# Effects of cupric ions on isolated guinea-pig ileum

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Moberg L-E, Appelgren B, Sjöstrand NO. Effects of cupric ions on isolated guinea-pig ileum. Acta Odontol Scand 1985; 43:223–229. Oslo. ISSN 0001–6357.

The possible biological significance of low concentrations of $Cu^{2+}$ on the guinea-pig ileum has been investigated. Responses to nerve stimulation by single electrical shocks, acetylcholine (ACh), and histamine and the response to ACh of depolarized muscle were examined. In summary, $Cu^{2+}$ has diverse and dose-dependent effects on the ileum. A stimulant effect dominates in intestine accommodated in physiological saline solution. The excitatory effect of the ion, in the concentration range 10 nM–1 μM, is probably due to stimulation of a depolarization-coupled initiation of the contraction. The inhibitory effect of $Cu^{2+}$ (10 nm–1 μM) is presumably due to a decreased $Ca^{2+}$ availability for the contractile process. In a higher concentration range (10 μM–100 μM) the stimulant action could also, in part, be related to a copper-induced release of $Ca^{2+}$ from a storage site. No effect that certainly could be ascribed to a neuronal site of action could be observed. The possible effect of $Cu^{2+}$, released during corrosion of dental alloys, on oral excitable tissue such as taste and pain receptors is discussed. □ *Cholinergic transmission; corrosion; dental alloys; smooth muscle*

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Metallic taste, burning sensations in the tongue, and other mucous irritations are symptoms that have been reported in connection with dental metallic restorations (1, 2). The question has arisen whether elements released from dental alloys in the mouth could be responsible for such effects.

In vivo investigations have shown increased concentrations of corrosion products from dental alloys in the hard and soft tissues in the mouth (3–7). Copper is a constituent in many gold alloys and in most amalgam alloys. The concentration of copper in gingiva or mucous membrane in contact with cast gold alloy restorations has been analyzed, and the maximal concentration was found to be about 1.6 mM (7).

In recent years increased interest has been paid to the corrosion of copper-rich amalgams. Corrosion attacks on the Cu phases in the amalgams have been shown in vivo (8) and in vitro (9, 10) and lead to regions containing an Sn-rich corrosion product without Cu. The corrosion of copper-rich amalgams in saline solutions has been shown to be associated with a substantial release of copper into the solution (11). Furthermore, the release of copper from copper-rich amalgams may increase considerably when these amalgams are in contact with gold alloys (12).

Publications concerning the effects of copper in low concentrations (< 1 mM) on isolated organs are sparse. Hazama (13) in 1925 studied the effect of copper on the rat intestine and found that in concentrations of around 100 μM an increase in resting tone occurred. Later studies on the effect of low $Cu^{2+}$ concentrations on the smooth-muscle activity of the Fallopian tube (10 μM) (14) and on the uterus (1–10 μM) (15, 16) showed a slight increase in motility. However, the influence of very low concentrations of $Cu^{2+}$ on autonomic neuromuscular transmission has apparently not been examined previously. It was therefore decided to study the effects of $Cu^{2+}$ in very low concentrations on the guinea-pig ileum. This is considered a suitable, convenient, and sensible model system for testing effects of agents on excitable tissue. A preliminary account of the investigation has been presented earlier (17).

## Materials and methods

Mottled, male or female guinea-pigs (400–700 g) were killed by a blow on the head. A segment of 2–5 cm was taken from the distal part of the guinea-pig ileum and placed in a 50-ml organ bath containing Tyrode's solution aerated with 6.5% $CO_2$ in $O_2$ and kept at 37°C. The Tyrode's solution had the following composition: 136.7 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$ 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.3 mM $NaHPO_4$ (all analytical grade) (E. Merck, Darmstadt, FRG) and 5.6 mM glucose (BDH Chemicals Ltd., Poole, England) (dissolved in deionized water). Depolarization of the muscle was achieved by replacing the NaCl with equimolar amounts of KCl. When $Ca^{2+}$ contraction of depolarized ileum was studied, $CaCl_2$ was replaced by equimolar amounts of KCl.

After being mounted, the muscle was allowed to equilibrate for at least 1 h and was slightly stretched (2–5 mN) before the experiment started. Contractions were recorded isometrically by a force-displacement transducer (Grass FT 03C) coupled to a polygraph (Grass model 7B).

Single electrical shocks of 0.5 msec duration were given every 60 sec with a strength (0.2–1.5 V) giving about half the maximum response. The neurogenic nature of the response was verified by its sensitivity to tetrodotoxin (TTX; 0.3 nM). ACh was added in concentrations of 50 nM–10 μM to non-depolarized and 5–100 μM to depolarized tissue. The test concentrations were selected to give about half the maximum response. Histamine was added in a similar manner to non-depolarized tissue (0.1–0.3 nM). Drugs were added every 5th min, followed by washing after 45 sec. In the $CaCl_2$-free organ bath solution, $CaCl_2$ (10 mM) was added every 12 min to induce a contraction and was allowed to act for 2 min. After the washing, EDTA (1 mM) was added for 5 min to deplete $Ca^{2+}$ from the preparation (18).

Added $CuCl_2$ was dissolved in bath medium to avoid dilution effects and added to the organ bath in a volume of 0.2–1.0 ml. At the start of testing $CuCl_2$ was added to a concentration of 0.1–10 nM, and after that the $CuCl_2$ concentration in the organ bath was repeatedly increased 10-fold by new additions.

The effects of $CuCl_2$ were also tested when $Ca^{2+}$ blockers—verapamil (5 μM), sodium nitroprusside (sodium nitroferricyanide, $C_5FeN_6Na_2O$, 10 or 20 μM), $CoCl_2$, and $NiCl_2$ (concentration ranges, 10 μM–1 mM)—were present in the organ bath.

Effects of hyperosmolarity were examined by comparing equiosmolar concentrations of $CuCl_2$ and NaCl. Preparations with high spontaneous activity were excluded from the study. Each type of experiment was performed on 5–20 preparations from 3–15 guinea-pigs. Usually two preparations were taken from each guinea-pig.

### *Drugs and salts*

Acetylcholine chloride (Hoffmann-La Roche & Co. AG, Basel, Switzerland), histamine chloride (Apoteksbolaget AB, Stockholm, Sweden), verapamil (Isoptin®-hydrochloride, Knoll AG, Chemische Fabriken Ludwigshafen, FRG), sodium nitroprusside, $CuCl_2$, $CoCl_2$, $NiCl_2$ (E. Merck), and tetrodotoxin (Sigma) were used.

## Results

In concentrations below 10 nM no clear effects of $Cu^{2+}$ were detected. At 10 nM an effect was seen in about 10% of the preparations, and a clear effect was seen at 1 μM in 50% of the preparations. At higher concentrations all preparations showed an effect. No effects were observed when equiosmolar concentrations of NaCl were added.

$Cu^{2+}$, in the concentration range 10 nM–1 μM, enhanced the neurogenic contractile response due to field stimulation (Fig. 1) and the response to ACh (Fig. 2) and histamine in preparations immersed in ordinary Tyrode's solution. This excitatory effect could not be reproduced in the same preparation unless the $Cu^{2+}$ concentration increased (range, 1–100 μM). The response to ACh and histamine could be divided into one rapid phase of contraction (1–10 sec)

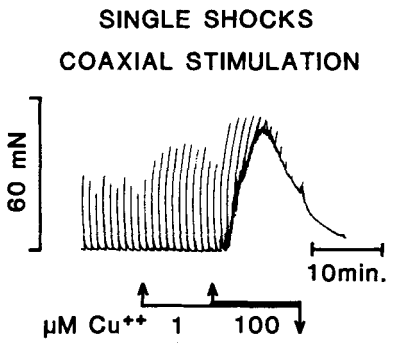


Fig. 1. Effect of $Cu^{2+}$ on field-stimulated guinea-pig ileum. A low concentration enhanced the spiking response, whereas a 100-fold increase of the $Cu^{2+}$ concentration enhanced the tonic activity.

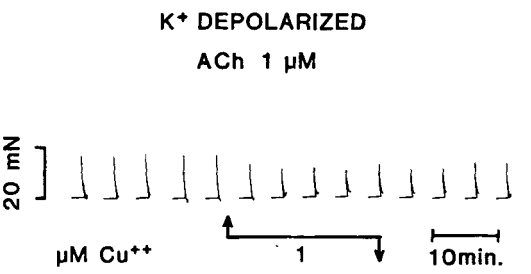


Fig. 3. Effect of $Cu^{2+}$ on acetylcholine (ACh)-induced contractions in depolarized tissue. The response is depressed by a low concentration of $Cu^{2+}$.

and one slow phase persisting throughout the drug exposure. The peak tension of the rapid phase was larger than that of the slow. The effect of $Cu^{2+}$ (10 nM–1 μM) was seen only on the rapid phase, which increased in tension.

The effects of $Cu(NO_3)_2$ and $CuBr_2$ on the intestine were compared with that of $CuCl_2$, but no differences could be observed in the reaction of intestine.

In higher concentrations (range, 10–100 μM) $Cu^{2+}$ increased the 'resting tone' of all the ileum (Figs. 1, 2, and 4). This increase in tone was not blocked by tetrodotoxin (0.3 nM). During the increase in tone the neurogenic or the ACh-induced contractions were also at times enhanced. These responses decreased after about 10 min and were reduced to below initial magnitude.

Reduction of the $CaCl_2$ concentration in the bath by 50% shifted the tonic response to $Cu^{2+}$ (1 μM) to the left, and this concentration produced a clear-cut increase in tone.

Depolarization of the tissue decreased the response to ACh and histamine but did not alter the shape of the contractions—that is, one rapid phase of short duration and one slow phase of long duration could still be separated. The relative difference in the strength of the phases was about the same as in the non-depolarized state. The rapid phase of contraction induced by ACh and histamine in depolarized tissue was depressed by $Cu^{2+}$ (10 nM–1 μM) (Fig. 3). However, a higher concentration of $Cu^{2+}$ (100 μM) increased the response to ACh.

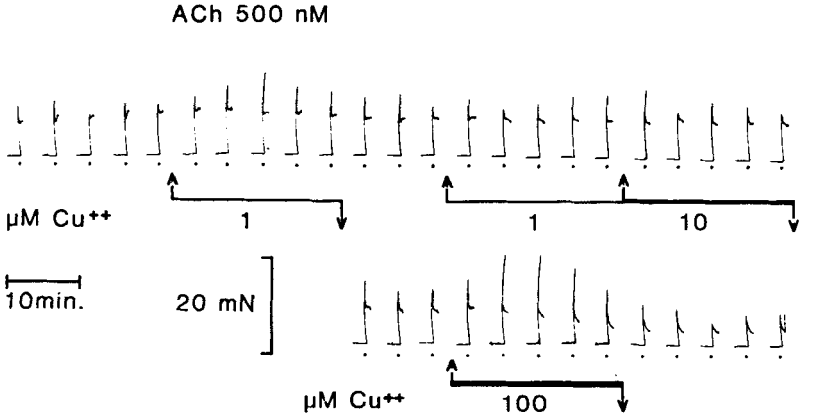


Fig. 2. Effects of $Cu^{2+}$ on acetylcholine (ACh)-induced contractions. The effect is tachyphylactic, and a higher concentration has to be used when the effect is repeated.

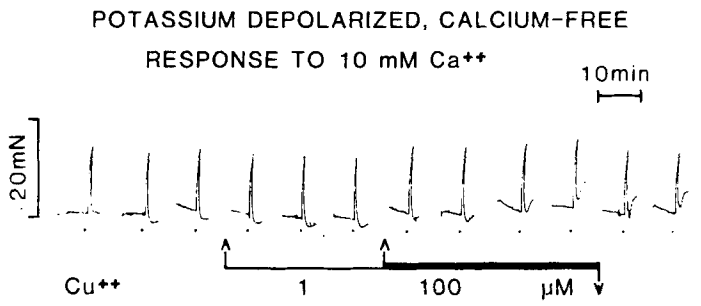


Fig. 4. Effects of $Cu^{2+}$ on $Ca^{2+}$-induced contractions on $K^+$-depolarized tissue ($Ca^{2+}$-free Tyrode's solution). A low concentration of $Cu^{2+}$ had a slight inhibitory effect, whereas the response to $Ca^{2+}$ was slightly increased by a high concentration of $Cu^{2+}$.

In depolarized and EDTA-treated tissue the response to added $CaCl_2$ (10 mM) was also divided into two phases. However, the slow long-lasting contraction was often stronger than that of the short phase. $Cu^{2+}$ (10 nM–1 μM) decreased the strength of both phases without changing the difference between them (Fig. 4). Higher concentration of $Cu^{2+}$ (100 μM) increased the $Ca^{2+}$ contracture.

*Effect of $Ca^{2+}$ blockers*

Verapamil (50 nM–50 μM) dose-dependently depressed both phases of the ACh-induced contraction, and both phases were virtually abolished with a concentration of 50 μM.

Sodium nitroprusside also depressed both phases of the ACh-induced contractile response, but compared with verapamil the effect on the rapid phase was more pronounced. In contrast to verapamil the effect of sodium nitroprusside showed a plateau at 10–20 μM, and higher concentrations gave no further reduction of the ACh-induced contractions.

The effects of the $Ca^{2+}$ blockers on the action of $Cu^{2+}$ are summarized in Table 1. Verapamil partially blocked the stimulant action of $Cu^{2+}$ (10 nm–1 μM). Usually 1–100 μM of $Cu^{2+}$ had to be used to obtain the same enhancement of the ACh-induced contractions as in untreated preparations. The tonic response to $Cu^{2+}$ (10–100 μM) was also blocked by verapamil, but a higher concentration of $Cu^{2+}$ (1 mM) could still increase the tension of the preparation.

Sodium nitroprusside also blocked the enhancing effect of $Cu^{2+}$ on ACh-induced contractions. It was, however, not possible to restore the effect of $Cu^{2+}$ by increasing the concentration of the ion. On the contrary, $Cu^{2+}$ often decreased the response to ACh in the presence of sodium nitroprusside. An increase in tension could still be elicited but with a higher concentration of $Cu^{2+}$ (1 mM) than without sodium nitroprusside in the bath.

With $Ca^{2+}$-depleted and depolarized tissue

Table 1. The influence of $Ca^{2+}$ blockers on the stimulant action of $CuCl_2$, in the two concentration ranges 10 nM–1 μM and 10–100 μM, on the contractile response to acetylcholine

| $Ca^{2+}$ blocker | $Cu^{2+}$ concentration range | |
|---|---|---|
| | 10 nM–1 μM | 10–100 μM |
| Verapamil, 5 μM | Partial block | Partial block |
| Sodium nitroprusside, 10 or 20 μM | Total block | Partial block |
| $CoCl_2$, 10 μM–1 mM | Total block | Partial block |
| $NiCl_2$, 10 μM–1 mM | Total block | Partial block |

both verapamil and sodium nitroprusside decreased the rapid phase of $Ca^{2+}$-induced contractions more than the slow phase. Addition of $Cu^{2+}$ (10 nM–1 μM) to the solution did not overtly change the $Ca^{2+}$ contraction. On the other hand, higher concentrations of $Cu^{2+}$ (100 μM) increased both phases of $Ca^{2+}$-induced contractions.

$CoCl_2$ and $NiCl_2$ (10 μM—1 mM) both inhibited the contractile response to ACh in normal Tyrode's and in potassium Tyrode's solution. With the highest concentration of $Co^{2+}$ and $Ni^{2+}$ (1 mM) the response to ACh in normal Tyrode's solution was almost abolished. When $Co^{2+}$ and $Ni^{2+}$ (range, 10 μM–1 mM) were present in the solution, $Cu^{2+}$ (10 nM–10 μM) had no effect. The increase in tone produced by 10–100 μM of $Cu^{2+}$ was blocked by $Co^{2+}$ and $Ni^{2+}$ (1 mM), but 1 mM $Cu^{2+}$ could still increase the tension of the preparation.

## Discussion

The guinea-pig ileum is a model system frequently used to study drug and ion effects on autonomic neuromuscular transmission. The results in this study showed that $Cu^{2+}$ in very low concentrations (10 nM–1 μM) had both stimulative and inhibitory effects at different levels in the neuromuscular transmission of the guinea-pig ileum.

In Fig. 5 a schematic drawing presents known actions of ACh on intestinal smooth muscle. Like most other agonists, such as histamine, ACh induces changes in membrane potentials and also in firing of action potentials. Depolarization in itself increases $Ca^{2+}$ influx to the cell. Action potentials also increase free intracellular $Ca^{2+}$. In addition, $Ca^{2+}$ seems to be more important than $Na^{+}$ as a current-carrying ion in the action potential of intestinal smooth muscle (19). Like most stimulants, however, ACh also increases intracellular $Ca^{2+}$ in depolarized tissue; that is, the stimulant could affect the contractile process without changes in the membrane potential.

Single electrical shocks of the kind used in this study are thought to produce contraction via excitation of postganglionic chol-

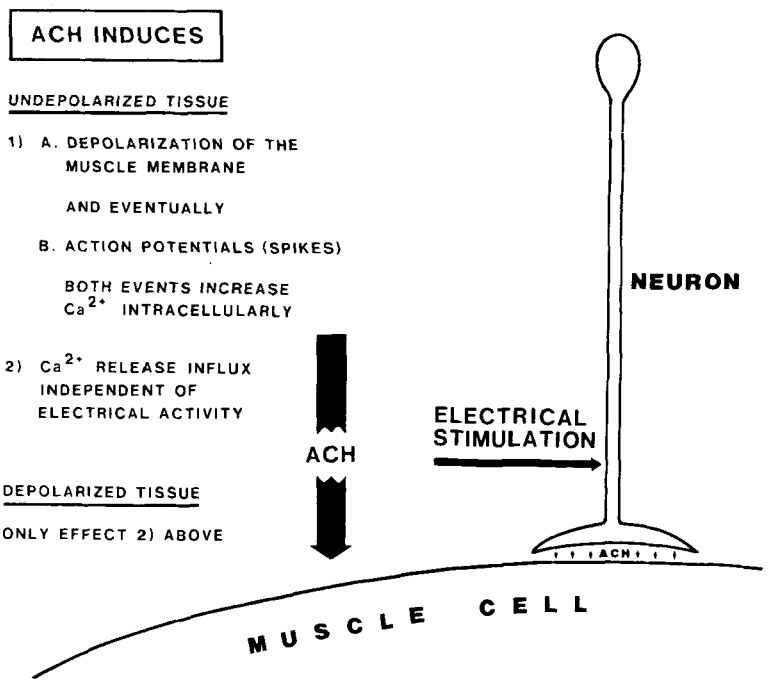


Fig. 5. Action sites of acetylcholine (ACh) in smooth muscle.

inergic nerves in the intestine (20). The facilitating effect of $Cu^{2+}$ in the low concentration range (10 nM–1 μM) was rather similar when the preparation was stimulated neurogenically or directly by ACh (or histamine). This similarity suggests that $Cu^{2+}$ in this low concentration range has no substantiated effect on the release of the cholinergic transmitter. Consequently, it is likely that the facilitating effect of $Cu^{2+}$ on the smooth-muscle contractions is exerted via a step in the neuromuscular transmission that involves depolarization of the muscle cell membrane.

$Co^{2+}$ and $Ni^{2+}$, which are supposed to block $Ca^{2+}$ channels (21), and verapamil and sodium nitroprusside, which are believed to block different $Ca^{2+}$ systems in smooth muscle (22), all blocked the stimulant action of low concentrations of $Cu^{2+}$. This suggests that the action of $Cu^{2+}$ is mediated via increased $Ca^{2+}$ availability for the contractile process. However, the exact point(s) of action of $Cu^{2+}$ remains unsolved. The experiments gave no clear hint of any specific site of action, although the tachyphylaxis of the $Cu^{2+}$ effect suggests an action on either a $Ca^{2+}$ store that is easily depleted or an easily deteriorated $Ca^{2+}$ transport mechanism. Heavy metals have earlier been suggested to increase the calcium permeability of the sarcoplasmic reticulum in vesicles derived from rabbit fast skeletal muscle (23). The capacity of heavy metals to form or initiate formation of disulfide groups from sulfhydryl groups was proposed as an explanation of the increased leakage of $Ca^{2+}$ through the sarcoplasmic reticulum membrane.

In depolarized muscle low concentrations of $Cu^{2+}$ (10 nM–1 μM) diminished contractions induced by ACh and also $Ca^{2+}$-induced contractions in $Ca^{2+}$-depleted muscle. This effect of $Cu^{2+}$ is most likely due to a decreased availability of $Ca^{2+}$ for the contractile process, although the possibility of a direct action on the contractile elements cannot be excluded. Furthermore, the antagonistic effect of $Cu^{2+}$ on $Ca^{2+}$ contracture suggests an interference with $Ca^{2+}$ influx into the cell. It is possible that the depressant action of low concentrations of $Cu^{2+}$ is also present in non-depolarized tissue but masked by the stronger stimulant action (see above).

$Cu^{2+}$ in high concentrations (100 μM) produced contraction by itself in non-depolarized tissue (and initially enhanced contractions produced by nerve stimulation or ACh). In depolarized tissue this concentration of $Cu^{2+}$ enhanced the contractile response to ACh and $Ca^{2+}$ (1 mM). It is possible that this effect of copper is in part related to a copper-produced release of bound $Ca^{2+}$ from a storage site.

The present study has shown that $Cu^{2+}$ could have diverse and dose-dependent biological effects. In this connection it is of particular interest that the concentration of copper in the oral mucous membrane, in contact with or located near copper-containing alloys, could well reach the levels studied here (7). Furthermore, the amounts of Cu in saliva have been shown to vary between 200 and 700 nM (24). However, no differences in Cu concentrations were found between subjects with or without oral mucosal symptoms. The effect of an element will be dependent on both concentration and state, ionic or bound, at the possible site of action. At present we do not know the fraction of free $Cu^{2+}$ ions or the proteins to which $Cu^{2+}$ is bound after corrosion in the oral cavity of man. In vitro the binding of corroded metallic ions to 'salivary-type proteins' has been shown to vary with the amount, composition, and pH of an artificial saliva (25). A decreased salivation could increase the concentration of free ion in the saliva. In the rat and rabbit inhibition of salivation has been shown to increase the penetration of dyes into the mucosa (26).

Changes in the oral epithelium could change the conditions for penetration of elements into the mucosa. Penetration into non-keratinizing epithelium is greater than into keratinized epithelium. Loading or mechanical wear of dental restorations could alter the barrier function and permeability of the oral mucosa (27). It is also possible that the ionic basis of excitation in sensory receptors could be altered with changed osmotic conditions. However, there appears to be a lack of knowledge about the ionic basis of excitation of taste and pain receptors

in the oral cavity. Nevertheless, it may be reasonable to believe that copper from dental alloys could, after corrosion, exert an action on oral excitable tissues. In this context the possible effect of $Cu^{2+}$ on taste and pain receptors is of particular interest and points to a field of further research.

*Acknowledgements.*—This work was supported by the Faculty of Odontology, Karolinska Institutet; The Foundation 'Lars Hiertas Minne'; and the Swedish Medical Research Council (project 06608), Stockholm. Sweden. We wish to thank Mrs Annika Rosén for excellent help in making the figures.

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Received for publication 5 December 1984