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Relation between Cell-to-Cell Junctions and Electrical Activities in the Dog and Frog Stomach

(electrical coupling/structure/stomach muscle)

TOKIO DOMOTO[a], E. E. DANIEL[b], MITSURU OKI[a]*, and OSAMU TANAKA[a]
[a]*Department of Anatomy, Shimane Medical University, Izumo 693, Japan and* [b]*Department of Neurosciences, McMaster University, Hamilton, Ontario, Canada*

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Electrical activities and cell-to-cell junctions in dog and frog stomach muscle were investigated. In frog stomach no repetitive spike discharges (burst spikes) superimposed on a slow wave, similar to those recorded from dog stomach, could be recorded using microelectrodes, pressure electrodes and suction electrodes. In general, nexuses are absent in the longitudinal muscle layer of dog stomach and everywhere in frog stomach. Close apposition contacts are common in both muscle layers of canine and frog stomach. Thus, present findings indicate that electrical coupling in the frog stomach can occur without nexuses, and the distribution of the close apposition contacts can provide the primary structural basis for coupling of electrical control activity. Rapid electrically coordinated contraction is unlikely in the fundus of dog stomach and at any regions in frog stomach. Nexuses may be required to provide an invariable pathway of current flow and the mechanical connection between cells.

Two basic types of electrical activity have been recorded from the mammalian stomach muscles. They are : 1) electric-control activity (ECA), also called basic electrical rhythm, pacesetter activity, or slow waves (1—5) and 2) electric-response activity (ERA), also known as fast activity, action potentials or spikes (1—4, 6). The former has been found to originate in the longitudinal muscle layer and then spreads passively to the segmental circular muscle layer (1—7). ERA or spike activity, which initiates contractions directly, is triggered by slow waves ; usual spikes occur near the peak of a slow wave, and most of them arise in the circular muscle (3, 5, 6, 8). Such evidence indicates that adjacent smooth muscle cells are electrically coupled. On the other hand, in frog stomach muscle there are no typical slow waves (or ECA) comparable to those recorded from mammalian stomach ; thus the longitudinal muscle generates spontaneous electrical activity consisting of rhythmic slow spikes (8), also called long spikes (9), with a duration of 10—15 sec, which propagate into the circular muscle. There is also no typical fast spike activity (so-called spike activity) comparable to that recorded from mammalian stomach (8—10).

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*To whom correspondence should be addressed.

Therefore, the structures which play a role of electrical coupling between the adjacent muscle cells, or between the longitudinal and circular muscle layer have to be clarified. It is generally accepted that nexuses or gap junctions are the site of low resistance to current flow between smooth muscle cells (7, 11). Ultrastructurally, they appear as a fusion of the outer leaflets of adjacent plasma membranes in ultrathin-sections (7, 11), or as aggregates of membrane particles in freeze-fracture electron microscopy (12). Such junctions have commonly been found in circular muscle, but are exceedingly rare or absent in the longitudinal muscle of canine gastrointestinal wall fixed *in situ* by perfusion with glutaraldehyde (13—16). The nexuses or gap junctions may be essential for propagation of ERA (or spike) (14—16) ; however, their relation to ECA (or slow wave) is uncertain.

The purpose of the present investigation was to study the intercellular junctions in the canine and frog stomach muscles and to consider what structures provide for electrical coupling among these smooth muscle cells.

MATERIALS AND METHODS

Electrical recording : Electrical activity was recorded from the isolated or non-isolated (*in situ*) bullfrog stomach, using either external electrodes or intracellular microelectrodes. The external electrodes were the silver-silver chloride (0.15 mm in tip-diameter) and the pressure electrodes (50—100 μ in tip-diameter) ; the pressure electrodes were the glass micropipettes filled with agar-3 M KCl solution. Intracellular potentials were recorded from the isolated frog stomach muscles, using so-called microelectrodes filled with 3 M KCl solution and with resistance between 30 to 60 megohms. The muscle strips obtained from stomach were perfused with Ringer's solution for at least 1 hr before the potential recordings. The standard Ringer's solution contained (mM per liter) : NaCl 112, KCl 2.0, $CaCl_2$ 1.1, and 2.0 Na-phosphate buffer to give pH 7.1. In some experiments, $NaHCO_3$ was used instead of Na_2HPO_4, but no appreciable differences between the results were observed.

Electron microscopy : Isolated tissues from frog stomach were fixed first in 2% glutaraldehyde, then in 1% osmic acid fixatives, both fixatives having been buffered with phosphate. The dog stomach was fixed with 1% glutaraldehyde in Krebs solution by means of an arterial cannulation, inserted via the left common iliac artery (the aorta was clamped above the coeliac artery). The stomach strips were fixed in the same fixatives for an additional 2 hr at 4°C and diced into several blocks and postfixed in 2% osmium tetroxide in phosphate buffer for 2 hr. The blocks were dehydrated in graded series of ethanols and propylene oxide, and embedded in Epon 812. Thin sections were cut on a Porter-Blum MT-2B ultramicrotome, mounted on uncoated 200-mesh copper grids, double stained with uranyl acetate and lead citrate, and examined with a JEM-7A microscope.

RESULTS

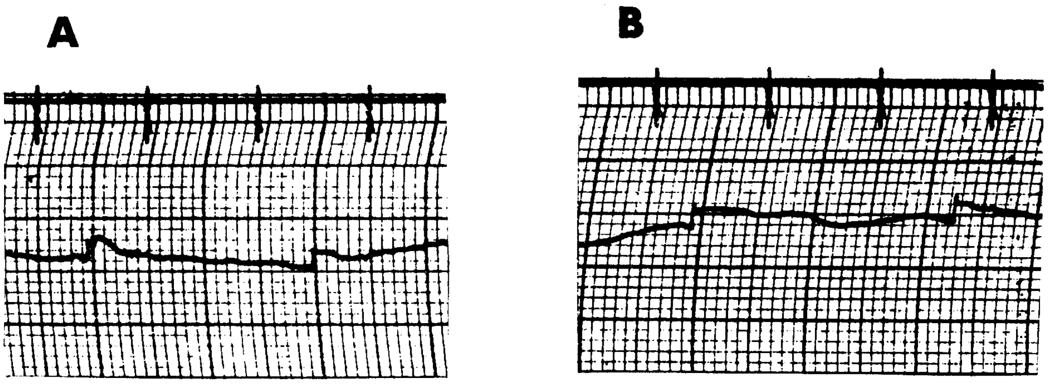
Electrical Activity

In bullfrog stomach, spontaneous electrical activity could be recorded from the longitudinal and the circular muscle layer, using the micro-electrode technique. Usually, spontaneous electrical activity recorded from the longitudinal muscle appeared as repetitive slow waves that lacked spike potentials (Fig. 1a). The amplitudes of the slow waves recorded from the longitudinal muscle, in most cases, varied from 5 to 10 mV, and the duration of the slow waves ranged from 3 to 5 sec. In these records, the resting membrane potentials were about −45.1 mV (SE=±4. 6, N=80). In Fig. 1a, the amplitudes of the slow waves were about 8.4 mV and the duration ranged from 4.0 to 4.25 sec, while the maximum membrane potential during the quiescent period was about −49 mV ; these slow waves repeatedly appeared at regular intervals of about 11—12 sec and more consistently in the longitudinal muscle than in the circular muscle. The other types of spontaneous slow-membrane changes were the long-term membrane potential fluctuation cycles ; they were recorded only from the longitudinal muscle ; their amplitudes, in many records, varied from 2 to 5 mV at the quiescent membrane potentials of −40 — −50 mV ; their durations, in most of the muscle cells, ranged from 20 to 30 sec (Gonda and Oki, data to be published). These slow wave-like fluctuations appeared to be correlated with cyclical changes in the tonus of the stomach muscles. Electrical stimulation of the vagus nerve could induce spike-like discharges, mainly in the circular muscle layer (Fig.

Fig. 1. Typical pattern of the membrane activity recorded from frog stomach muscle, using different types of electrodes. 1a and 1b : microelectrode ; 1c : pressure electrode. Also, 1a : spontaneous activity of the longitudinal muscle, 1b : record induced by electrical stimulation of the vagus, from cicular muscle. No repetitive spikes (burst spikes) were recorded, and slow waves showing long duration were common everywhere in stomach. Vertical bars show 50 mV and time scales, 10 sec, respectively.

1b). The usual stimulus parameters were about 10 V, 1—2 msec in duration, and roughly 15—20 sec intervals of a single stimulation. In Fig. 1b, the spike-like component may be preceded by slowly depolarizing potentials ; hence the spike occurred at the peak of slow waves which seemed to be the generator potentials, although the critical firing level for spike generation was not studied. These spike-like components measured 35.4—37.5 mV. In some preparations a small notch, such as an aborted spike, rarely occurred in the initial rising phase of a slow wave. It is conceivable that this phenomenon might occur as a result of the electrotonic influence of the potential changes that originated in the adjacent muscle cells. It is emphasized that no repetitive spike discharges (or burst spikes) superimposed on a slow wave have been recorded from the frog stomach muscle ; in addition, the spike-like discharges were recorded from the subcardiac regions of the stomach.

Fig. 1c shows the electrical activity recorded from the circular muscles in the immediate vicinity of the pressure electrode-tip. The indifferent electrode was set in contact with the Ringer's solution at some distance from the preparation. As can be seen in Fig. 1c, the polarity of the potential changes was the same as that recorded with microelectrodes (Figs. 1a and 1b). The configuration of the potential was similar to the monophasic action potential (1, 17), which consisted of two components ; the first rapid and the second slow components of the slow waves (1), often with initial spike. When the spike appeared, it was followed by the plateau phase which resembled that recorded from the atrial muscle ; the plateau often consisted of the first rapid phase of repolarization followed by the slower phase of repolarization. In some records the two components, as described above, were less clearly defined. In Fig. 1c, there were also abortive monophasic-action potentials or oscillatory potentials. These events, as have been discussed by Bortoff and Weg (17), probably imply that membrane currents, generated from several cells in the immediate vicinity of the recording electrode, do not spread much further through the muscle layer.



Figs. 2A and 2B. The frog stomach electrical activity recorded with suction electrode. Fig. 2B was recorded immediately after application of Ba^{++}. Time scale : 10 sec.

Fig. 2 shows electrical activity recorded from the longitudinal muscle layer of the body (Corpus ventriculi) using the suction electrode. The electrode consists of a silver-plated (Ag-AgCl) syringe-needle with an outer diameter of about 3.5 mm. The proximal lumen of the electrode was connected to a suction pump with a negative pressure of about 100—150 mm mercury. A silver plate (1×3 cm^2) was used as the indifferent electrode and was connected to the abdominal skin.

In Fig. 2A, the potential changes, which probably correspond to the demarcation potentials, consisted of a first rapid depolarization and a second slow repolarization of the slow waves ; these electrical activities may imply action currents induced by electrotonic currents derived from active cells. Their magnitudes and total durations are variable even in the control (Fig. 2A). When 2 or 3 drops of 2 mM $BaCl_2$ solution were applied over the longitudinal muscle layer in the immediate vicinity of the suction electrode, the second phase, so-called plateau, showed a prolongation in duration as well as a reduction in membrane potentials. After application of $BaCl_2$, there was no initiation of spikes in the circular muscle layer, although Papasova *et al.* (1) recorded spike discharges in cat stomach. It is conceivable that the electrical properties of the frog stomach muscle probably differ from those of the cat stomach (18).

Cell-to-Cell Junction

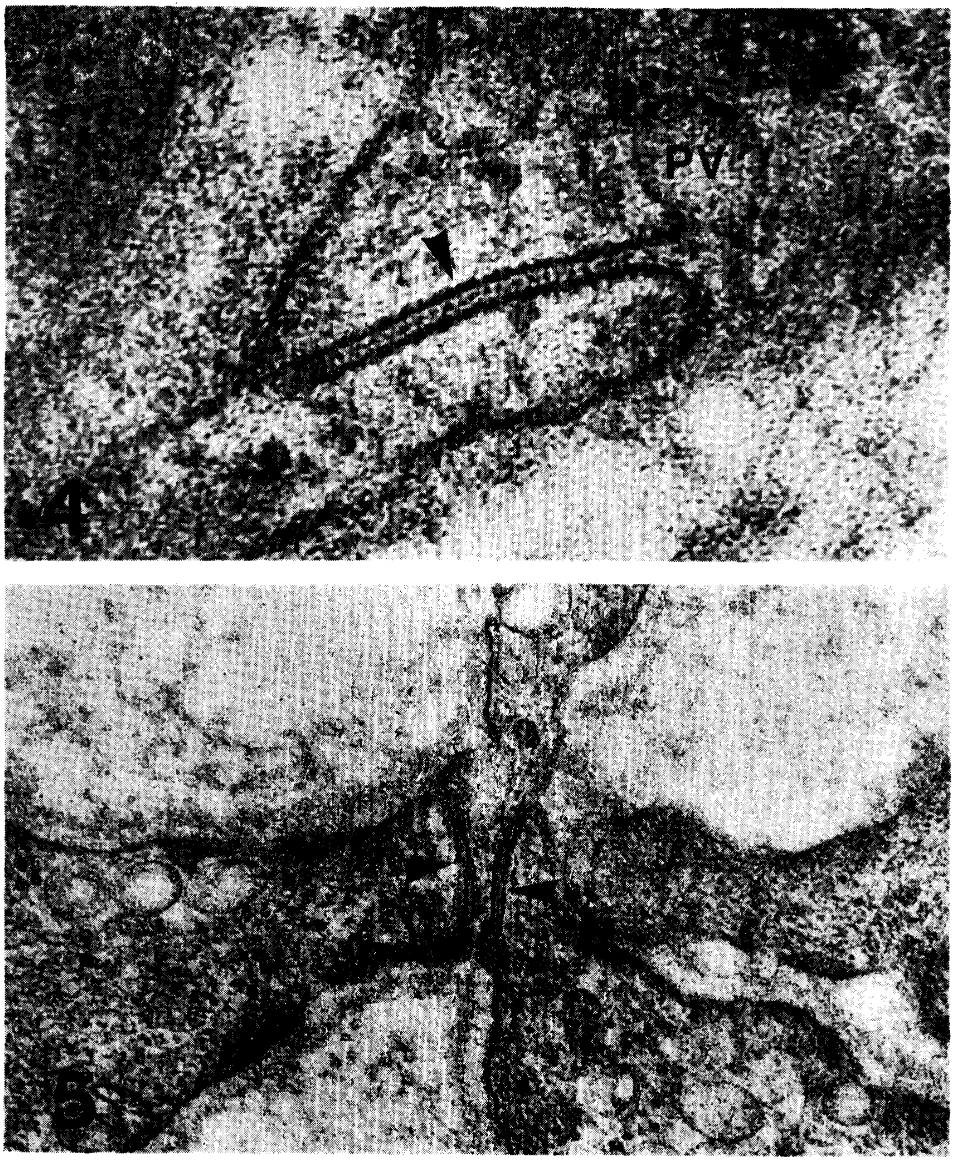
It has been reported that both the dog and the frog stomach have no longitudinal muscle, in the gastric angulus (Incisura angularis) near the lesser curvature (19) or in the middle region (9) respectively. However, in the

Fig. 3. Nexus and close apposition contact seen in the circular muscle of the canine antrum. (×26000)

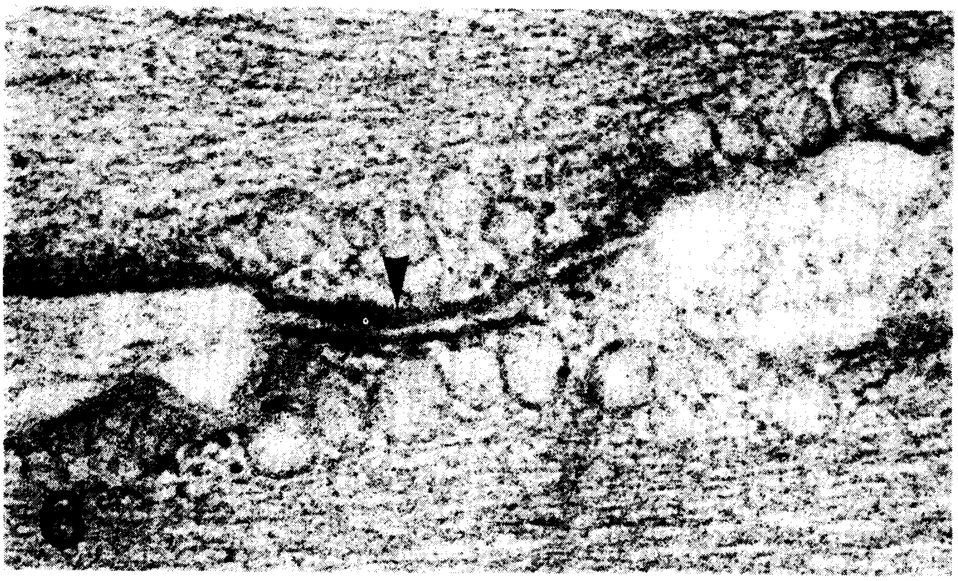
present study, a thin longitudinal muscle layer was found in these regions in both species (data will be published separately).

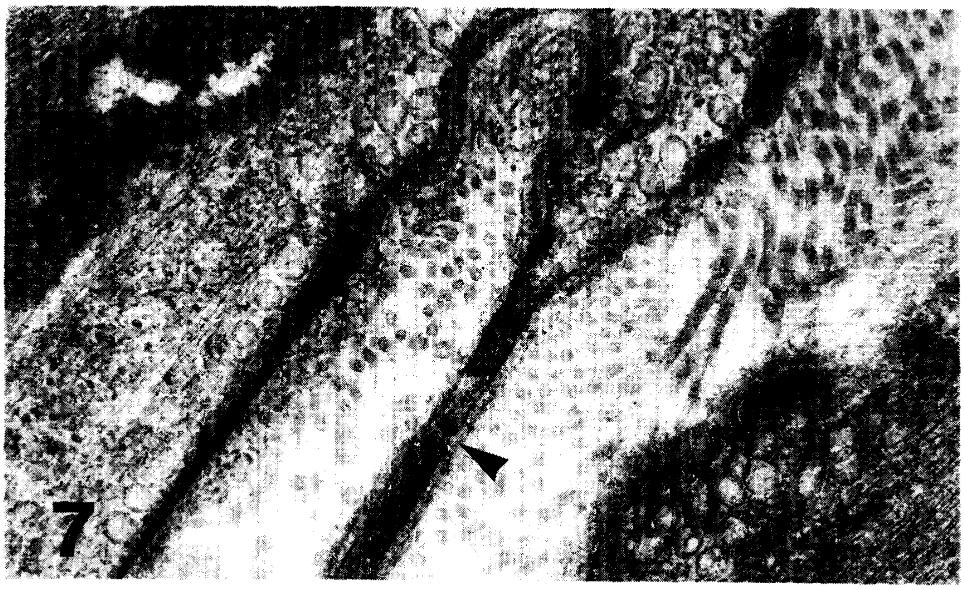
Dog Stomach

It has been ascertained, that intra-arterial perfusion with glutaraldehyde fixatives followed by post-osmication is most reliable in preserving cell-to-cell junctions (20). Ultrastructurally, in the circular muscle layer of the dog stomach there were two types of typical cell-to-cell contacts ; that is, nexus and close apposition contact (Fig. 3). Fig. 4 shows a nexal contact where the three dense lines separated by two clear spaces are visible ; thus, the outer leaflets of the plasma membranes appeared to fuse with each other, so that intercellular spaces seem to be absent ; the inner leaflets of the membranes are apparently electron-dense and thicker than elsewhere. The total average



Figs. 4 and 5. Nexual contacts (arrow-heads) seen in circular muscle of dog stomach : they were generally absent in longitudinal muscle of dog stomach and in every area in frog stomach. (Fig. 4 ; ×170000, Fig. 5 ; ×75000)





Figs. 6 and 7. Close apposition contacts (arrow-head) appeared in dog stomach, e. g. by simple protrusions (Fig. 6 ; ×80000) and finger-like processes protrusions (Fig. 7 ; ×40000). Such are common types of intercellular contacts in both muscle layers and in both dog and frog stomach.

widths of the nexuses, including plasma membranes, were 200 Å. Fig. 5 shows that two or more nexus-contacts appear among several cells in convergent state. The nexuses were common in the circular muscle and their distributions were consistent with the electrically active regions, as previously reported (14). Usually, nexuses were absent in that region of the circular muscle layer where the slow waves were absent. In addition, they were absent or rare everywhere in the longitudinal muscle layer and were found only in the antrum. Indeed, in one arbitrary experiment, there were 2 nexuses in 560 cells from longitudinal muscle cut in cross section (0.004 nexus/cell, in relative frequencies), while in circular muscle of the same region there were 168 nexuses in 560 cells (0.3 nexus/cell). Virtually it is

Fig. 8. Nexus-like contact (arrow-heads) seen in the circular muscle of frog stomach. This structure often appeared in the stomach after immersion-fixation. pv : pinocytotic vesicle. (×100000)

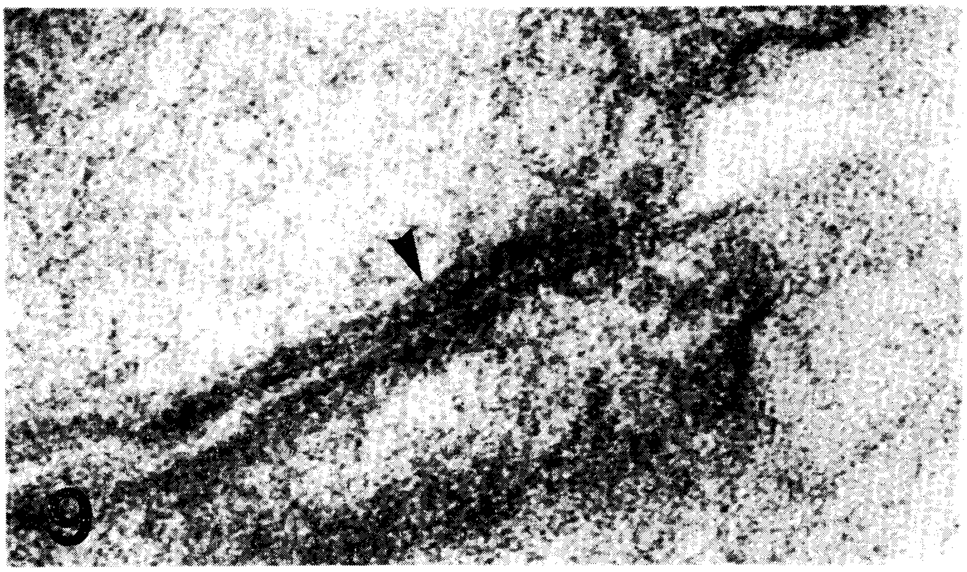


Fig. 9. Intermediate contact (arrow-head) in frog stomach. This type of contact which rarely was seen in either frog or dog stomach, usually was found in the longitudinal muscle. (×60000)

likely that in the longitudinal muscle layer the nexus has no functional role. Figs. 6 and 7 show "simple close apposition" contacts. They are the main type of cell-to-cell contacts in the longitudinal muscle layer. Oki and Daniel have reported several types of their contacts (14) ; e. g. by simple protrusion (Fig. 6), by simple projection (Fig. 7), by bulbous projection, by invagination, etc. They are characterized by a close contact of the adjacent muscle cells where the plasma membranes run in parallel, and are separated by an intercellular space of approximately 200—250 Å. The subplasmalemmal cytoplasm at these contacts is more electron-dense than elsewhere. Usually, they lack the basement membranes between the junctions. Such structures between the smooth muscle cells may be one of the sites of electrical transmission by the electrotonic spread. The other type of cell-to-cell contact was the intermediate contacts, which were invariably few in dog stomach, as will be described later.

Frog Stomach

In the stomach of bullfrog, a nexal structure was never apparent, although a "nexus-like" structure was often found, mostly in the circular muscle, after perfusion-fixation. As can be seen in Fig. 8, the nexus-like contacts never exhibited membrane fusions between the adjacent cells. Such contacts were characterized by the presence of four membrane leaflets between the cells and of the pinocytotic vesicles in close association with cell membranes; thus, they are not gap junctions. We also found that, after presoaking the non-perfused stomach in low calcium Ringer's solution (containing 0.2 mM $CaCl_2$), nexus-like contacts were often produced in both longitudinal and circular muscles. Bozler *et al.* have reported that the frog stomach muscles presoaked in the Ringer's solution showed an increase of intracellular sodium ions (28).

In Fig. 9, this type of cell-to-cell contact may be the intermediate contact where the intercellular spaces are usually 300—600 Å. The center of the intercellular spaces usually consists of an electron-dense line or band, often of granular appearance. The cytoplasm under the cell membrane at such contacts is invariably occupied by electron-dense substances. This type of contact is occasionally seen mainly in the longitudinal muscle layer of both the frog and the dog stomach. It is emphasized that in the frog stomach, only the close apposition contacts were common in both muscle layers.

DISCUSSION

In dog stomach, electrically active and inactive regions have been defined (14). In the active regions, the gastric corpus and antrum showed regular control potentials (also called slow waves); and spike activities when present were at the frequency of control potentials. In the inactive regions, the slow waves were not initiated by the electrical stimulation of the stomach wall, using square pulses of about 100 msec (14). On the other hand, it is also known that in the isolated guinea-pig stomach, the antrum generates slow potential changes with the generation of the spikes, but such potential changes are not observed in the fundus and the cardia (21). It has been accepted that the slow waves originating in the longitudinal muscle of the orad corpus spread circumferentially to the contralateral gastric wall and also distally to the gastroduodenal junctions (2—5). In the human stomach, the pacemaker region is also located in the midcorpus along the greater curvature; and the pacemaker potentials (slow waves) propagate circumferentially and aborally from the pacemaker (22). Bedi *et al.* (23) reported that selective excision (myomectomy) of a band of the longitudinal muscle layer, but not of the circular muscle layer, mostly resulted in the permanent uncoupling of the slow waves across the operated sites ; in contrast, circular myomectomy temporarily interrupted the propagation of the slow waves. Therefore, they concluded that the canine, gastric longitudinal muscle layer propagated the slow waves. It has also been reported that in guinea-pig stomach, the slow waves and superimposed spikes are recorded from the circular muscle of the antrum more consistently than from the longitudinal muscle (24). In the small intestine, the slow waves also appear to be generated in the longitudinal muscle and spread electrotonically to the underlying circular muscle (25). It has been reported that the slow wave behaves like a generator potential (24). Such a hypothesis has been confirmed by Sarna *et al.* (5).

Ultrastructurally, the results of the present study showed that in the dog stomach, nexuses were absent or rare in any region of the longitudinal muscle layer and, if present, were found only in the antrum. Close apposition contacts were common between the longitudinal muscle cells along the greater curvature and occurred most frequently in the antrum, whereas their occurrence above the antrum and nearer the lesser curvature was rare. If longitudinal muscle provides the structural basis for coupling of the slow waves, the current flow must occur at the close apposition contacts between the cells or at similar structures providing a low resistance pathway for the current flow. This hypothesis may be supported by the fact that frog stomachs have no nexuses in the longitudinal and circular muscle where only close apposition contacts were observed. Shuba (29) recorded electrotonic potentials in the frog stomach muscles using extracellularly applied currents and concluded that the spread of the action current from one muscle fiber to the next was due to the electrotonic coupling based on the presence of a certain continuous morphological link between the cells.

The fact that many close apposition contacts and occasional nexus-like contacts, but not nexuses, are found in the frog stomach is consistent with the hypothesis that electrical coupling can occur without nexuses. It is assumed that nexus-like structures may be labile and easily disrupted, because they were produced when muscle cells were swollen by metabolic inhibition (20). Intra-arterial perfusion with glutaraldehyde resulted in good fixation of the dog stomach in which nexus-like structures were never found. Thus nexus-like contacts appear to be structurally, highly labile depending on the fixation of the tissues. In the present study, the intermediate contacts rarely appeared between the cells. Their functions, however, remain unknown. It has been suggested that nexuses, as well as intermediate contacts, may have a degree of mechanical strength and may serve for attachment between cells (13, 26). Thus, in the dog stomach, there was a tight coupling of electrical control activity, with the generation of spikes along the greater curvature and throughout the antrum ; and there were corresponding high frequencies of close apposition contacts in the longitudinal and of the nexuses in the circular muscle.

The nature of the spontaneous electrical activity in stomach of the lower vertebrates is poorly understood. In the present study, the spontaneous electrical activity of the frog stomach muscle showed repetitive slow waves that lacked spike potentials resembling those seen in dog stomach. Berger (27) recorded the membrane activity of the frog stomach using the double sucrose gap method, and reported that the circular muscle showed only slow potentials. Prosser (8, 18) recorded spontaneous electrical activity from the toad stomach, using the pressure electrode and the sucrose gap methods; each of these showed rhythmic "long spikes" which consisted of the spike-like depolarizations of several seconds' duration ; he also reports that in toad stomach, the spontaneous long spikes occur in the longitudinal muscle. It has also been reported that in *Rana pipiens* the slow spike originates in a pacemaker in the longitudinal muscle layer located at the cardiac end of the stomach and the spike is transmitted to the circular muscle layer ; this author also reported that not every slow spike was associated with contraction, except in a short segment of stomach (10). These slow spikes correspond well to the slow waves described in this report. In addition, we failed to elicite the burst-spikes superimposed on the slow waves. Hence, it is postulated that electrical coupling in the frog stomach can occur without nexuses, and the distribution of the close apposition contacts can provide the primary structural basis for coupling of the electrical control activity. Nexuses may be required to provide an invariable pathway of the current flow and the mechanical connection between cells.

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