Praca oryginalna *Original paper*

Papaverine should not be used as an indicator for controlling the ability of the gastrointestinal tract muscle to relax in vitro

MAGDALENA CHŁOPECKA, NATALIA DZIEKAN, ADAM BĄKAŁA, MARIA WIECHETEK, MARTA MENDEL

Division of Pharmacology and Toxicology, Department of Preclinical Sciences, Faculty of Veterinary Medicine, Warsaw Agricultural University, ul. Ciszewskiego 8, 02-786 Warszawa

Chłopecka M., Dziekan N., Bąkała A., Wiechetek M., Mendel M.

Papaverine should not be used as an indicator for controlling the ability of the gastrointestinal tract muscle to relax in vitro

Summary

The aim of this research was to study the possibilities of using papaverine as a reference substance to control the ability of isolated gastrointestinal (GI) tract strips to relax. The effects of papaverine hydrochloride (0.001-100 μM) dissolved in distilled water, or in DMSO (0.5%), on the mechanical activity of isolated rat GI strips (stomach fundus and corpus, duodenum and jejunum) were studied. The obtained results show that papaverine provoked various responses of the examined muscle strips dependent on the part of GI tract and papaverine solvents used (water or DMSO). Papaverine applied as water solution caused muscle relaxation of all investigated gastrointestinal strips: the lowest effective (induced relaxation) concentration of papaverine was 10 μM for gastric corpus, jejunum and duodenum and 100 μM for gastric fundus. However, there was no dependence between the concentration of papaverine and the degree of muscle relaxation of the studied GI strips. Moreover, in case of gastric fundus strips, papaverine applied as 0.5% DMSO solution provoked different muscle responses: in the presence of 0.1 and 1 μM papaverine contraction occurred; administering papaverine at higher concentrations (10 and 100 μM) resulted in relaxation. The obtained results clearly indicate that papaverine does not fulfil the criteria set for the reference substance and should not be used as an indicator for controlling of gastrointestinal tract muscle relaxation in vitro.

Keywords: papaverine, gastrointestinal tract

The rapid progress in laboratory techniques and the present ethics require inventing and developing new toxicological research models that would be an alternative to experiments performed on animals. Being an alternative means in this case that they would reduce or eliminate the use of laboratory animals. One of the possibilities for the study of the toxic effect of un-known substances on gut motility is performing experiments on isolated gastrointestinal (GI) strips (3, 8, 15). Such investigations are intended to study the effect of different chemicals on smooth muscle motility. They require the use of reference substances that exhibit well known contractile or relaxant activity. In order to control the ability of isolated GI strips to contract different contractile agents are used. Among a number of others the most frequently used chemicals are: acetylcholine chloride, and carbachol chloride (3, 5, 6, 8). Substances applied relatively often as relaxant factors in experiments on the isolated gastrointestinal tract are isoproterenol (5, 8) and papaverine hydrochloride (3, 5, 6). However, it is noteworthy that isolated gastrointestinal strips must initially be carefully standardized and validated before they will become wide spread as an alternative experimental model. One of the most important stages in the standardization process is choosing the proper reference substances whose contractile or relaxant impact is well known. Every reference substance must fulfil several criteria. One of the most important criteria for the reference substance is its ability to dissolve well in an incubation medium (the most common is Krebs--Henseleit solution, pH 7.4). It must also provide universal, reproducible and repeatable reactions in all examined gastrointestinal strips. Furthermore, a reference substance must provoke a dose–effect dependence. The relation between the administered dose and the reaction of each strip should become a basis for nominating an optimal dose (recommended for use) of this substance for every strip. The optimal dose of a reference substance can be neither the first nor the last effective dose on a dose-effect curve. Moreover, after being exposed to this dose, every strip should be able to return to its spontaneous activity. The optimal dose of a reference substance might be different for each gastrointestinal part. While acetylcholine, carbachol and isoproterenol do fulfil the first mentioned requirement, namely to dissolve well in the incubation medium, papaverine fails to fulfil it. Thus, the usefulness of papaverine (from the very beginning) in *in vitro* studies is quite controversial. Papaverine hydrochloride is dissolvable only in acidic conditions with super-saturation at pH values 3.9-4.5 (12, 14). The increase of the pH value leads to such a rapid precipi-

tation that the process already reaches approximately 100% at a pH of about 5. (10) So, physicochemical properties of papaverine hydrochloride actually exclude the possibility of using it as a reference substance in all *in vitro* experiments that require the use of an incubation medium with a pH above 4.5. Nevertheless, considering the fact that papaverine hydrochloride is commonly used in experiments on isolated GI strips, it was decided to examine the possibilities of using it as a reference substance to control the ability of isolated GI strips to relax.

Material and methods

Male Wistar rats weighing 200-250 g were used. The animals were kept under laboratory conditions with free access to food and water. Rats were euthanized using carbon dioxide according to the ethics committee recommendations (1, 4, 11). Immediately after euthanasia the stomach, the duodenum and the first part of the jejunum were removed from the animal body and were placed into a cuvette field with a warm (37°C) modified Krebs-Henseleit solution (M K-HS) of the following composition (in mM): NaCl – 123.8; KCl – 5.04; $CaCl_2$ – 2.5; $MgSO_4$ – 1.20; $NaHCO_3$ – 14.5; KH_2PO_4 – 2.75 and glucose – 12.5. Additionally, M K-HS was gassed with carbogen (95% O_2 + 5% CO_2), causing a final pH between 7.35 and 7.45 at 37°C. The surrounding connective tissues were removed. The stomach was divided into two parts, namely corpus and fundus. The stomach strips were approximately 5 mm wide and 15 mm long. The content of duodenum and jejunum was carefully removed by flushing with warm M K-HS. In the next step the natural shape segments (15 mm long) were cut out. Each strip was kept in a separate bath vessel (Schuler Organ Bath of Hugo Sachs Electronic, Germany), containing aerated (95% O_2 + 5% CO_2), warm (37°C) M K-HS. The strips were bound to the tissue holders and silk threads, which were connected with lever transducers. The changes in strip lengths were recorded by an isotonic transducer (B40 type 373, Hugo Sachs Electronic, Germany) under 0.5 g load. The changes in length were amplified with a bridge circuit (DBA type 660, Harvard Apparatus, Germany) and recorded on a computer using an analogue-digital recorder (PowerLab, ADInstruments, Australia). Experimental data was analyzed using PowerLab Chart v 5.0 and Excel (MS Office XP Professional). The effects of papaverine hydrochloride (Sigma, St. Louis, Mo, USA), dissolved in distilled water or in 0.5% dimethylsulphoxide (DMSO) (Sigma, St. Louis, Mo, USA), on the motility of gastrointestinal strips were studied. Papaverine (in concentrations of 0.001 μM, 0.01 μM, 0.1 μM, 1 μM, 10 μM and 100 μM) was directly administered into tissue chambers in a volume of 50 μl. All experiments were performed according to the following protocol: 1) 60 min of equilibration with some flushings every 15-20 minutes, 2) M K-HS (50 μl) application in order to check the response of examined GI strips, 3) administering papaverine hydrochloride (dissolved in distilled water or in 0.5% DMSO) to study its effect on GI strips' motility. Each concentration of papaverine hydrochloride was maintained in the bath chambers for 5 min. After administering each dose of papaverine hydrochloride the chambers were flashed with M K-HS as long as it was needed for the strips to again reach the base line of muscle spontaneous activity level similar to the one prior the chemical application.

The results are expressed as a mean of 4-6 independent experiments (± SD); values of relaxation or contraction are given as changes in strip lengths (mm) before and after application of the studied substances. Statistical analysis was performed using Statistica for Windows (version 6.0 Pl) by means of one-way ANOVA when more than two groups are compared or Student t-test to compare two experimental groups. When ANOVA indicated that significant differences existed, multiple comparisons between groups was carried out by the Fischer LSD test. For all statistical tests, values were considered significantly different at $p \leq 0.05$.

The study was approved by the III Local Ethics Committee of Warsaw by Warsaw Agricultural University (Nr 31/2003).

Results and discussion

Papaverine provoked various responses of the examined muscle strips dependent on the part of GI tract and applied papaverine solvents (water or 0.5% DMSO). Papaverine applied as a water solution caused muscle relaxation of all investigated gastrointestinal strips (fig. 1). The effective (induced relaxation) concentration of papaverine was 10 μM for gastric corpus, jejunum and duodenum (fig. 1A, C and D) and 100 μM for stomach fundus (fig. 1B). These observations are in agreement with the results obtained by other authors (2, 7, 9-11, 16) according to which only relatively high (10-1000 μM) doses of papaverine hydrochloride are recommended to be used in *in vitro* experiments performed on isolated smooth muscles. The suggestion of using such high doses of papaverine probably results from the fact that administering papaverine to the incubation medium (M K-HS, pH 7.4) causes rapid precipitation and its inactivation.

Furthermore, while analyzing the results obtained in experiments with distilled water used as a solvent, it must be pointed out that exposition of gastric corpus, duodenum and jejunum strips to papaverine hydrochloride in a ten fold higher concentration (100 μM) did not significantly change the degree of smooth muscle relaxation in comparison to the muscle reaction observed in the presence of 10 μM papaverine (fig. 1A, C and D). The lack of dependence between the papaverine concentration and the degree of muscle relaxation of the mentioned GI strips contradicts the use of papaverine as an indicator for controlling the ability of GI tract muscle to relax in *in vitro* experiments.

In case of using papaverine dissolved in 0.5% DMSO, the effective relaxant concentration of papaverine was significantly lower than that observed in the presence of papaverine water solution. In these conditions (papaverine DMSO solution), applying papaverine at a concentration as low as 0.001 μM already resulted in a significant relaxation of duodenum and jejunum strips (fig. 1C and D); whereas the lowest effective relaxant concentration of papaverine for stomach corpus muscle strips was 0.1 μM (fig. 1A). Moreover, in experiments using papaverine DMSO solution the degree of stomach corpus, duodenum and jejunum strips' relaxation correlated with the papaverine concentration. A significant dependence between papaverine concentration and muscle relaxation was stated for papaverine concentrations in the range of 0.1 μM – 100 μM for stomach corpus, and 0.001 μM – 100 μM for jejunum and duodenum (fig. 1A, C and D).

The stronger reaction of stomach corpus, duodenum and jejunum muscle strips to papaverine dissolved in DMSO than to papaverine water solution might be explained by the following thesis. Dissolving papaverine in DMSO solution slows down the process of papaverine precipitation and, therefore, the studied muscle strips were actually exposed to higher concentrations of papaverine. Papaverine, dissolved in distilled water, underwent fast precipitation which finally resulted in the strips reacting with much lower doses of papaverine. However, it is noteworthy to point out that DMSO at a concentration of 0.5% did not show any effect on the spontaneous activity of the isolated gastrointestinal strips (data not shown here). Thus, the observed changes in muscle reactivity to the application of

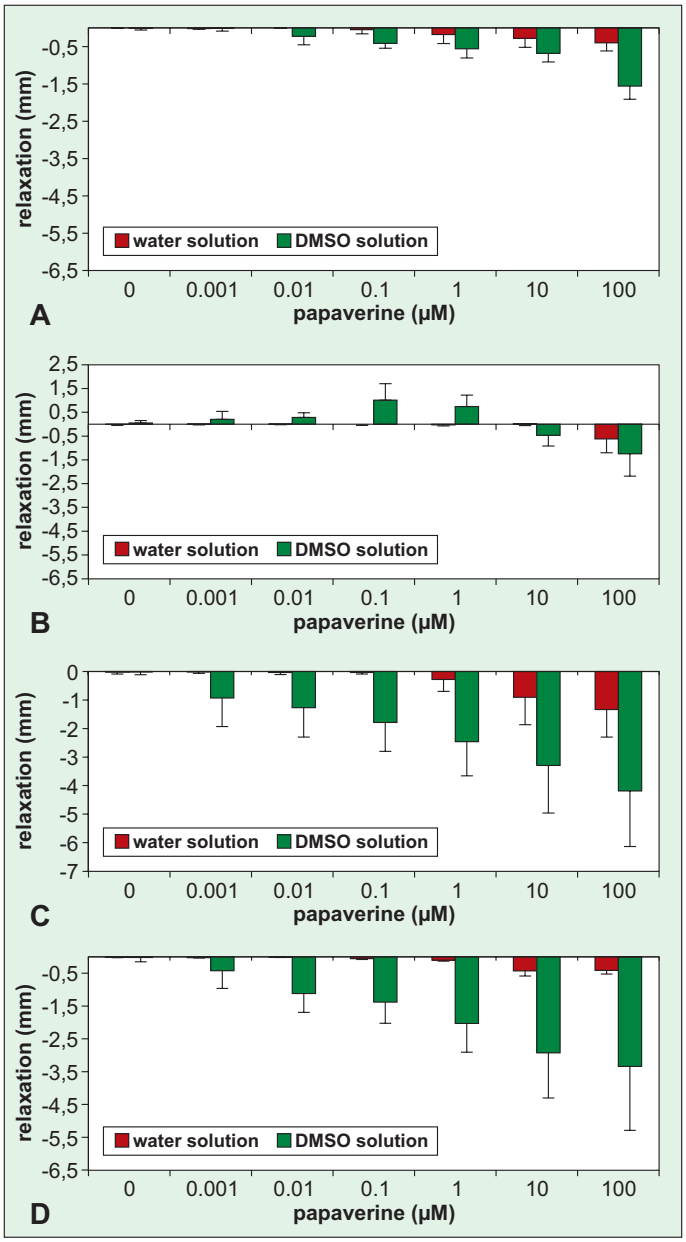

Fig. 1. Effect of papaverine on mechanical activity of stomach corpus (A), stomach fundus (B), jejunum (C) and duodenum (D) smooth muscle.
The data represent changes in strips' length (mm) before and after the application of papaverine water and DMSO solution (x ± SD; n = 6)

papaverine dissolved in DMSO certainly did not come from the solvent. Furthermore, our observations indicate that all substances used in *in vitro* researches on isolated smooth muscle strips should be dissolved in an incubation medium because preparing water solution of examined compounds and administering them in a small volume to the incubation vessels may not make it possible to observe their precipitation.

In contrast to the results obtained for the stomach corpus, duodenum and jejunum strips, the application of DMSO solution of papaverine resulted in completely different responses of the stomach fundus smooth muscle. Unexpectedly, the type of reaction of the stomach fundus muscle varied dependent on the concentration of papaverine (0.5% DMSO solution). When applied at low concentrations (0.1 μM-1 μM) it provoked a contractile response of the stomach fundus muscle, whereas at higher concentration (10 and 100 μM) it induced muscle relaxation (fig. 1B).

The fact that stomach fundus strips react differently to papaverine (with relaxation after papaverine water solution application and with contractile or relaxant response after administering papaverine DMSO solution in a concentration dependent manner) demonstrates that papaverine should definitely not be used as a reference substance.

Summing up, we conclude that papaverine hydrochloride does not fulfil the criteria set for a reference substance. Despite the fact that it is insoluble in the incubation medium (modified Krebs-Henseleit solution, pH 7.4), papaverine water solution does not show dose-effect dependence – one of the most important requirements for a reference substance. Furthermore, the application of papaverine DMSO solution did not deliver repeatable results: administering papaverine DMSO solution also provoked contraction as well as relaxation of the stomach fundus. The obtained results clearly indicate that papaverine cannot generally be recommended as a reference substance for controlling the ability of smooth muscle to relax and therefore should never be used to compare and express the miorelaxant activity of unknown substances.

Acknowledgements: The study was supported by the State Committee for Scientific Research Nr 3 P06 K02423.

References

1. 2000 Report of the AVMA Panel on Euthanasia. J. Am. Vet. Med. Assoc. 2001, 218, 669-696.
2. *Baccari M. C., Bani D., Bigazzi M., Calamai F.*: Influence of relaxin on the neurally induced relaxant responses of the mouse gastric fundus. Biol. Reprod. 2004, 71, 1325-1329.
3. *Ceregrzyn M., Ozaki T., Kuwahara A., Wiechetek M.*: 1: Sodium nitrite – a potent relaxant of rat stomach fundus: in vitro evidence. Can. J. Physiol. Pharmacol. 1998, 76, 9-12.
4. *Danneman P. J., Stein S., Walshaw S. O.*: Humane and practical implications of using carbon dioxide mixed with oxygen for anesthesia or euthanasia of rats. Lab. Anim. Sci. 1997, 47, 376-385.
5. *Etus V., Tugay M., Utkan T., Muezzinoglu B., Ceylan S.*: Foregut smooth muscle reactivity changes in the hydrocephalus-induced infantile rats. J. Surg. Res. 2004, 118, 197-202.
6. *Fujimoto S., Mori M.*: Characterization of capsaicin-induced, capsazepine-insensitive relaxation of ileal smooth muscle of rats. Eur. J. Pharmacol. 2004, 487, 175--182.
7. *Harrison W. E., Mellor A. J., Clark J., Singer D. R. J.*: Vasodilator pre-treatment of human radial arteries; comparison of effects of phenoxybenzamine vs papaverine on norepinephrine-induced contraction in vitro. Eur. Heart J. 2001, 22, 2209-2216.
8. *Hazelhoff B., Malingre Th. M., Meijer D. K. F.*: Antispasmodic Effects of Valeriana Compounds: an in vivo and in vitro Study on the Guinea-Pig Ileum, Arch Int. Pharmacodyn. 1982, 257, 274-287.
9. *Huddart H., Saad K. H.*: Papaverine-induced inhibition of electrical and mechanical activity and calcium movements of rat ileal smooth muscle. J. Exp. Biol. 1980, 86, 99-114.
10. *Kaneda T., Shimizu K., Nakajyo S., Urakawa N.*: The difference in the inhibitory mechanisms of papaverine on vascular and intestinal smooth muscles. Eur. J. Pharmacol. 1998, 55, 149-157.
11. Report of the International Consensus Meeting on Carbon Dioxide Euthanasia of Laboratory Animals, ANZCCART News 2006, 19.
12. *Serajuddin A. T. M., Rosoff M.*: pH-solubility profile of papaverine hydrochloride and its relationship to the dissolution rate of sustained-release pellets. J. Pharm. Sci. 1984, 73, 1203-1208.
13. *Shimuzu K., Ichikawa T., Urakawa N., Nakajyo S.*: Inhibitory mechanisms of papaverine on the smooth muscle of guinea pig urinary bladder. J. Pharmacol. 2000, 83, 143-149.
14. The Merck Index. An Encyclopedia of Chemicals, Drugs and Biologicals. 14th Edition. O'Neil M. J. (red.). Merck&Co., Inc., Whitehouse Station, N.J., USA 2006, 6973.
15. *Trute A., Gross J., Mutschler E., Nahrstedt A.*: In Vitro Antispasmodic Compounds of the Dry Extract Obtained from Hedera helix. Planta Med. 1997, 63, 125-129.
16. *Tugay M., Utkan T., Utkan Z.*: Effects of caustic lye injury to the esophageal smooth miscle reactivity: in vitro study. J. Surg. Res. 2003, 113, 128-132.

Author's address: Magdalena Chłopecka, ul. Pileckiego W. Rtm. 110 m 38, 02-795 Warszawa; e-mail: magdalena_chlopecka@sggw.pl