	156.6	2872.0	0.05	38.0

Ca^{2+} medium), receptor-bound cyclic AMP was slightly higher after the preincubation (see the Materials and Methods section) and remained high during incubation in absence of Ca^{2+} (see Table 4). Some corticosterone was also released during preparation of primed cells.

Comparison of our results with cells primed for Ca^{2+} and corticotropin-induced adrenocortical cells led us to conclude that binding of corticotropin to its receptor may bring about a change in membrane properties, e.g. increase in fluidity, as described for other hormone-responsive tissue (Rimon *et al.*, 1978), or in Ca^{2+} permeability, an effect which may be partly mimicked by preincubation of the cells in a Ca^{2+} -poor medium. In the subsequent step extracellular Ca^{2+} may now have facilitated access to the adenylate cyclase complex; in cells primed for Ca^{2+} this event is facilitated by presentation of Ca^{2+} in the form of a metastable complex of small size. Ca^{2+} at the right place and in the right sub-millimolar concentration may then activate the adenylate cyclase complex, leading to increased production of cyclic AMP. It is likely that Ca^{2+} supports the activation by corticotropin and GTP of the cyclase and may affect the coupling of the hormone-receptor-cyclase complex.

It is concluded that the stimulation of steroidogenesis in adrenal cells by either corticotropin or Ca^{2+} involves mainly the activation of the adenylate cyclase complex by Ca^{2+} , and that cyclic AMP mediates steroidogenesis in both cases.

Our thanks are due to Dr. W. Rittel, Ciba-Geigy Ltd., for the supply of corticotropin-1-24-tetracosapeptide (Synacthen), Dr. R. Kübler and Dr. H. R. Blattmann, Ciba-Geigy Ltd., for Ca^{2+} titrations of glutathione and calculations of pCa^{2+} , and F. Martin for excellent technical help. We are grateful for valuable advice on cell

separation techniques to Dr. G. Gerisch, Biocenter, Basel, and Dr. R. Hinnen, Friedrich Miescher-Institut, and on the adenylate cyclase assay to Dr. M. Towbin, Friedrich Miescher-Institut.

References

- Arthur, J. R., Mason, J. I. & Boyd, G. S. (1976) *FEBS Lett.* **66**, 206–209
- Bär, H. P. & Hechter, O. (1969) *Biochem. Biophys. Res. Commun.* **35**, 681–686
- Birmingham, M. K., Elliott, F. H. & Valère, P. H. L. (1953) *Endocrinology* **53**, 687–689
- Birmingham, M. K., Kurlents, E., Lane, R., Mulstock, B. & Traikov, H. (1960) *Can. J. Biochem. Physiol.* **38**, 1077–1085
- Bower, F. & Kitabchi, A. E. (1974) *Biochem. Biophys. Res. Commun.* **57**, 100–105
- Dazord, A., Gallet, D. & Saez, J. M. (1975) *Horm. Metab. Res.* **7**, 184–189
- Farese, R. V. (1971) *Science* **173**, 447–450
- Farese, R. V. & Prudente, W. J. (1978) *Endocrinology* **103**, 1264–1271
- Gasko, O. D., Knowles, A. F., Shertzer, H. G., Suolinna, F. M. & Racker, E. (1976) *Anal. Biochem.* **72**, 57–65
- Gerisch, G. & Wick, U. (1975) *Biochem. Biophys. Res. Commun.* **65**, 364–370
- Haksar, A. & Peron, F. G. (1973) *Biochim. Biophys. Acta* **313**, 363–371
- Harding, B. W. & Nelson, D. H. (1964) *Endocrinology* **75**, 506–513
- Jaanus, S. D. & Rubin, R. P. (1971) *J. Physiol. (London)* **213**, 581–598
- Jaanus, S. D., Carchman, R. A. & Rubin, R. P. (1972) *Endocrinology* **91**, 887–895
- Jeffcoate, C. R. & Orme-Johnson, W. H. (1975) *J. Biol. Chem.* **250**, 4671–4677
- Kelly, L. A. & Koritz, S. B. (1971) *Biochim. Biophys. Acta* **237**, 141–155
- Kowal, J., Srinivasan, S. & Saito, T. (1974) *Endocrinol. Res. Commun.* **1**, 305–319

- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Lefkowitz, R. J., Roth, J. & Pastan, I. (1970) *Nature (London)* **228**, 864–866
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lymangrover, J. R. & Martin, R. (1978) *Life Sci.* **23**, 1193–1200
- Matthews, E. K. & Saffran, M. (1973) *J. Physiol. (London)* **234**, 43–64
- Neher, R. & Milani, A. (1976) *Clin. Endocrinol.* **5**, Supplement, 29S–39S
- Neher, R. & Milani, A. (1978a) *Mol. Cell. Endocrinol.* **9**, 243–253
- Neher, R. & Milani, A. (1978b) in *Hormones and Cell Regulation* (Dumont, J. & Nunez, J., eds.), vol. 2, pp. 71–73, North-Holland Publishing Co., Amsterdam
- Peron, F. G., Guerra, F. & McCarthy, J. L. (1965) *Biochim. Biophys. Acta* **110**, 277–289
- Pfeiffer, D. R. & Tchen, T. T. (1975) *Biochemistry* **14**, 89–97
- Podesta, E. J., Milani, A., Steffen, H. & Neher, R. (1979) *Biochem. J.* **180**, 355–363
- Rae, P. A., Gutman, N. S., Tsao, J. & Schimmer, B. P. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1896–1900
- Rimon, G., Hanski, E., Braun, S. & Levitzki, A. (1978) *Nature (London)* **276**, 394–396
- Sala, G. B., Hayashi, K., Catt, K. J. & Dufau, M. L. (1979) *J. Biol. Chem.* **254**, 3861–3865
- Salomon, Y., London, C. & Rodbell, M. (1974) *Anal. Biochem.* **58**, 541–548
- Sayers, G., Beall, R. J. & Seelig, S. (1972) *Science* **175**, 1131–1133
- Schwarzenbach, G., Senn, H. & Anderegg, G. (1957) *Helv. Chim. Acta* **40**, 1886–1900
- Sulimovici, S. & Boyd, G. S. (1967) *Biochem. J.* **103**, 16P–17P
- Warner, W. & Carchman, R. A. (1978) *Biochim. Biophys. Acta* **528**, 409–415
- Whysner, J. A., Paule, W. J., Nelson, D. H. & Harding, B. W. (1966) *Clin. Res.* **14**, 180
- Wickramasinghe, R. H. (1974) *Bull. Environ. Contam. Toxicol.* **12**, 99–103
- Yanagibashi, K., Kamiya, N., Lin, G. & Matsuba, M. (1978) *Endocrinol. Jpn.* **25**, 545–551