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Dilazep: an inhibitor of adenosine uptake with intrinsic calcium antagonistic properties

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Concentrations of dilazep which were ineffective in altering the muscular tone of the guinea-pig taenia caeci (0·03, 0·3 μM) or the phasic mechanical activity of the rabbit proximal ileum (0·03 μM) markedly potentiated the inhibitory action of adenosine on both these parameters. Dilazep, 0·3 μM or greater, dose-dependently inhibited the mechanical activity of the proximal ileum. This inhibitory action was probably mediated by more than one mechanism, as shown by the fact that theophylline (50, 100 μM) antagonized the effect at lower dilazep concentrations (up to 3 μM) leaving essentially unchanged the response to higher concentrations (6, 10 μM). Similarly, the responses to low doses of dilazep were reduced after desensitization of the organ to adenosine, whilst the responses to higher doses were unaffected by this procedure. In a Ca^{2+}-free, high-K^+ medium, dilazep (1–10 μM) caused a parallel shift to the right of the Ca^{2+}-induced contractions of the guinea-pig taenia caeci. Adenosine showed only slight Ca^{2+}-antagonistic properties within the mM range of concentrations. These findings suggest that, at the higher concentrations tested, dilazep exhibits Ca^{2+}-antagonistic properties unrelated to its adenosine-mediated mode of action.

Dilazep is a tertiary diamine derivative of trimethoxybenzoic acid which possesses coronary vasodilator action both in animals and in man (Hilger et al 1968; Rau et al 1968; Nonaka & Ueno 1978). This action is assumed to be mainly adenosine-mediated. In fact, the drug blocks the uptake of adenosine into cardiac cells (Mustafa 1979), enhances the responsiveness of coronary vascular smooth muscle to exogenous adenosine (Buyniski et al 1972) and induces a dilatation of small coronary vessels which is prevented by the adenosine antagonist theophylline (Spieckermann et al 1969). An additional mode of action is suggested by the recent finding that the drug is able to cause a sustained dilatation of the human large coronary vessels (L'Abbate et al 1981) which are known to be insensitive to adenosine (Rubio et al 1979). In this respect, the observation that dilazep possesses local anaesthetic (Tamura et al 1974) and Ca^{2+}-antagonist properties (Tamura et al 1975) is of particular interest. The latter effect, however, is not necessarily independent of adenosine modulation since adenosine can decrease transmembrane Ca^{2+} movements in vascular (Schnaar & Sparks 1972; Herlihy et al 1976) and atrial muscles (Schrader et al 1975) probably by interacting with specific adenosine receptor sites (Schrader et al 1977).

The aims of this study were to re-assess the adenosine-mediated action of dilazep on guinea-pig taenia caeci and rabbit proximal ileum and to evaluate the nature of the Ca^{2+}-antagonistic action of the drug. To differentiate dilazep activity from that of adenosine, Ca^{2+} antagonism was assessed by using a preparation (e.g. guinea-pig taenia caeci in Ca^{2+}-free, K^+-depolarizing Tyrode solution) in which Ca^{2+}-induced contractions are expected to be mediated via voltage-dependent channels (Spedding 1982).

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MATERIALS AND METHODS

Guinea-pigs and rabbits of either sex (350–450 and 1500–1800 g respectively) were killed by cervical dislocation and bleeding. Intestinal preparations were set up as described below, and left to equilibrate for at least 30 min before any drug addition.

Guinea-pig

Strips of taenia (2·5–3 cm) dissected from the caecum were used to evaluate the potentiating effect of dilazep on adenosine responses and to test the Ca^{2+}-antagonistic properties of the drug. In the adenosine experiments the preparation was mounted isometrically (tension applied 0·8 g) in a 10 ml organ bath containing Tyrode solution kept at 36 °C, and bubbled continuously with a mixture of 95% O_2 + 5% CO_2. Since adenosine did not cause desensitization in this preparation, cumulative concentration-relaxation curves were obtained in strips submaximally contracted by previous administration of carbachol (25 nM). Adenosine was added immediately after a steady response to the preceding concentrations had been obtained. The response to a

given dose of adenosine was calculated as percentage of the maximal relaxation induced by 200 μM adenosine. Dose-response curves for adenosine were repeated in the presence of dilazep (0·03, 0·3 μM: incubation period 20 min) which by themselves were ineffective in altering the muscular tone.

In the experiments designed to assess the Ca^{2+}-antagonistic effect of dilazep, the same preparation was set up isotonically but with a tension of 1 g in a 20 ml organ bath containing Ca^{2+}-free, K^+-depolarizing Tyrode solution. Cumulative concentration-response curves to $CaCl_2$ (0·03–3 mM) were obtained by increasing the Ca^{2+} concentration at 3 min intervals. A 20 min washout was allowed between curves. The 100% response was taken as the maximum contractile response of the strip during the second concentration-response curve, and all subsequent contractions were calculated as a percentage of this value (Spedding 1982). Dose-ratios were calculated at the EC50 level with and without dilazep (1–10 μM). The apparent pA_2 value was calculated by the method of Arunlakshana & Schild (1959). In experiments evaluating the Ca^{2+}-antagonistic effect of adenosine, the nucleoside (1, 1·5 mM) was added 2 min before, to assess the Ca^{2+}-induced contractions.

Rabbit

Segments of proximal ileum (2–2·5 cm) were suspended isometrically (tension applied 1 g) in a 50 ml organ bath containing Tyrode solution kept at 37 °C and gassed with oxygen (95%) and carbon dioxide (5%). Concentration-response curves for the inhibitory effect of adenosine on spontaneous phasic contractions of the longitudinal muscle were obtained by adding increasing concentrations of the nucleoside to the bath. A 10 min washing after each drug addition prevented desensitization (Weston 1973; McKenzie et al 1977). When concentration-response curves were obtained in the presence of dilazep (0·03 μM: incubation period 20 min), the drug was added to the rinse fluid at the same concentration (dilazep at this concentration did not modify the mechanical activity of the ileum). Responses to adenosine in the absence and in the presence of dilazep were calculated as percentage of inhibition of the basal phasic contractions of the longitudinal muscle.

At concentrations higher than 0·03 μM dilazep decreased the amplitude of phasic contractions. Cumulative concentration-response curves for dilazep (0·3–10 μM) were obtained by increasing the drug concentration stepwise. To assess whether the inhibitory effect of dilazep was mediated by its potentiating action on endogenous adenosine, concentration-response curves were repeated (a) after the preparation had been exposed to theophylline (50, 100 μM) or (b) immediately after the preparation had been desensitized to adenosine by adding adenosine repeatedly at 1–2 min intervals (1, 5 and 10 μM, 4, 3, and 3 times respectively). The maximum inhibitory effect (about 70% inhibition of basal contractions) was achieved after the first administration (1 μM). Thereafter, the amplitude of the mechanical activity recovered to 80–90% of the control value in spite of the subsequent additions of larger adenosine concentrations. Responses to dilazep before and after theophylline exposure or adenosine desensitization were calculated as percentage inhibition of the basal contractions.

Statistical analysis

Student's *t*-test was used for comparisons of mean values. Values here refer to mean ± s.e.m.

Solutions and drugs

The Tyrode solution had the following composition (mM): NaCl 136·9, KCl 2·7, $CaCl_2$ 1·8, $MgCl_2$ 1·04, $NaHCO_3$ 11·9, NaH_2PO_4 0·4, glucose 5·5. The composition of the Ca^{2+}-free, high-K^+ Tyrode solution was (mM): NaCl 97, KCl 40, $NaHCO_3$ 11·9, NaH_2PO_4 0·4, glucose 5·5. The following drugs were used: dilazep dihydrochloride (Schering), adenosine (BDH), theophylline (BDH), carbamylcholine chloride (carbachol) (Sigma), (±)-propranolol hydrochloride (ICI), piperoxan hydrochloride (Rhône-Poulenc) and tetrodotoxin (Sankyo).

RESULTS

Assessment of the effect of adenosine and dilazep in the guinea-pig taenia caeci

In the guinea-pig taenia caeci, adenosine (1–80 μM) caused a dose-dependent decrease of the muscular tone. At relatively low concentrations (0·03, 0·3 μM) dilazep had no effect on the tone but was found after 20 min exposure to cause a dose-dependent displacement to the left of the adenosine concentration-response curve (Fig. 1). The pD_2 values for adenosine were increased from 5·05 ± 0·07 in the control state to 5·45 ± 0·10 ($P < 0·05$) and to 6·04 ± 0·10 ($P < 0·01$) in the presence of dilazep 0·03 and 0·3 μM respectively (n = 7 in all experiments).

Concentrations of dilazep ranging from 0·6 to 10 μM caused a slight dose-dependent inhibition of the muscular tone. These responses were not affected by pretreating the preparation for 10 min with

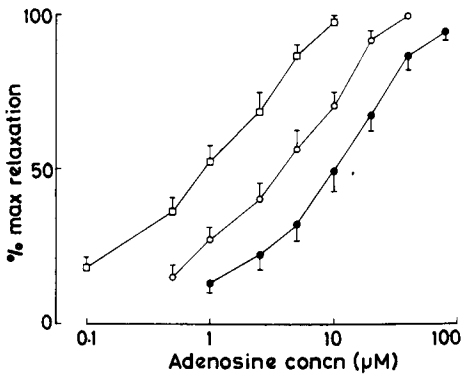

FIG. 1. Adenosine dose-response curves in the guinea-pig taenia caeci, in the absence (●) and in the presence of dilazep 0·03 μM (○) and 0·3 μM (□) respectively. Ordinate muscular relaxation expressed as percent of the maximal response induced by 200 μM adenosine. Each point represents the mean (± s.e.m.) of 7 experiments.

tetrodotoxin (0·7 μM) or with a combination of piperoxan (3·7 μM) and propranolol (3·3 μM). The possible role of endogenous adenosine in mediating the dilazep-induced responses in the taenia caeci could not be investigated under our experimental conditions, because this organ does not undergo adenosine desensitization (at least for the nucleoside concentrations used in the present study) and because theophylline has been shown to be a poor adenosine antagonist in this preparation (Small & Weston 1979).

Assessment of the effect of adenosine and dilazep in the rabbit ileum

In the longitudinal muscle coat of the rabbit ileum, adenosine (0·1–3 μM) caused a dose-dependent inhibition of the amplitude of spontaneous phasic contractions. As observed for the taenia caeci, dilazep at a low concentration (0·03 μM) had no effect on mechanical activity but displaced to the left the adenosine-concentration response curve (pD_2 6·18 ± 0·08 in the control state vs 6·57 ± 0·10 after dilazep, n = 7, $P < 0·05$).

At concentrations ranging from 0·3 to 10 μM, dilazep (added to the medium at 3–4 min intervals) caused a dose-dependent submaximal inhibition of the amplitude of phasic contractions. The maximal effect (suppression of mechanical activity) was obtained with 50 μM dilazep. The effect of the drug was not affected by pretreatment of the preparation with tetrodotoxin (0·7 μM) or a combination of piperoxan (3·7 μM) and propranolol (3·3 μM). Theophylline (an effective adenosine antagonist in this preparation: Ally & Nakatsu 1976; Small & Weston 1979) at 50 and 100 μM significantly reduced, after a 30 min exposure, the responses to the lower dilazep concentrations (up to 3 μM) leaving essentially unchanged those to higher (6, 10 μM) concentrations (Fig. 2). Similar results were obtained after the organ had been desensitized to adenosine. In fact, desensitization resulted in depression of responses to the low dilazep concentrations but had no effect on the inhibition of phasic contractions seen at the higher concentrations (Fig. 3). This finding could not be ascribed to a temporal change (i.e. reduction) in the degree of desensitization (Crema et al 1982) for at least two reasons. First, the response curves obtained after desensitization were constructed by using a random sequence of dilazep doses. Second, no more than two doses of dilazep were tested for each preparation which meant that the determination of the responses to the drug were carried out within a short time from the desensitization procedure.

Assessment of Ca^{2+}-antagonistic properties of dilazep and adenosine

Guinea-pig taenia caeci strips equilibrated in Ca^{2+}-free, K^+-depolarizing medium, dose-dependently contracted in response to cumulative additions of $CaCl_2$ (0·03–3 mM). A steady response to a given Ca^{2+} concentration was usually achieved within 3 min. This was the minimum interval between two consecutive administrations. Concentrations of dilazep lower than 1 μM did not show any antagonistic effect on Ca^{2+} responses.

Pretreatment of the preparation with 1, 5, 10 μM dilazep (20 min exposure) resulted in a displacement

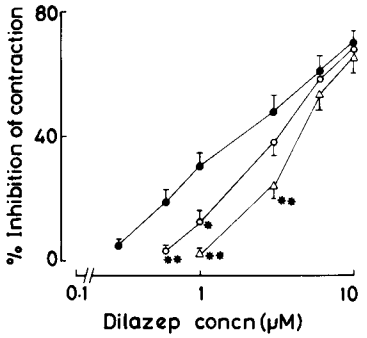

FIG. 2. Dilazep dose-response curves in the rabbit proximal ileum, in the absence (●) and in the presence of theophylline 50 μM (○) and 100 μM (△) respectively. Ordinate: submaximal decrease in amplitude of spontaneous phasic activity of the longitudinal coat (expressed as percent of the pretreatment value). Each point represents the mean (± s.e.m.) of at least 5 experiments. * $P < 0·05$; ** $P < 0·01$.

of the Ca^{2+} curves to the right in a concentration-related manner without depression of the maximum response (Fig. 4). The effect of dilazep (1 and 5 μM) was reversed by washing the preparations with Ca^{2+}-free, K^+-Tyrode for 30–60 min. However, in 3 out of 7 strips the effect of 10 μM dilazep was not completely reversed despite prolonged (>60 min) rinsing. The slope of the Arunlakshana & Schild plot (−1·26 ± 0·28) was not significantly different from −1, expected for competitive antagonism. Determination of the apparent pA_2 gave a value of 5·74 ± 0·09. Adenosine also displaced to the right the Ca^{2+} dose-response curve. The minimal concentration of the nucleoside at which Ca^{2+}-antagonism was apparent was 1 mM. The degree of antagonism was, in any case, relatively small (dose ratios 1·36 ± 0·14, n = 5, and 2·18 ± 0·40, n = 5, at adenosine concentrations of 1 and 1·5 mM respectively). No Ca^{2+}-antagonistic effect was seen when subliminal (0·1, 0·2 mM) doses of adenosine were tested after pretreatment with dilazep at a concentration (0·3 μM) that had been shown to cause a ten-fold increase in adenosine potency in taenia strips bathed in normal medium.

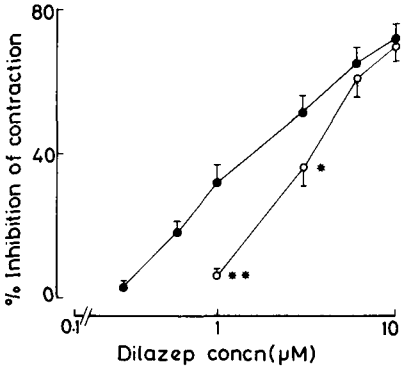

FIG. 3. Dilazep dose-response curves in the rabbit proximal ileum, before (●) and after desensitization to adenosine (○). Ordinate: submaximal decrease in amplitude of spontaneous phasic activity of the longitudinal muscle coat (expressed as percent of the pretreatment value). Each point represents the mean (± s.e.m.) of at least 5 experiments. * $P < 0·05$; ** $P < 0·01$.

In strips submaximally contracted by 0·1 mM Ca^{2+}, addition of dilazep (10 μM) caused rapid relaxation. The time required to obtain 50% of the maximum response (t50) was 3·3 ± 0·2 min (n = 5), with complete recovery of the basal (pre-Ca^{2+}) tone being reached in 15 min. The reversal of the response to 0·1 mM Ca^{2+} after changing the bathing medium to Ca^{2+}-free, K^+-Tyrode (Spedding 1982) exhibited a similar time course (t50 3·1 ± 0·2 min,

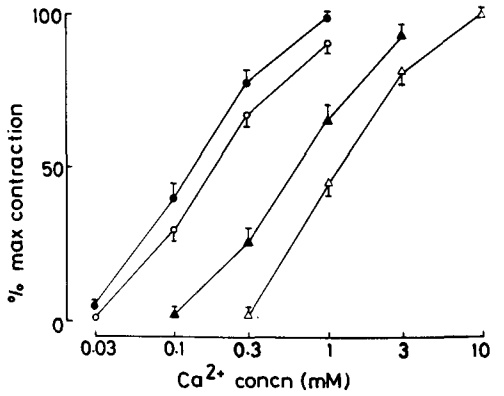

FIG. 4. Ca^{2+} dose-response curves in the guinea-pig taenia caeci, in the absence (●) and in the presence of dilazep 1 μM (○), 5 μM (▲) and 10 μM (△ respectively. Ordinate: increase in muscular tone expressed as percentage of the maximal response to Ca^{2+} (3 mM). Each point represents the mean (± s.e.m.) of 6 experiments.

n = 5, time necessary to full relaxation about 15 min) (Fig. 5). In the same model, adenosine (0·1, 1 mM) also caused relaxation of strips submaximally contracted by Ca^{2+}. As shown in Fig. 5, the response was relatively small with the 0·1 mM dose (maximum relaxation in tone 18·4 ± 2·0%, n = 5) but was appreciably greater with the larger dose (maximum reduction in tone 79 ± 4%, n = 5). The time course of the effect was markedly different from that observed with dilazep; not only did the peak relaxation occur earlier (t50 0·6 ± 0·1 min at 1 mM) but also the duration of action was shorter, with full or partial recovery of the initial (Ca^{2+}-induced) tone occurring during the 15 min observation period.

DISCUSSION

As previously shown for other intestinal (Gustafsson et al 1977) and non-intestinal structures (Buyniski et al 1972; Mustafa 1979), dilazep at low concentrations (0·03, 0·3 μM in the taenia caeci and 0·03 μM in the proximal ileum) markedly increased the inhibitory action of exogenously applied adenosine in both preparations studied. Neither the muscular tone (taenia caeci) nor the spontaneous phasic mechanical activity (ileum) was affected by these concentrations, while higher dilazep doses caused a dose-dependent inhibition of both parameters. These inhibitory responses to dilazep were not adrenergic in origin and were not nerve-mediated since they persisted unchanged in the presence of tetrodotoxin.

In the rabbit ileum, theophylline antagonized the responses to low doses of dilazep only suggesting that the relaxation seen at higher doses of dilazep were

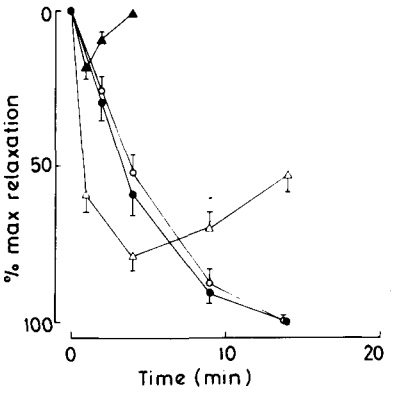

FIG. 5. Comparison of the relaxant effect of dilazep (10 μM ○) and adenosine (0·1 mM ▲; 1 mM △) with the effect of changing to a Ca^{2+}-free, K^+-Tyrode solution (● control relaxation) on established submaximal contraction to Ca^{2+} (0·1 mM) in the guinea-pig taenia caeci. Ordinate: degree of muscular relaxation (complete recovery of the tone observed before Ca^{2+} addition was taken as 100% response). Each point represents the mean (± s.e.m.) of 5 experiments.

unrelated to potentiation of endogenous adenosine. Confirmation of such an hypothesis could be obtained in ileal preparations densensitized to adenosine. In fact, the desensitization procedure (Weston 1973; MacKenzie et al 1977) reduced the responsiveness of the preparation to lower doses of dilazep but did not modify the response seen at high doses.

In the K^+-depolarized guinea-pig taenia caeci, dilazep (1–10 μM) showed Ca^{2+}-antagonistic properties as demonstrated by the fact that pretreatment of the preparation with the drug caused a parallel displacement to the right of the concentration-response curve to Ca^{2+}-induced contractions. The shift was consistent with a 'competitive' type of antagonism with the slope of the Arunlakshana & Schild (1959) plot being close to the hypothetical value of −1. In a separate series of experiments, dilazep was found to relax K^+-depolarized strips submaximally contracted by previous addition of Ca^{2+}. The time course of this relaxation was similar to that observed with diltiazem and verapamil in a previous study aimed at differentiating antagonist sub-groups and conducted under the same experimental conditions (Spedding 1982). Thus, dilazep behaves as a typical Ca^{2+}-antagonist, even though its potency is lower than that of the above mentioned drugs.

These experiments clearly rule out the possibility that the Ca^{2+}-antagonistic properties of dilazep in our system were exerted through an adenosine-mediated mechanism. Indeed, in a preparation like the K^+-depolarized taenia caeci, which is particularly suitable for assessing the antagonism on voltage-dependent Ca^{2+}-channels (Golenhofen et al 1977; Bolton 1979; Brading & Sneddon 1980), adenosine showed only slight Ca^{2+}-antagonistic properties (and only at very high concentrations) and behaved differently from dilazep in relaxing preparations submaximally contracted by Ca^{2+}. Moreover, the antagonistic action of the nucleoside was not enhanced by concentrations of dilazep which in separate experiments had been shown to potentiate the effects of exogenously applied adenosine in taenia strips bathed in normal medium. The intrinsic Ca^{2+}-antagonistic effect of dilazep explains the theophylline-resistant inhibition of the spontaneous mechanical activity in rabbit ileum. In fact, it is known that Ca^{2+}-antagonists such as nifedipine and verapamil are especially effective in suppressing the phasic components of mechanical activity in spontaneous active smooth muscle preparations (Golenhofen 1981).

The use of inhibitors of adenosine uptake in the treatment of coronary artery disease is justified by the fact that they potentiate the vasodilatory action of adenosine released from the hypoxic myocardium or during excessive β-adrenergic stimulation (Gerlach et al 1979); this dilatation, however, is confined to small resistance vessels of the coronary bed (Needleman & Johnson 1980). The Ca^{2+}-antagonistic properties of dilazep may explain its additional vasodilatory action on large coronary vessels as demonstrated in animals (Buyniski et al 1972) and in man (L'Abbate et al 1981). These vessels are known to be insensitive to adenosine and to be dilated electively by Ca^{2+}-antagonistic drugs (Fleckenstein 1977; Hillis & Braunwald 1978).

REFERENCES

Ally, A. I., Nakatsu, K. (1976) J. Pharmacol. Exp. Ther. 19: 208–215

Arunlakshana, O., Schild, H. O. (1959) Br. J. Pharmacol. Chemother. 14: 48–58

Bolton, T. B. (1979) Physiol. Rev. 59: 606–718

Brading, A. F., Sneddon, P. (1980) Br. J. Pharmacol. 70: 229–240

Buyniski, J. P., Losada, M., Bierwagen, M. E., Gardier, R. W. (1972) J. Pharmacol. Exp. Ther. 181: 522–528

Crema, A., D'Angelo, L., Frigo, G. M., Lecchini, S., Onori, L., Tonini, M. (1982) Br. J. Pharmacol. 75: 311–318

Fleckenstein, A. (1977) Ann. Rev. Pharmacol. Toxicol. 17: 149–166

Gerlach, E., Schrader, J., Nees, S. (1979) in: Baer, H. P., Drummond, G. I. (eds) Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides. Raven Press, New York, pp 127–136

Golenhofen, K. (1981) in: Bülbring, E., Brading, A. F., Jones, A. W., Tomita, T. (eds) Smooth Muscle: an assessment of current knowledge. Edward Arnold, London, pp 157–170

Golenhofen, K., Wagner, B., Weston, A. H. (1977) in: Casteels, R., Godfraind, T., Rüegg, J. C. (eds) Excitation-contraction coupling in Smooth Muscle. Elsevier, Amsterdam, pp 131–136

Gustafsson, L., Hedqvist, P., Fredholm, B. B. (1977) Acta Pharmacol. Toxicol. 41: 49

Herlihy, J. T., Bockman, E. L., Berne, R. M., Rubio, R. (1976) Am. J. Physiol. 230: 1239–1243

Hilger, H. H., Behrenbeck, D. W., Hellwig, H., Wagner, J. (1968) Pharmacol. Clin. 1: 77–89

Hillis, L. D., Braunwald, E. (1978) New Eng. J. Med. 13: 695–702

L'Abbate, A., Simonetti, I., Trivella, M. G. (1981) Atti del XLII Congresso Italiano di Cardiologia, Rome May 23–26, Abstract, pp 241–249

McKenzie, S. G., Frew, R., Bär, H. P. (1977) Eur. J. Pharmacol. 41: 183–192

Mustafa, S. J. (1979) Biochem. Pharmacol. 28: 2617–2624

Needleman, P., Johnson, E. M. (1980): in: Goodman Gilman, A., Goodman, L. S., Gilman, A. (eds) The Pharmacological Basis of Therapeutics. 6th edn, Macmillan, New York, pp 819–833

Nonaka, H., Ueno, A. (1978) Appl. Pharmacol. 16: 863–870

Rau, G., Tauchert, M., Bruckner, J. B., Eberlein, H. J., Bretschneider, H. J. (1968) Verh. Deut. Ges. Kreislaufforsch. 34: 385–393

Rubio, R., Belardinelli, L., Thompson, C. I., Berne, R. M. (1979) in: Baer, H. P., Drummond, G. I. (eds) Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides. Raven Press, New York, pp 167–182

Schnaar, R. L., Sparks, H. V. (1972) Am. J. Physiol. 223: 223–228

Schrader, J., Nees, S., Gerlach, E. (1977) Pfluegers Arch. 369: 251–257

Schrader, J., Rubio, R., Berne, R. M. (1975) J. Mol. Cell. Cardiol. 7: 427–433

Small, R. C., Weston, A. H. (1979) Br. J. Pharmacol. 67: 301–308

Spedding, M. (1982) Naunyn-Schmiedeberg's Arch. Pharmacol. 318: 234–240

Spieckermann, P. G., Hellberg, K., Kettler, D., Reploh, H. D., Strauer, B. (1969) Pfluegers Arch. 312: R15–R16

Tamura, H., Nakamura, M., Wada, S., Kusaji, T., Hayashi, H., Yamauchi, Y., Ishii, T., Kirihara, J. (1974) The Clinical Report 8: 1354–1373

Tamura, H., Shirosawa, Y., Hori, H., Kondo, A. (1975) Folia Pharmacol. Jpn. 71: 757–768

Weston, A. H. (1973) Br. J. Pharmacol. 48: 302–308