Table 2. Concentration of phenytoin required to inhibit drug metabolizing enzymes in liver by 50% (IC_{50})

Enzymes	IC_{50} Value*
Aniline hydroxylase	31.6 μM
Aminopyrine-*N*-demethylase	79.2 μM

* Average of three experiments in triplicate.

bition of both APN-D and AH which metabolize type I and II substrates respectively. Iinhibition of these enzymes may also impair the metabolism of various other drugs or xenobiotics which are inactivated by the same pathway rendering the developing organism more sensitive to drugs and chemicals.

In summary, present studies demonstrate that neonatal (2–14-day-old) phenytoin exposure (40 mg/kg/day orally) inhibited aniline hydroxylase and aminopyrine-*N*-demethylase in postnatally maturing animals, which persisted up to 12 weeks of age. *In vitro* studies showed that phenytoin inhibited the enzyme activities competitively. These effects were sex dependent. Inhibition of these enzymes may be responsible for reduced phenytoin metabolism and hepatic damage seen under clinical conditions.

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Muscarinic regulation of Ca^{2+} mobilization in a human salivary myoepithelial cell line

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Secretion of saliva is considered to be under the control of the autonomic nervous system [1, 2]. Studies concerned with the neuroreceptor regulation of salivary secretion have largely been confined to acinar cell preparations (e.g. Refs 1 and 3–5) or to excretory duct segments [6]. Myoepithelial cells represent an additional component of salivary and other exocrine glands [7]. While the exact function of these cells in secretory events is not clearly established, they are

contractile [8, 9] and are believed to play a role in facilitating the extrusion of viscid secretions, preventing any fluid backflow, and physically supporting other parenchymal elements [8, 10]. Generally, functional studies of myoepithelial cells have been somewhat indirect (e.g. Refs 8 and 9). In the human submandibular gland, myoepithelial cells represent only ~ 2.5% of the total gland volume [8]. In typical studies, following electrical or pharmacological stimuli, morphological features of myoepithelial cells are then assessed. Such experimental approaches, while useful, are limiting, and convenient preparations for more direct and detailed studies have not been available.

Recently, Sato and colleagues reported the establishment of a cell line (HSG-MY), by treatment with sodium butyrate of a parent human salivary gland adenocarcinoma cell line (HSG-PA) [11]. The HSG-MY cell line exhibits a phenotype similar to myoepithelial cells including immunocytochemical staining for marker proteins such as myosin, β-chain of S-100 protein, myofilaments and the oxytocin receptor [11]. It is the contractile elements of true myoepithelial cells and their purported contraction during autonomic secretory stimuli that appear to be of the most biological significance (see Refs 8 and 9). Emmelin and his colleagues [8, 12, 13] and Nishiyama *et al.* [9] have suggested that cholinergic stimulation can result in gland tissue contraction likely due to contraction by myoepithelial cells. Furthermore, a lack of Ca^{2+} in the extracellular perfusing solution reduces this contractile effect [9]. To study directly the suggested important role for cholinergic-stimulated Ca^{2+} mobilization in myoepithelial cell function, we have utilized the HSG-MY cell line. Previously, we have similarly studied the nature of cholinergic-stimulated Ca^{2+} mobilization in the parent HSG-PA cell line [14]. In the present paper, we report that high-affinity muscarinic-cholinergic receptors coupled to calcium mobilization were found in HSG-MY cells and displayed differences in their characteristics from those observed in HSG-PA cells [14].

Materials and methods

Cell culture. Experiments were performed on HSG-MY cells, a gift from Dr Mitsunobu Sato. HSG-MY cells were cultured in Eagle's minimum essential medium supplemented with 10% newborn calf serum, 100 units/ml penicillin G, and 100 μg/mL streptomycin sulfate (all from Biofluids, Rockville, MD) at 37° in a humidified 5% CO_2 atmosphere. HSG-MY cells used in these studies were at passages 9 to 17 and were subcultured twice weekly.

Measurement of muscarinic receptors. HSG-MY cell membranes were prepared, and muscarinic receptors were measured, by a [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding assay, as described [14]. Specific binding of [^{3}H]QNB was defined as the difference between total binding and non-specific binding ([^{3}H]QNB binding measured in the presence of 10 μM atropine). Specific binding typically accounted for more than 60% of total radioligand binding at [^{3}H]QNB concentrations near the K_d. The K_d was obtained by analyzing the specific binding of different concentrations of [^{3}H]QNB according to the method of Scatchard [15].

Measurement of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). The $[Ca^{2+}]_i$ was measured using the fluorescent dye quin2 (Calbiochem, LaJolla, CA) essentially as described before [14, 16]. Fluorescence was measured at 37° in an SLM-8000 spectrofluorimeter (microprocessor controlled) in ratio mode as previously described [5]. Calibration of the fluorescence signal and calculation of $[Ca^{2+}]_i$ were as described by Tsien *et al.* [17].

Protein assay. Protein was determined by the method of Lowry *et al.* [18] with bovine serum albumin as the standard.

Results and discussion

Specific binding of [^{3}H]QNB to HSG-MY membranes was rapid, reaching equilibrium values within 20 min at 37° (data not shown). The specific binding of [^{3}H]QNB was concentration dependent and was saturable. [^{3}H]QNB appeared to interact with a single class of binding sites since the calculated Hill coefficient was 1.04 ± 0.07 (mean ± SE, N = 3). When these data were analyzed according to the method of Scatchard [15], a K_d of 0.35 ± 0.06 nM and a B_{max} of 73 ± 7 fmol/mg protein were determined.

As shown in Fig. 1, carbachol (Cch, 100 μM) induced a rapid increase in $[Ca^{2+}]_i$ of HSG-MY cells. On the average this corresponded to an increase in $[Ca^{2+}]_i$ from 173 ± 15 nM to 535 ± 82 nM (N = 7). $[Ca^{2+}]_i$ returned to initial levels within 5 min after stimulation with the agonist (Fig. 1). The muscarinic antagonist atropine (10 μM) reversed Ca^{2+} mobilization if given subsequent to Cch exposure and, if cells were exposed to atropine prior to Cch stimulation, the Ca^{2+} response was inhibited (data not shown).

When cells were preincubated with the inorganic Ca^{2+} entry blocker La^{3+}, the Cch-induced rise of $[Ca^{2+}]_i$ was blunted and declined to initial levels within 3 min (Fig. 2a). Similar results were seen when cells were incubated in a nominally Ca^{2+}-free medium (Fig. 2b and c). If Ca^{2+} (final concentration, 1.5 mM) was then added to the incubation medium, a second rise in $[Ca^{2+}]_i$ was evoked, from 113 ± 18 to 240 ± 46 nM (N = 3, Fig. 2b). This second Ca^{2+} response was inhibited completely by 10 μM atropine (Fig. 2c).

As shown in Fig. 3a, membrane depolarization, induced by addition of KCl (55 mM) to the incubation medium, appeared to have no obvious effect, by itself, on $[Ca^{2+}]_i$ in HSG-MY cells, but profoundly inhibited the Cch-induced Ca^{2+} response (compare with Fig. 1). In the presence of 55 mM KCl, the $[Ca^{2+}]_i$ at rest was 137 ± 24 nM and increased to 237 ± 63 nM (N = 4) with 100 μM Cch stimulation. When cells were incubated in the presence of 5 mM tetraethylammonium chloride, a putative K^+ channel blocker, and then exposed to Cch (100 μM), a similar inhibitory effect on Ca^{2+} mobilization was observed (data not shown). Gramicidin (1 μM), which should depolarize cell membranes, also moderately blunted the Cch-induced Ca^{2+} mobilization (Fig. 3b, peak $[Ca^{2+}]_i$ was 303 ± 108 nM, N = 4). Neither verapamil (Fig. 3c), diltiazem or nifedipine (not shown) had any obvious effects on the ability of Cch to mobilize Ca^{2+} in HSG-MY cells.

Our findings have demonstrated that HSG-MY cells, possess high-affinity muscarinic–cholinergic receptors which are functionally coupled to Ca^{2+} mobilization. Previous studies with various mammalian submandibular glands [8, 9, 12] suggested that myoepithelial cell function (contraction) was regulated by cholinergic stimuli, and was dependent on the existence of Ca^{2+} in the extracellular fluid [9]. The current studies, by showing the presence of a

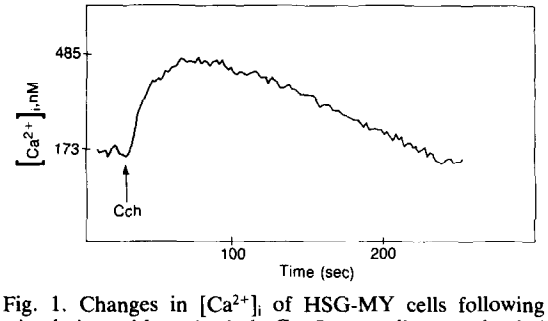

Fig. 1. Changes in $[Ca^{2+}]_i$ of HSG-MY cells following stimulation with carbachol. Confluent cells were loaded with a 50 μM concentration of the acetoxymethylester of quin2, and $[Ca^{2+}]_i$ was determined as described in the text. Cells were incubated in a complete medium, and carbachol (Cch, 100 μM) was added at the arrow. The data shown are typical of results obtained with three different cell preparations.

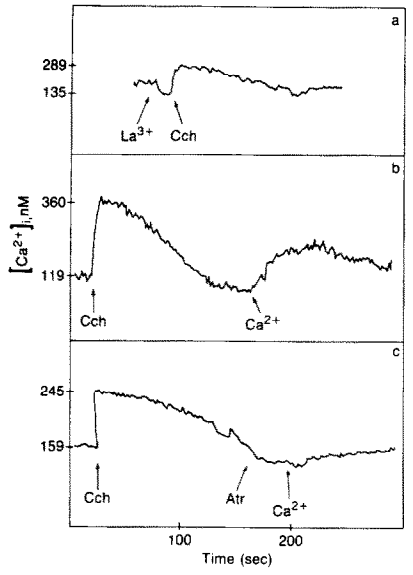

Fig. 2. Two components of Ca^{2+} mobilization induced by carbachol in HSG-MY cells. Confluent cells were treated as in Fig. 1, and $[Ca^{2+}]_i$ was determined as described in the text. Cells were incubated in a complete medium (a) or a nominally Ca^{2+}-free medium (b and c). La^{3+} (25 μM), carbachol (Cch, 100 μM), atropine (Atr, 10 μM) and Ca^{2+} (1.5 mM) were added at the arrows. The data shown are typical of results obtained with three different cell preparations. The decline in baseline following addition of La^{3+} likely results from quench of fluorescence signal from extracellular (leaked) quin 2.

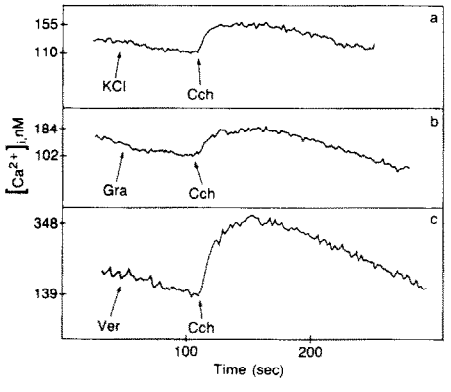

Fig. 3. Effect of membrane depolarization and an organic Ca^{2+} channel blocker on Ca^{2+} mobilization in HSG-MY cells. Quin2-loaded cells were incubated in a complete medium, and $[Ca^{2+}]_i$ was measured as in Figs 1 and 2. KCl (55 mM), Cch (100 μM), gramicidin D (Gra, 1 μM) and verapamil (Ver, 10 μM) were added at the arrows. The data shown are typical of results obtained with three different cell preparations.

muscarinic–cholinergic receptor-activated Ca^{2+} entry pathway in HSG-MY cells, provide support for the earlier suggestions.

HSG-MY cells are derived by treatment with sodium butyrate of the parent HSG-PA cell line [11]. The sodium butyrate treatment, besides altering the morphological and immunocytochemical appearance of the parent cells, considerably changes several characteristics of the muscarinic receptor/Ca^{2+} mobilizing system. For example, HSG-MY cells have approximately twice the number of muscarinic receptors ([^{3}H]QNB binding sites) present in HSG-PA cells, 73 vs 37 fmol/mg protein [14], and exhibit a difference in ligand binding affinity (K_d = 0.35 vs 0.17 nM). Cch (100 μM) can mobilize Ca^{2+} from an intracellular store and via a receptor-operated Ca^{2+} entry pathway in both cell types [14]. However, HSG-MY cells showed a clear functional difference from HSG-PA cells in the latter mechanism. Membrane depolarization (with either 55 mM KCl or 1 μM gramicidin D) blunted the Ca^{2+} entry response in HSG-MY cells, but has no obvious effect in HSG-PA cells under similar incubation conditions [14]. Although the Ca^{2+} entry pathway of HSG-MY cells was clearly influenced by membrane potential, it appeared not to be a classical voltage-operated mechanism, such as seen in electrically-coupled cells, since verapamil, diltiazem and nifedipine were all without effect.

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Isolation and characterization of a novel dihydrofolate formylating enzyme from human MCF-7 breast cancer cells

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Methotrexate (MTX*) is a clinically important drug for the treatment of a variety of neoplasms [1]. MTX is felt to produce its cytotoxic effects through a potent direct inhibition of the enzyme dihydrofolate reductase (DHFR) [2]. Recent investigations using MCF-7 breast cancer cells and normal human myeloid precursor cells have shown that following exposure to MTX, the intracellular levels of dihydrofolate (H_2folate) increase rapidly [3]. Over time, a novel intracellular folate identified as formyl-H_2 folate appears and reaches concentrations equal to that of H_2 folate within 12 hr [3]. The effect of this new folate on several folate-requiring enzymes, including thymidylate synthase [TS] and the *de novo* purine enzymes AICAR and GAR transformylase, was examined [4]. These studies revealed that formyl-H_2folate pentaglutamate was an inhibitor of both GAR transformylase and TS, and an alternate formyl donor for AICAR transformylase. Enzymatic conversion of tetrahydrofolate (H_4folate) to 10-formyl-H_4folate (the required cofactor for both GAR and AICAR transformylase) has been demonstrated using cytosolic extracts of porcine liver [5], but the possible role of H_2folate as a substrate for this reaction has not been examined. In this report, we describe the enzymatic conversion of H_2folate to 10-formyl-H_2folate by a cytoplasmic activity that appears to be distinct from the enzyme responsible for formylation of H_4folate.

Materials and methods

MCF-7 cells were maintained in RPMI-1640 medium (Biofluids, Rockville, MD) enriched with 10% dialyzed fetal calf serum (Gibco, Grand Island, NY). Cells were harvested at 80% confluency and stored at −70° until used. Cytosolic preparations were made by lysing cells with three 5-sec bursts from a sonicator (Branson model 350) in hypotonic buffer (0.05 M Tris buffer, pH 8.5) followed by centrifugation at 100,000 *g* for 30 min. An extract of *Lactobacillus casei* bacterial cytosol was purchased from the New England Enzyme Center (Boston, MA).

H_2folate and H_4folate were purchased from the Sigma Chemical Co. (St Louis, MO). [3′,5′,7,9-^{3}H]Folic acid (20 Ci/mmol; 95–98% pure by HPLC) was supplied by Moravek Biochemicals (Brea, CA).

[^{3}H]H_2folate was prepared by dithionite reduction of [^{3}H]folic acid using a modification of the method of Blakley [6]. The final product was determined by HPLC analysis to be 80–85% H_2folate with 15–20% residual folic acid. A second crystallization was not performed in the preparation of the radiolabeled H_2folate; thus, the preparation used contained residual folic acid. Further purification was not performed as the yield of H_2folate after a second crystallization was poor, and the residual folic acid would not interfere with these experiments. While folic acid may be formylated during these experiments, formyl-folic acid is easily separable from formyl-dihydrofolate by HPLC. The residual folic acid in the commercial preparations and the radiolabeled preparation is evident in the HPLC separations shown in Fig. 1. Standard formyl-H_2folate was prepared by first formylating folic acid using formic acid according to the method of Blakley [7]. Formylfolic acid was then reduced to formyl-H_2folate using the dithionite reaction followed by alcohol precipitation of the product as previously described [8]. The purity of the formyl-H_2folate was greater than 95% as determined by HPLC [4].

All other chemicals were the highest grade obtainable and were purchased from the Sigma Chemical Co.

Measurement of H_2folate formylating activity. Dialyzed cytosolic preparations of either MCF-7 cells or *L. casei* were incubated in a shaking water bath at 37° with H_2folate (0 to 0.4 mM), [^{3}H]H_2folate (0.5 μCi), 100 mM sodium formate, 20 mM $MgCl_2$, 100 mM potassium chloride, 50 mM ATP, and 250 mM 2-mercaptoethanol in 0.05 M Tris–HCl buffer, pH 8. MTX (10^{-5} M) was included in each assay to prevent the reduction of H_2folate or of the formyl-H_2folate product by DHFR present in the enzyme preparations. Reactions were terminated by immersing reaction tubes in boiling water for 60 sec in preparation for analysis by HPLC (*vide infra*). [^{3}H]H_2folate incubated at 37° with 0.5 mM 2-mercaptoethanol for 4 hr was found to be 97% preserved and had a half-life of 26 hr under the assay conditions.

Folate extraction and identification. Separation, identification, and quantitation of formyl-H_2folate, formyl-H_4folate, H_2folate, H_4folate, and folic acid were accomplished by using a reverse-phase HPLC assay as previously described [3] (see Fig. 1). After termination of the enzymatic reaction by immersion of assay tubes into a boiling water bath for 1 min, the folates in the reaction mixture were concentrated using a Waters C_{18} SepPak as previously described [3]. Standards and samples were separated on a Waters high-pessure liquid chromatograph using a C_8 radial

* Abbreviations: MTX, methotrexate; H_2folate, dihydrofolate; H_4folate, tetrahydrofolate; TS, thymidylate synthase; GAR, glycinamide ribotide; AICAR, aminoimidazolecarboxamide ribonucleotide; and DHFR, dihydrofolate reductase.