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Mechanism of smooth muscle relaxation by tiropramide

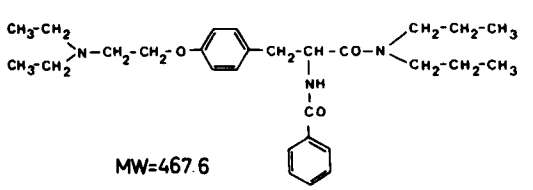
R. R. VIDAL Y PLANA, A. CIFARELLI* AND I. SETNIKAR

Rotta Research Laboratorium S.p.A. 20050 Monza, Milan, Italy

Experiments on rabbit isolated colon show that a carbachol-induced contraction is accompanied by a decrease in cAMP content of the smooth muscle. (±)-α-(Benzoylamino)-4-[2-(diethylamino)-ethoxy]-*NN*-di-propylbenzen-propanamid (tiropramide, CR 605), a new tyrosine derivative with antispastic properties, increases cAMP concentrations within the same dose range that produces smooth muscle relaxation with or without carbachol (0·1 μM). These effects are potentiated by 1 mM theophylline, a phosphodiesterase inhibitor. In a purified microsomal preparation from rabbit colon smooth muscle, corresponding to sarcoplasmic reticulum, tiropramide induced a dose-dependent increase Ca^{2+} binding in the presence of ATP and Mg^{2+}. Tiropramide inhibited phosphodiesterase activity in rabbit colon homogenates in a range of doses about ten times that producing relaxation, cAMP content enhancement and increase Ca^{2+} binding to sarcoplasmic reticulum. The effects of tiropramide on the carbachol-stimulated rabbit colon in the presence of theophylline, indomethacin or PGE_1 are more in agreement with an action of tiropramide as inhibitor of cAMP catabolism than as a result of a prostaglandin-mediated effect. These observations suggest that the smooth muscle relaxant activity of tiropramide arises from drug-induced increase of cAMP concentrations possibly because of inhibition of cAMP catabolism. This effect is accompanied by the binding to the sarcoplasmic reticulum of Ca^{2+}, preventing its interaction with the contractile proteins of the smooth muscle. A direct effect of tiropramide-enhanced cAMP content on contractile proteins in the smooth muscle cannot be excluded.

Cyclic AMP (cAMP) (Andersson 1972; Andersson & Nilsson 1972; Andersson et al 1975; Carsten 1969; Kukovetz et al 1975; Pöch & Kukovetz 1972; Somlyo et al 1972) and Ca^{2+} (Andersson et al 1972; Andersson & Nilsson 1972; Carsten 1969) are among the main final regulators of smooth muscle activity. Papaverine, a classical antispasmodic, is a potent inhibitor of phosphodiesterase activity in several tissues and this mechanism is common to many other smooth muscle relaxants (Pöch & Kukovetz 1971, 1972). Other "calcium antagonists', like verapamil and nifedipine, have been shown to inhibit the calcium activation systems in the membrane of smooth muscle cells (Golenhofen 1976). We set out to determine the mechanism of action of (±)-α-(benzoylamino)-4-[2-(diethylamino)-ethoxy]-*NN*-di-propylbenzenpropanamid (tiropramide, CR 605), a new tyrosine derivative showing a broad spectrum of antispasmodic activity (Makovec et al 1975; Senin et al 1978).

Tiropramide (CR-605)



MW=467.6

* Correspondence.

MATERIALS AND METHODS

Most reagent grade chemicals were purchased from Merck, Darmstadt, Germany, prostaglandin PGE_1 from Sigma, St. Louis, Missouri, U.S.A., theophylline from BDH, Poole, U.K., radioactive compounds from the Radiochemical Centre, Amersham, U.K., and ready for use scintillation cocktails Filter-Count and Dimilume-30 from Packard, Downers Grove, Illinois, U.S.A.

Tiropramide, CR 605 (empirical formula: $C_{28}H_{41}N_3O_3$ with a molecular weight of 467·6) was supplied by the chemical laboratories of the Rotta Research Laboratorium S.p.A. The Krebs solution used (Andersson 1972), had the following composition (mM): 122 NaCl, 4·7 KCl, 2·6 $CaCl_2$, 1·2 $MgCl_2$, 15·4 $NaHCO_3$, 1·2 KH_2PO_2 and 11·5 glucose.

Measurement of Muscle Tension

Experiments were performed on rabbit (male New Zealand Whites, 1 kg) isolated colon muscle according to Andersson (1972).

Specimens were mounted in an organ bath filled with Krebs solution (Andersson 1972) maintained at

37 °C and continuously gassed with 95% oxygen + 5% CO_2. Muscular tension was recorded isometrically by an Ugo Basile (Comerio, Italy) force-displacement transducer, type 7003, on a Ugo Basile microdinamometer, type 7051.

Usually after a 2 min pre-incubation with the test drugs, a submaximal dose of carbachol (0·1 μM) was added; the drugs were washed out after a further 2 min with pre-warmed fresh Krebs solution for at least 3 min and until the complete recovery of basal motility. In the experiments using indomethacin, measurements were begun 15 min after its first addition; the drug was not washed out. When exogenous cAMP was used, the preparation was pre-incubated with the other compounds as described and cAMP was added with the carbachol.

Determination of cyclic AMP content

Transverse sections of rabbit colon, approximately 100 mg, were equilibrated for 1 h at 37 °C in aerated Krebs solution, then transferred to individual tubes containing 2 ml Krebs solution and the test drug (previously added) and maintained at 37 °C. At the indicated times, the tubes were transferred into a water bath (100 °C) for 5 min. The denaturated proteins were sedimented by centrifugation, and extracted cAMP determined in the supernatants with a specific protein binding assay (Gilman 1970) by using the cAMP assay kit (The Radiochemical Centre, Amersham, U.K.).

Protein determination

The method according to Lowry et al (1951), was used. Fast preliminary measurements were made with the Bio-Rad (Richmond, California) protein assay kit. All samples were dissolved in NaOH and then neutralized with HCl before assay. Bovine serum albumin was used as standard protein.

Estimation of phosphodiesterase activity

Phosphodiesterase activity was determined on rabbit colon homogenates, according to Andersson (1972), with radioactively labelled cAMP (10^{-4} M ^{3}H-cAMP) as substrate in the enzyme reaction. Protein concentration was 1 mg ml^{-1}. The rate of hydrolysis of labelled cAMP to labelled 5′-AMP was determined after removal of 5′-AMP by $ZnSO_4$ + $Ba(OH)_2$ precipitation, according to Pöch (1971).

Preparation of sarcoplasmic reticulum

The sarcoplasmic reticulum microsomal fraction was isolated, as described by Andersson et al (1972), from rabbit colon muscle by differential centrifugation and subsequent separation on a sucrose density gradient (Carsten 1969). Experiments were on the microsomal fraction that layered between 35 and 45% sucrose.

Calcium uptake by sarcoplasmic reticulum

The uptake of $^{45}Ca^{2+}$ by the sarcoplasmic reticulum was in an incubation mixture containing 1 mg ml^{-1} protein and 10^{-5} M calcium (specific activity: 1 mCi $mmol^{-1}$) at 37 °C. The rate of calcium uptake was measured by the filtration method of Carsten (1969), by filtering aliquots at specified times through Millipore (Bedford, Massachussets) filters, type HAMK-0·45 μm pore size, using as prefilters glass fibre filters, type GF/B (Whatman, Maidstone, Kent, U.K.). Blanks did not contain vesicular protein.

Two parallel series of samples, with and without 0·5 mM ATP were run. ATP-dependent counts were determined by difference between them. Calcium-binding was computed from the counts in the filtrates; no allowance being made for any intrinsic calcium in the preparations.

Mathematical analysis of experimental results

The ED50 of the drugs (50% of the maximal response with 95% confidence limits) were calculated by the log dose-probit method (Miller & Tainter 1944). The regression lines and the confidence limits were calculated according to Armitage (1973).

RESULTS AND DISCUSSION

Tiropramide caused dose-dependent inhibition of basal tone in rabbit isolated colon preparation (Fig. 1) in the same dose ranges effective in preventing the 0·1 μM carbachol-induced spasm, the ED50's of tiropramide being 47·5 (26·9–83·7) and 32·5 μM (18·2–58·1) respectively. The addition of 1 mM theophylline (Fig. 1) or 0·1 mM cAMP, potentiated the relaxant effect of tiropramide on the carbachol-induced spasm in rabbit colon giving ED50's of 1·4 (0·6–3·5) and 9·5 μM (3·8–23·8) respectively. By contrast, 10 μM indomethacin lowered the sensitivity to tiropramide, giving an ED50 of 42·7 μM (33·3–54·6). The difference between the ED50 values of tiropramide and tiropramide + theophylline on the carbachol-induced spasm is statistically significant ($P \leqslant 0.05$).

Fig. 2 shows, in a double-reciprocal plot, the effects of tiropramide on the carbachol-induced spasm of colon when constant amounts of other drugs were also given. The intercept of the regression line obtained for tiropramide alone and for tiro-

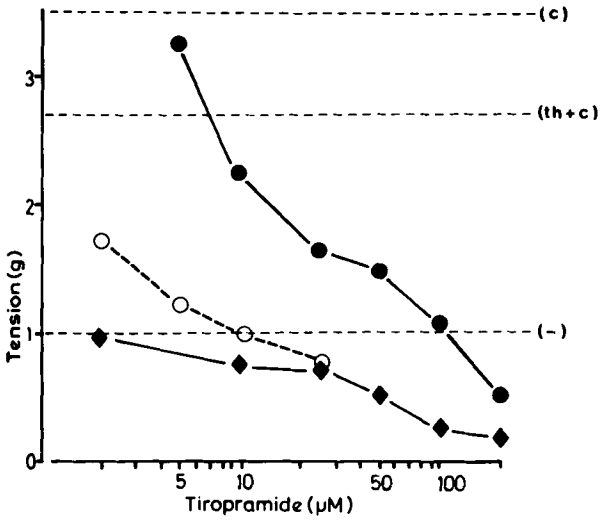


FIG. 1. Inhibition by tiropramide alone (◆) of the tension in the rabbit isolated colon and with 0·1 μM carbachol (●) stimulation, and influence of 1 mM theophylline (○) on it. Each point represents one experimental value. Tested concentrations of tiropramide lower or higher than those represented in this figure showed 0% or 100% effects.

pramide + 1 mM theophylline crosses close to the 1/E. axis. In contrast, the intercepts with the regression lines for tiropramide + 10 μM indomethacin or + 10 μM PGE_1 were away from the 1/E axis. The intercept of these two latter regression lines was close to 1/E axis.

Carbachol, 0·1 μM, lowered the cAMP content of the tissue as determined after an interval as short as 15 s after its addition, and the cAMP concentration remained depressed for not less than 5 min (Fig. 3).

Tiropramide induced a dose-dependent increase of cAMP within the dose range that influenced the tension of the isolated muscle, an effect not significantly influenced by carbachol. On addition of theophylline (1 mM) and the same doses of tiropramide to the tissues, the effects on cAMP concentrations were additive. Tiropramide produced a dose-dependent inhibition of phosphodiesterase activity in rabbit colon homogenates (Fig. 4) only at concentrations higher than those causing muscle relaxation. In a purified fraction of rabbit colon sarcoplasmic reticulum, tiropramide produced a dose-dependent increase of the Ca^{2+} binding, in the presence of ATP and Mg^{2+}, in the same dose range as relaxed the isolated organ (Fig. 5). Table 1 shows that theophylline, 0·1–1·0 mM induces a dose-dependent increase of cAMP in 0·1 μM carbachol-

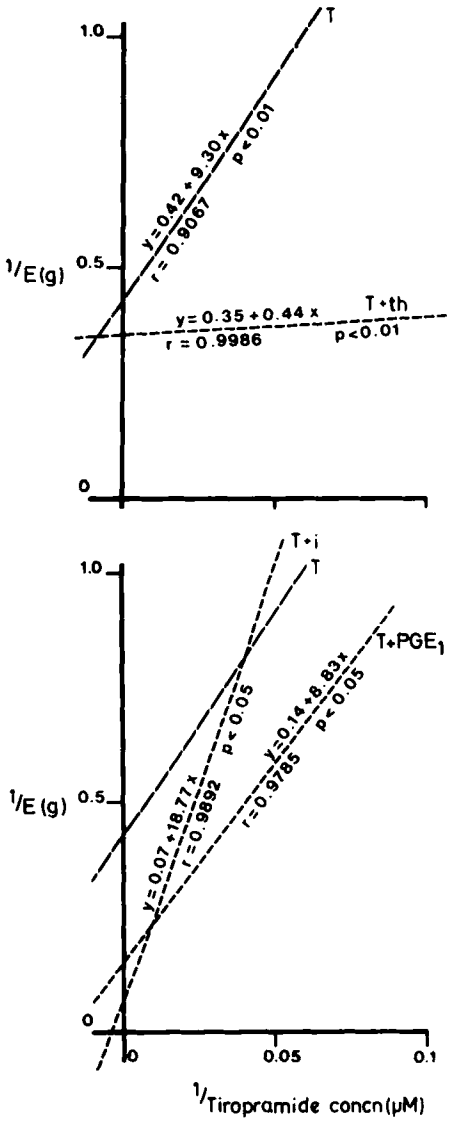


FIG. 2. Double-reciprocal plot of the activity of tiropramide (T) alone or in presence of 1 mM theophylline (th), PGE_1, or indomethacin (10 μM) (i), in preventing the 0·1 μM carbachol-induced spasm in the isolated rabbit colon. E = decrease of tension (g) enhancement (by 0·1 μM carbachol).

stimulated colon, but not in the unstimulated organ.

Basal tension is not influenced by theophylline but carbachol-stimulated contraction is enhanced by 0·1 mM and depressed by 1 mM theophylline.

Theophylline 0·1 mM, a dose ten times lower than that showing an antispastic effect on smooth muscle, potentiated the 0·1 μM carbachol-induced spasm, despite the marked increase of cAMP content

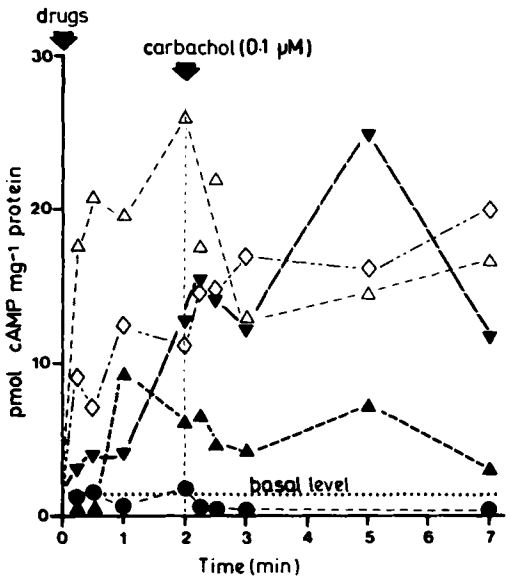


FIG. 3. Effects of tiropramide (▲ 25, ▼ 100 μM), theophylline (◇ 1 mM) and tiropramide + theophylline (25 μM + 1 mM) △ and 0·1 μM carbachol on the cAMP contents in rabbit colon. Each point represents one experimental value. ● none.

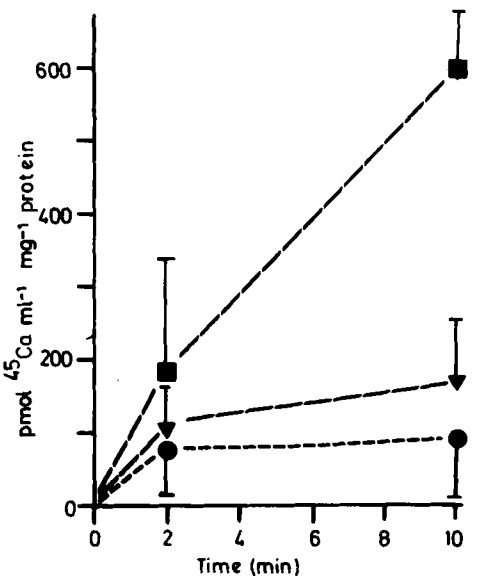


FIG. 5. Enhancement by tiropramide of the ATP-dependent Ca^{2+} uptake, measured in vitro, by a sarcoplasmic reticulum fraction purified from rabbit colon. Each value is the mean of three separate experiments. Vertical bars represent the standard error. Tiropramide ■, 100 μM, ▼ 10 μM. ● none.

observed (Table 1), thereby indicating the existence of other unknown more complex mechanisms, as suggested by Andersson (1972).

Exogenously added cAMP 0·1 mM, showed an inhibitory effect on the 0·1 μM carbachol-induced spasm and potentiated the antispastic activity of tiropramide. The high concentration of exogenous cAMP needed, according to Andersson (1972), is probably due to its slow penetration of the cell membrane and rapid hydrolysis by phosphodiesterases.

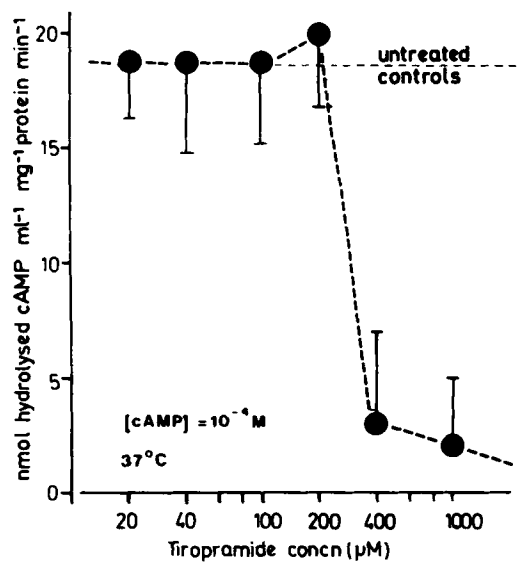


FIG. 4. Inhibition of phosphodiesterase activity in rabbit colon homogenates by tiropramide. Each value is the mean of three separate experiments. Vertical bars represent the standard error.

Prostaglandins stimulated adenylate cyclase activity in smooth muscle and other tissues (Shaw et al 1972; Butcher & Baird 1968; Kuehl et al 1972). Both 10 μM PGE_1 and 10 μM indomethacin, an inhibitor of prostaglandin synthesis (Flower 1974), influenced the inhibitory effect of tiropramide on the 0·1 μM carbachol-induced spasm of rabbit colon (Fig. 2). The intercepts, in a double reciprocal plot

Table 1. Effect of theophylline on the tension (isometrically measured) and cAMP contents of the rabbit colon, before and after 0·1 μM carbachol stimulation. Reported values are averages of two measurements.

0·1 μM carbachol	theophylline mM	tension g	cAMP pmol mg^{-1} protein
	—	1·0	2·65
–	0·1	1·0	6·02
	1·0	1·0	5·51
	—	3·5	1·85
+	0·1	4·7	7·09
	1·0	2·7	10·09

(Lineweaver & Burk 1934; Lehninger 1975) of the regression line for tiropramide alone and when additional constant doses of theophylline, indomethacin or PGE_1 were also given, may indicate that tiropramide inhibits the carbachol-induced spasm of rabbit colon by acting on the same mechanisms as are affected by theophylline but not prostaglandin.

However, according to Lineweaver & Burk (1934), the double-reciprocal plot indicates which mechanism or mechanisms may be involved, while not necessarily proving the mechanism involved in given cases. In fact the rate of onset of the theophylline-induced effect on cAMP content is faster than that induced by tiropramide (Fig. 3) and the addition of 0·1 μM carbachol depresses the cAMP concentrations in the presence of tiropramide, but not of theophylline. These results may indicate a similar rather than identical mode of action. Also the effects of the prostaglandins on smooth muscle tone, according to Eckenfels & Vane (1972), probably vary in different organs.

The in vitro dose-dependent inhibition of phosphodiesterase activity in rabbit colon homogenates by tiropramide (Fig. 4) occurred at higher doses than those producing an increase of cAMP content when the drug was given to the whole smooth muscle (Fig. 3). Under the same experimental conditions, 100 μM papaverine totally inhibited the phosphodiesterase activity in agreement with Pöch & Kukovetz (1971, 1972). Papaverine exerted its smooth muscle-relaxing effect in concentrations that were comparable to those that inhibited phosphodiesterase activity in vitro, but other weak phosphodiesterase inhibitors, like etafenone, were effective at concentrations from 10 to 100 fold lower than those needed for in vitro inhibition (Pöch & Kukovetz 1972). Our results suggest that tiropramide belongs to this second category of antispastic drugs.

The release and storage of calcium in the sarcoplasmic reticulum (Carsten 1969) regulates relaxation and contraction in skeletal, heart and smooth muscle. Andersson et al (1972) presented further evidence regarding a connection between cAMP and Ca^{2+} metabolism in intestinal smooth muscle, and showed that cAMP and drugs relaxing smooth muscle via cAMP, increased the Ca^{2+} binding capacity of a subcellular microsomal fraction isolated from rabbit colon.

Our results on tiropramide (Fig. 5) support Andersson's theory: tiropramide, in presence of Mg^{2+} and ATP, caused a dose-dependent increase of the binding of Ca^{2+} to a microsomal fraction purified from rabbit colon, corresponding to the sarcoplasmic reticulum, within the same dose ranges which relax the isolated organ.

The exact mechanism by which cAMP controls calcium storage or release in the smooth muscle sarcoplasmic reticulum is not known: a recent report by Tada et al (1979) supports the existence of a regulatory protein controlling the active calcium transport by sarcoplasmic reticulum, the activity of this protein being dependent upon its phosphorylation by a cAMP-dependent protein-kinase.

Recent data by Adelstein et al (1978) also suggest that cAMP has a direct effect on actin-myosin interaction in smooth muscle by phosphorylation of smooth muscle myosin light chain kinase via a cAMP-dependent protein kinase.

Therefore, from our experimental results on rabbit colon, we conclude that tiropramide relaxes smooth muscle by inducing an increase of the cAMP content of the tissue and enhancing the capacity of the sarcoplasmic reticulum to bind Ca^{2+}, in the presence of ATP and Mg^{2+}, an activity which has been said to be dependent upon increased cAMP concentrations (Andersson et al 1972). Thus, the concentration of free myoplasmic Ca^{2+} able to interact with the contractile proteins of the smooth muscle is reduced. Moreover, according to the hypothesis of Adelstein et al (1978), a direct effect of the tiropramide-enhanced cAMP content on the interaction of contractile proteins in the smooth muscle cannot be excluded.

REFERENCES

Adelstein, R. S., Conti, M. A., Hathaway, D. R., Klee, C. B. (1978) J. Biol. Chem. 253: 8347–8350

Andersson, R. (1972) Acta Physiol. Scand. Suppl. 382: 1–59

Andersson, R., Lundholm, J., Mohme-Lundholm, E., Nilsson, K. (1972) in: Greengard, P., Robinson, G. A., Paoletti, R. (eds) Advances in Cyclic Nucleotide Research Vol. I, Raven Press, New York, pp 213–229

Andersson, R., Nilsson, K. (1972) Nature New Biol. 238: 119–120

Andersson, R., Nilsson, K., Wikberg, J., Johansson, S., Mohme-Lundholm, E., Lundholm, L. (1975) in: Drummond, G. I. et al (eds) Advances in Cyclic Nucleotide Research Vol. 5, Raven Press, New York, pp 491–518

Armitage, P. (1973) in: Medical Research, Blackwell, Oxford

Butcher, R. W., Baird, C. E. (1968) J. Biol. Chem. 243: 1713–1717

Carsten, M. E. (1969) J. Gen. Physiol. 53: 414–426

Eckenfels, A., Vane, J. R. (1972) Br. J. Pharmacol. 45: 451–462

Flower R. J. (1974) Pharmacol. Rev. 26: 33–67

Gilman, A. G. (1970) Proc. Natl. Acad. Sci. USA 67: 305–312

Golenhofen, K. (1976) in: Bulbring, E., Shuba, M. F. (eds) Physiology of Smooth Muscle, Raven Press, New York, pp 197–202

Kuehl, F. A., Hames, J. L., Cirillo, V. J., Harm, E. A. (1972) in: Greengard, P. et al (eds) Advances in Cyclic Nucleotide Research Vol. 1, Raven Press, New York, pp 493–502

Kukovetz, W. R., Pöch, G., Wurm, A. (1975) in: Drummond, G. I. et al (eds) Ibid. Vol. 5, pp 395–414

Lehninger, A. L. (1975) Biochemistry, Worth Publishers, New York

Lineweaver, H., Burk, D. (1934) J. Am. Chem. Soc. 56: 658–666

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) J. Biol. Chem. 193: 265–285

Makovec, F., Rovati, A. L., Senin, P. (1975) Chem. Abs. 83: 193728

Miller, L. C., Tainter, M. L. (1944) Proc. Soc. Exp. Biol. Med. 57: 261–264

Pöch, G. (1971) Naunyn-Schmiedeberg's Arch. Pharmacol. 268: 272–299

Pöch, G., Kukovetz, W. R. (1971) Life Sci. 10: 133–144

Pöch, G., Kukovetz, W. R. (1972) in: Greengard, P., Robinson, G. A., Paoletti, R. (eds) Advances in Cyclic Nucleotide Research Vol. 1, Raven Press, New York, pp 195–211

Senin, P., Makovec, R., Rovati, A. L., Casula, P. L. (1978) in: Duthie, H. L. (ed.) Gastrointestinal Motility in Health and Disease. MTP Press Limited, Lancaster, England, pp 417–427

Shaw, J. E., Jessup, S. J., Ramwell, P. W. (1972) in: Greengard, P. et al (eds) Advances in Cyclic Nucleotide Research. Vol. 1, Raven Press, New York, pp 479–491

Somlyo, A. P., Somlyo, A. V., Smieslo, V. (1972) in: Greengard, P. et al (eds) Ibid. pp 479–491

Tada, M., Ohmori, F., Yamada, M., Abe, H. (1979) J. Biol. Chem. 254: 319–326