Physiol. Res. 47: 463–470, 1998

Time Course of Isolated Rat Fundus Response to Muscarinic Agonists: A Measure of Intrinsic Efficacy

S. M. JANKOVIC, D. KOUVELAS[1], V. MIRTSOU-FIDANI

Department of Pharmacology, Medical Faculty, Kragujevac, Serbia, Yugoslavia and [1]Department of Experimental Pharmacology, Medical Faculty, Thessaloniki, Greece

Received August 19, 1997
Accepted June 12, 1998

Summary

The establishment of a dose-response relationship and its quantification is the usual procedure for analysing drug action on an isolated organ. However, the time course of the effect seems to be an inherent characteristic of the agonist which produces it. In our study, we have analyzed the time-response curves of four cholinergic agonists (acetylcholine, methacholine, carbachol and bethanechol) which produce tonic contractions of the isolated rat gastric fundus. The order of affinity of agonists to muscarinic receptors on the rat fundus were carbachol > bethanechol > methacholine > acetylcholine (K_A values: 46±12, 84±21, 380±110 and 730±120 nM, respectively). The effective concentrations which produced 60 % of the maximal response (EC_{60}) were used for establishing the time-response curves. The time-response curves were also recorded after partial alkylation of muscarinic receptors with phenoxybenzamine, after exposure of the isolated rat fundus to physostigmine and after addition of supramaximal concentrations of the agonists. The experimental time-response curve for acetylcholine was on the extreme left, followed by curves for methacholine, bethanechol and carbachol, respectively. Phenoxybenzamine and supramaximal doses of the agonists did not change the order of response development in time, but supramaximal doses shifted all curves to the left and phenoxybenzamine shifted all time-response curves to the right. Only physostigmine shifted the time-response curve for methacholine to the right. The results of our study suggest that the response rate of the isolated rat gastric fundus to cholinergic agonists depends on the intrinsic activity of these agents, but not on their affinity for muscarinic receptors.

Key words
Choline esters – Rat – Gastric fundus – Dose-response relationship – Contraction

Introduction

The responses of cells or tissues to various agonists differ not only in their magnitude, but also in their time course. It has been hypothesized that the time-course of cellular response development to an agonist depends primarily on the type or capacity of intracellular signalling mechanisms (Mewes and Ravens 1994). Even if the whole cell response was changed by experimental conditions or disease (e.g. contraction of heart myocytes in failing hearts), the time course of the responses should not differ from that in control cells, providing that there was no impairment of the signal transduction cascade above the level of receptor for the agonist (Mewes and Ravens 1994). When the effects of agonists on isolated organs are examined, the development of the response in time will depend not only on the type of receptor

Fax +420 2 293 894
E-mail physres@sun1.biomed.cas.cz
http://www.lf2.cuni.cz/physiolres/physiolres.htm

and intracellular signalling, but also on the diffusion constants of the agonists in a bath medium and the tissue (Kenakin 1984). However, if the agonists with similar molecular structure and polarity were used, the role of diffusion could be disregarded (Ushio-Fukai *et al.* 1995).

The analysis of time-dependent kinetics of relaxation or contraction of isolated organs could be a useful subsidiary tool for studying the effect of agonist(s) action, as has been proved previously for phenylephrine and the isolated rabbit aorta (Wiener and Thalody 1993).

The aim of the present study was to analyze the time course of isolated rat fundus tonic contractions produced by four choline esters and to assess if the rate of responses produced by the four muscarinic receptor agonists was related to their intrinsic activity. According to previous studies, M_3 muscarinic receptors mediate contractions of the rat fundus muscle (Eglen *et al.* 1992, Jankovic *et al.* 1994). It is known that muscarinic agonists differ according to the receptor reserve, the ability to induce a conformational receptor change and/or stimulus-response coupling (Eglen *et al.* 1996). However, the time course of their effects has not been evaluated up to now.

Methods

Isolated preparations of the rat fundus

After fasting for 12 h, 12 Wistar rats of either sex, weighing between 200 g and 350 g, were killed by cervical dislocation and exsanguinated. The abdomen was opened along the midline and the gastric fundus was excised and put into a dissecting dish filled with Krebs solution (in mM: NaCl 94.7, KCl 4.7, $MgSO_4$ 2.4, $CaCl_2$ 2.52, KH_2PO_4 1.18, $NaHCO_3$ 24.88 and glucose 11.7). The fundus was at first flattened by two parallel incisions along the great curvature. According to the method of Vane (1957), several parallel sections at 1.5 mm intervals with the great curvature were made in the fundus producing very long and narrow isolated preparations (40 mm in length and 1.5 mm in width) containing all the layers of the gastric wall. Usually, two isolated preparations of the gastric fundus were prepared from one animal.

The strips were mounted in an isolated 100 ml organ bath, filled with Krebs solution. The bath was gassed continuously with 95 % O_2 and 5 % CO_2, and maintained at 37 °C. One end of the preparation was attached to the bottom of the bath, and the other to the lever of an isotonic transducer (T_3 isotonic transducer, Palmer Bio Science, USA). All strips were loaded with 1.0 g, and an additional stretching weight of 1.0 g. Tonic contractions of isolated preparations were recorded on a Linseis recorder, model L6522.

Agonists and antagonists

After mounting the isolated preparation in an organ bath, 45 min were allowed for the preparation to become stabilised before any drug was added.

In the first series of experiments, agonists were added to the bath cumulatively. Between the addition of each agonist, there was a 20 min break. After the first series had been finished, the concentration-response curves were determined and the concentration producing 60% of the maximal response (EC_{60}) for each agonist was assessed.

In the second series of experiments, EC_{60} of each agonist was added to the bath. In order to follow the time course of each isolated contraction, the speed of the recorder was set at 50 mm/min.

In the third series of experiments, a supramaximal concentration of each agonist (concentration of the agonist higher than the concentration producing maximal response, 50–100 multiple of the EC_{50}) was added to the bath. In order to follow the time course of the contraction of an isolated preparation, the speed of the recorder was also set at 50 mm/min.

The first two series of experiments were repeated after exposure of the isolated preparations to antagonists. The preparations were exposed to phenoxybenzamine for 30 min, and then thoroughly washed every 10 min for one hour before the agonists were added. When physostigmine was used, it was constantly present in the bathing solution (10 mM).

Compounds

The following substances were used: acetylcholine chloride, bethanechol chloride, carbamylcholine chloride (carbachol), methacholine chloride, physostigmine salicylate (Sigma Chemical Co., St. Louis, USA) and phenoxybenzamine hydrochloride (Smith Kline & French, Brentford, UK).

Statistical analysis

Every concentration of each agonist was administered to isolated preparations from six different animals. The concentration-response curves were constructed using linear regression according to least-squares analysis. Effective concentrations of each agonist that produced a 50 % response of the maximum (EC_{50}) was calculated for each agonist together with its confidence limits (1.96 x standard error). The absolute maximal responses to the agonists were compared among themselves by Student's t-test. The results were considered statistically significant when $P<0.05$.

The dissociation constants (K_A) for the cholinergic agonists were calculated according to the procedure described by Furchgott and Bursztyn (1967): after partial receptor inactivation by an irreversible antagonist, phenoxybenzamine, equally effective

concentrations of a cholinergic agonist before [A] and after treatment [A'] were determined. Then, 1/[A] was plotted against 1/[A']. The slope of the regression line and the y-intercept were used to calculate the dissociation constant: K_A = (Slope-1)/intercept. Receptor reserve (R) for the agonists was calculated according to the formula: $R = K_A/EC_{50}$.

The differences in time-development of mean responses to EC_{60} of the agonists were tested by two-way analysis of variance. The difference was considered significant if $P<0.05$.

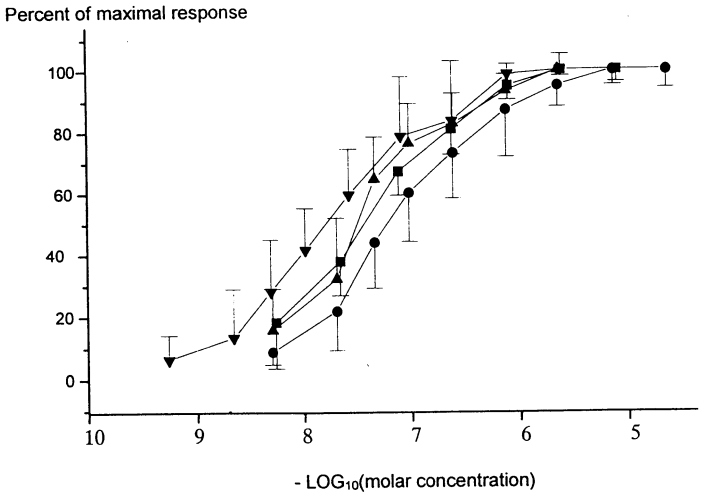

Fig. 1. *Concentration-response curves for choline esters used in the study. Each point represents the mean from 6 experiments. Error bars = standard deviations, full squares – acetylcholine, full circles – bethanechol, upward full triangle – methacholine, downward full triangle – carbachol.*

Results

Acetylcholine (5.5 nM–7.9 μM), methacholine (5.0 nM–2.3 μM), bethanechol (5.0 nM–1 μM) and carbachol (5 nM–790 nM) induced concentration-dependent contractions of the isolated rat fundus (Fig. 1). The order of potency of the agonists was: carbachol = acetylcholine > methacholine > bethanechol (EC_{50} values: 18±1, 19±1, 35±1 and 77±1 nM, respectively). In contrast, the maximal responses obtained with different agonists (the absolute values on recordings: acetylcholine 98.2±15.3 mm, methacholine 89.7±14.2 mm, bethanechol 99.7±12.8 mm and carbachol 88.0±20.5 mm) were not significantly different. In order to determine the dissociation constant (K_A) of agonists we used an irreversible antagonist of muscarinic receptors, phenoxybenzamine (10 mM). Examples of these experiments are presented in Fig. 2. The order of affinity of agonists to muscarinic receptors in the fundus were carbachol > bethanechol > methacholine > acetylcholine (K_A values: 46±12, 84±21, 380±110. 730±120 nM, respectively). The receptor reserve, expressed as K_A/EC_{50}, was 39.2 for acetylcholine, 10.9 for methacholine, 2.6 for carbachol and 1.1 for bethanechol.

The time for developing the maximum of tonic contractions in the isolated rat fundus induced by EC_{60} of acetylcholine (47 nM), methacholine (71 nM), bethanechol (160 nM) and carbachol (38 nM) is shown in Figure 3. The responses to carbachol and bethanechol did not significantly differ, while significant differences were found between responses to acetylcholine, methacholine and bethanechol (or carbachol) ($p<0.001$).

The development of tonic contractions of the isolated fundus in time produced by EC_{60} of acetylcholine (11 mM), methacholine (17 mM), bethanechol (20.4 mM) and carbachol (2.6 mM) after exposure to phenoxybenzamine (10 mM) is shown in Figure 4. The difference between responses to acetylcholine, methacholine and bethanechol (or carbachol) was significant ($P<0.05$).

The development of tonic contractions of the isolated rat fundus produced by supramaximal concentrations of acetylcholine (5.5 mM), methacholine (5.1 mM), bethanechol (5.1 mM) and carbachol (1.6 mM) is shown in Figure 5. The differences between responses to acetylcholine, bethanechol and carbachol (or methacholine) were significant ($P<0.05$). There was no significant difference between responses to carbachol and methacholine.

In the presence of the acetylcholinesterase-inhibiting agent physostigmine (10 mM), the concentration-response curve for acetylcholine was significantly shifted to the left ($P<0.05$; EC_{50} = 1.3±0.3 nM). On the other hand, physostigmine (10 mM) did not affect the concentration-response curves for methacholine, bethanechol and carbachol ($P>0.05$; EC_{50} values: 30±2, 89±2 and 17±2 nM, respectively). The development of tonic contractions of the isolated rat fundus in time produced by EC_{60} of acetylcholine (47 nM), methacholine (71 nM), bethanechol (160 nM) and carbachol (38 nM) in the presence of physostigmine (10 mM) is shown in Figure

6. According to the two-way analysis of variance, the responses to carbachol and methacholine were not significantly different, while a significant difference was found between responses to acetylcholine, bethanechol and methacholine (or carbachol) ($P<0.01$).

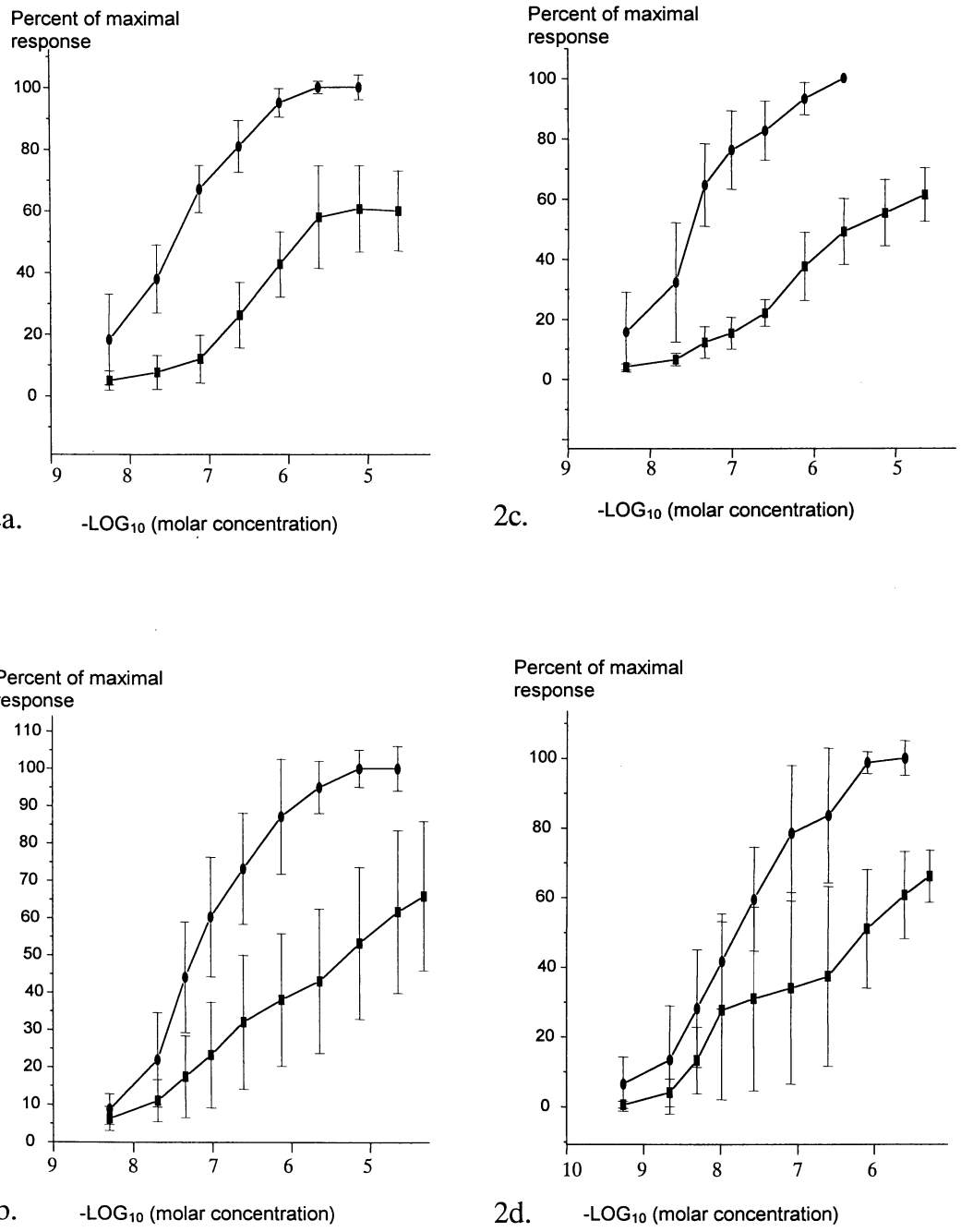

Fig. 2. *Concentration-response curves for choline esters before and after incubation of isolated rat fundus with 10 mM phenoxybenzamine. Each point represents the mean from 6 experiments. Error bars = standard deviations, 2a – acetylcholine, full squares – acetylcholine + 10 mM phenoxybenzamine, full circles – acetylcholine (control), 2b – bethanechol, full squares – bethanechol + 10 mM phenoxybenzamine, full circles – bethanechol (control), 2c – methacholine, full squares – methacholine + 10 mM phenoxybenzamine, full circles – methacholine (control), 2d – carbachol, full squares – carbachol + 10 mM phenoxybenzamine, full circles – carbachol (control).*

Discussion

Although the analysis of the time-dependence of macrophenomena has become quite usual and a matter of course (e.g. growth of malignant tumours, postprandial time-course of blood glucose concentrations, secretory response of endocrine glands), this does not apply to the effects of drugs. The usual procedure of analyzing the effects of drugs on isolated tissues is to establish the magnitude of the

response. Although the theories of both Clark and Paton differed in their explanation of the cause of the relative efficacy of the given drug (whether it is due to the proportion of occupied receptors in the first case or to the rate of interaction between the drug and receptor in the second), they considered only the link between drug concentration and effect without paying attention to the time course of the effect. However, there has been recent renewal of interest in the time course of drug effects which turned out to be worth analyzing (Bova *et al.* 1992). Indeed, it has been recently shown at the cellular level, that changes of the rate of response to certain agents, without changes of the magnitude of response, may significantly affect the final outcome of the response (Alekseev *et al.* 1996). From this aspect, it has been hypothesized that the rate of the response to an agonist could reflect the type and/or capacity of the intracellular signalling system at the cellular level (Naqvi *et al.* 1994).

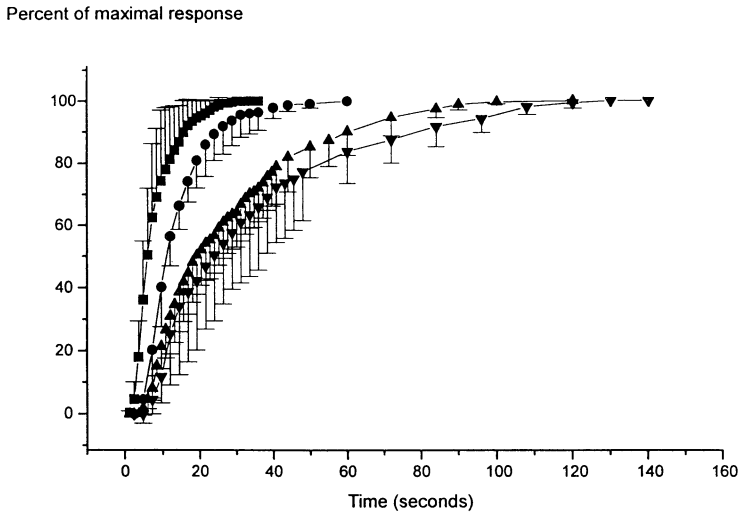

Fig. 3. *The time course of isolated rat fundus tonic contractions after addition of EC_{60} of acetylcholine, methacholine, bethanechol or carbachol into the bath. Each point represents the mean from 6 experiments. Error bars = standard deviations. full squares – acetylcholine, full circles – methacholine, upward full triangle – bethanechol, downward full triangle – carbachol.*

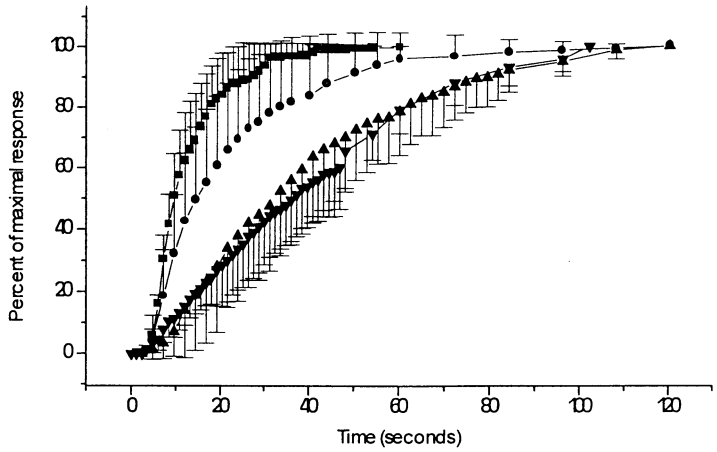

Fig. 4. *The time course of isolated rat fundus tonic contractions produced by EC_{60} of acetylcholine, methacholine, bethanechol or carbachol after the exposure to phenoxybenzamine (10 μM). Each point represents the mean from 6 experiments. Error bars = standard deviations, full squares – acetylcholine, full circles – methacholine, upward full triangle – bethanechol, downward full triangle – carbachol.*

In principle, when an agonist is added to the bath solution with an isolated tissue, a sequence of events ensues resulting ultimately in the pharmacological effect. The following steps are well recognized in the agonist action on an isolated tissue: 1) dispersion through the bath solution and contact with the isolated preparation, 2) movement through the interstitium of the isolated preparation and reaching its receptors, in the present study receptors on the surface of smooth muscle cells, 3) binding of agonist and receptor activation, 4) the activated receptor triggers an intracellular signalling system followed by a cascade of enzymatic reactions within the cells, and 5) which leads to a response, i.e. to muscle contraction in our study. Therefore, the rate of the response depends on the following aspects (Benet *et al.* 1990): (1) molecular weight of the agonist and its electrostatic charge; (2) agonist affinity for receptors; (3) the type of intracellular signalling system and its activity. It thus seems that the rate of response development of an isolated organ depends on the molecular characteristics of an agonist (molecular weight, degree of its dissociation in a bath solution at a given pH), on its affinity for the receptor and the type of intracellular signalling system (Limbird 1988).

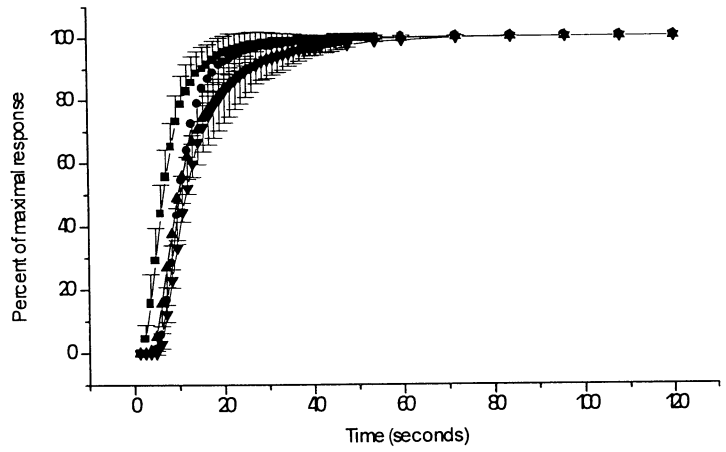

Fig. 5. *The time course of isolated rat fundus tonic contractions produced by supramaximal concentrations of acetylcholine, methacholine, bethanechol or carbachol. Each point represents the mean from 6 experiments. Error bars = standard deviations, full squares – acetylcholine, full circles – methacholine, upward full triangle – bethanechol, downward full triangle – carbachol.*

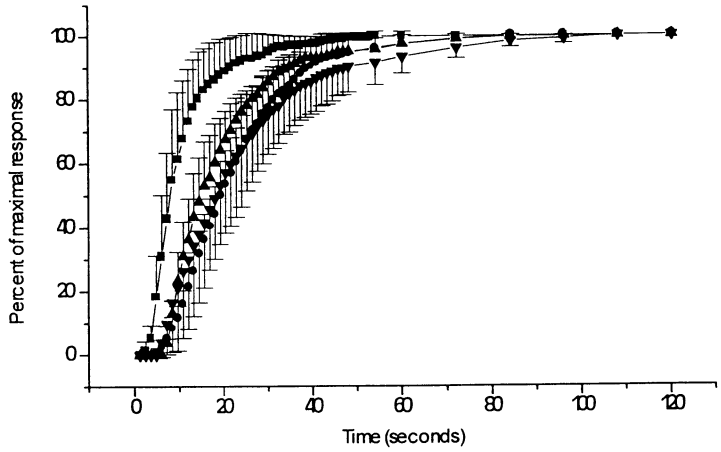

Fig. 6. *The time course of isolated rat fundus tonic contractions produced by EC_{60} of acetylcholine, methacholine, bethanechol or carbachol after the exposure to physostigmine (10 mM). Each point represents the mean from 6 experiments. Error bars = standard deviations, full squares – acetylcholine, full circles – methacholine, upward full triangle – bethanechol, downward full triangle – carbachol.*

In our experiments we used four cholinergic agonists with similar molecular characteristics: acetylcholine (molecular weight 181.7), carbachol (molecular weight 182.6), bethanechol (m.w. 196.7) and methacholine (m.w. 195.7), all four being quaternary ammonium compounds. This means that all four agonists have approximately similar diffusion rates in the bath solution and in the intercellular spaces of the isolated preparation. All four compounds are muscarinic agonists which bind to the M_3 receptor on smooth muscle cells of the isolated rat fundus producing tonic contractions (see Figs 1–4). Therefore, the differences in the rates of response of gastric fundus to cholinergic agonists can not be ascribed to different molecular characteristics.

When elimination of the agonists from the isolated organ was blocked by physostigmine, the time course of the contraction development did not change significantly for acetylcholine, bethanechol and carbachol. Although the response to methacholine on the whole became slower, it still developed along the same pattern as without physostigmine. If the time course of the contraction still depended significantly on the rate of agonist arrival at the receptors, the time-response curves in the presence of physostigmine (at least for acetylcholine) should have shifted to the left. However, this did not happen.

In order to test the possibility that the observed differences are due to the different affinities, we determined K_A values for all the agonists studied. For the muscarinic receptors that mediate contraction of the gastric fundus in response to the agonists studied, the order of affinity was carbachol > bethanechol > methacholine > acetylcholine. However, the order of affinities did not correspond to the order of response rates, suggesting that the affinity of the cholinergic drug is not the only important factor that determines the time course of gastric fundus contractions.

In general, the receptor reserve expressed as K_A/EC_{50} (Ruffolo 1982), reflects the efficacy of the coupling and/or receptor density (Kenakin 1984). In

the present study, since all the agonists studied acted on the same tissue with the same receptor density, the receptor reserve can be considered as an indirect indicator of the receptor coupling efficacy. The order of receptor reserves observed in our study was acetylcholine > methacholine > carbachol > bethanechol. This order corresponds to the order of rates, suggesting that the type and efficacy of coupling and consequently of the binding of the agonists to the receptor could be the decisive factors that determine the time course of gastric fundus contractions in response to cholinergic drugs.

The decrease in the total number of muscarinic receptors produced by phenoxybenzamine slowed down all responses, affecting more the responses of bethanechol and carbachol the receptor reserves of which were originally lower. However, the differences in the time course of contractions became even greater between acetylcholine and methacholine on one side and bethanechol and carbachol on the other. Obviously, the qualitative characteristics of intracellular reactions following receptor activation did not change; however, it is not surprising considering that the mechanism of phenoxybenzamine action does not extend further than the cell surface. Since the receptor reserves for bethanechol and carbachol were the lowest, the total intensity of intracellular signalling should be seriously affected by a diminished number of functionally available receptors. Consequently, the time-response curves should be shifted to the right, and this was actually the case.

When supramaximal concentrations of cholinergic agonists were used, the difference in time-response curves decreased. The time-response curves for bethanechol and carbachol shifted to the left and came closer to the curves of acetylcholine and methacholine. If the higher gradient for diffusion of bethanechol and carbachol was the cause for this shift, the same should have happened to the time-response curves for acetylcholine and methacholine. Since it had not, some changes of the receptors induced by high concentrations of an agonist, with a subsequent increase in intrinsic activity, could be a plausible explanation. In any case, if the time course of a response is to be used for evaluation of an agonist, supramaximal concentrations of the agonist should not be employed instead of EC_{60}.

In conclusion, this study has shown that the rate of response of rat isolated gastric fundus to cholinergic agonists depends rather on the characteristics of intracellular signalling induced by these agents, than on their affinity for muscarinic receptors. We therefore suggest that measurements of the time course of drug action could be a valuable adjunct to tissue-pharmacology studies, when determination of agonist intracellular signalling is of importance.

Acknowledgments

We thank Dr Aleksandar Jovanovic for reviewing the final version of the manuscript.

References

ALEKSEEV A.E., JOVANOVIC A., LOPEZ J.R., TERZIC A.: Adenosine slows the rate of K^+-induced membrane depolarization in ventricular cardiomyocytes: possible implications in hyperkalemic cardioplegia. *J. Mol. Cell. Cardiol.* **28:** 1193–1202, 1996.

BENET L.Z., MITCHELL J.R., SCHEINER L.B.: Pharmacokinetics: the dynamics of drug absorption, Distribution and Elimination. In: *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 8th ed., A.G. GILMAN, T.W. RALL, A.S. NIES, P. TAYLOR, (eds), Pergamon Press, New York, 1990, pp. 33–48.

BOVA S., PADRINI R., GOLDMAN W.F., BERMAN D.M., CARGNELLI G.: On the mechanism of vasodilating action of berberine: possible role of inositol lipid signaling system. *J. Pharmacol. Exp. Ther.* **261:** 318–323, 1992.

EGLEN R.M., HARRIS G.C., COX H., SULLIVAN A.O., STEFANICH E., WHITING R.L.: Characterization of the interaction of the cervane alkaloid, imperialine, at muscarinic receptors in vitro. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **346:** 144–151, 1992.

EGLEN R.M., HEGDE S.S., WATSON N.: Muscarinic receptor subtypes and smooth muscle function. *Pharmacol. Rev.* **48:** 531–565, 1996.

FURCHGOTT R.F., BURSZTYN P.: Comparison of dissociation constants and of relative efficacies of selected agonists acting on parasympathetic receptors. *Ann. N. Y. Acad. Sci.* **144:** 882–899, 1967.

JANKOVIC M.S., KOUVELAS D., ARGIRIOU A., MIRTSOU-FIDANI V., PAVLIDIS T., PAPANIKOLAU CH., PAPAIOANNIDOU P., PARADELIS A.G.: Muscarinic receptor subtypes. *Epitheor. Klin. Farmakol. Farmakokinet. EI.* **8:** 118–126, 1994.

KENAKIN T.P.: The classification of drugs and drug receptors in isolated tissues. *Pharmacol. Rev.* **36:** 165–222, 1984.

LIMBIRD L.E.: *Cell Surface Receptors: A Short Course on Theory and Methods.* Martinus Nijhoff, Boston, 1988.

MEWES T., RAVENS U.: L-type calcium currents of human myocytes from ventricle of non-failing and failing hearts and from atrium. *J. Mol. Cell. Cardiol.* **26:** 1307–1320, 1994.

NAQVI R.U., DEL MONTE F., O GARA P., HARDING S.E., MACLEOD K.T.: Characteristics of myocytes isolated from hearts of renovascular hypertensive guinea pigs. *Am. J. Physiol.* **266:** H1886–H1895, 1994.

RUFFOLO R.R.Jr.: Review: important concepts of receptor theory. *J. Auton. Pharmacol.* **2:** 277–295, 1982.

USHIO-FUKAI M., NISHIMURA J., KOBAYASHI S., KANAIDE H.: Endothelin-1 and endothelin-3 regulate differently vasoconstrictor responses of smooth muscle of the porcine coronary artery. *Br. J. Pharmacol.* **114:** 171–179, 1995.

VANE J.R.: A sensitive method for the assay of 5-hydroxytryptamine. *Br. J. Pharmacol.* **12:** 344–349, 1957.

WIENER H.L., THALODY G.P.: Kinetic characterization of adenosine A2 receptor-mediated relaxation in isolated rabbit aorta. *Eur. J. Pharmacol.* **238:** 65–74, 1993.

Reprint requests

Prof. Dr Slobodan M. Jankovic, Department of Pharmacology, Medical Faculty, P.O.Box 124, Kragujevac, 34000 Serbia, Yugoslavia.