CMLS, Cell. Mol. Life Sci. 57 (2000) 149–157
1420-682X/00/010149-09 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

Loperamide: novel effects on capacitative calcium influx

J. W. Daly* and J. Harper

Laboratory of Bioorganic Chemistry, Bldg 8, Rm 1A17, National Institutes of Health Bethesda (Maryland 20892, USA), Fax + 1 301 402 0008, e-mail: johnd@intra.niddk.nih.gov

Received 22 July 1999; received after revision 17 September 1999; accepted 5 October 1999

Abstract. Loperamide is a widely used antidiarrheal that primarily acts at nanomolar concentrations through activation of opioid receptors in the gastrointestinal tract. At somewhat higher concentrations, loperamide blocks calmodulin activity, calcium channels, N-methyl-D-aspartate-receptor channels, and maitotoxin-elicited calcium influx. Loperamide at micromolar concentrations has now been shown to have a remarkable stimulatory effect on the capacitative calcium influx that is triggered in many cells by depletion of the inositol-trisphosphate-sensitive stores of calcium in the endoplasmic reticulum. The mechanism whereby loperamide enhances levels of intracellular calcium elevated by capacitative calcium influx is, as yet, undefined.

Key words. Loperamide; calcium channels; calcium stores; maitotoxin; inositol trisphosphate; thapsigargin; ATP.

Tincture of opium, containing morphine, has a long history of effective use as an antidiarrheal. Attempts to discover agents without the undesirable central effects of morphine led to the discovery of the selective antidiarrheal activity of the opioid agonist diphenoxylate (fig. 1) in the 1950s. The diphenoxylate series consisted of compounds combining features of pethidine analgesics and isoproamide anticholinergics [1]. Diphenoxylate, in combination with the muscarinic antagonist atropine, was marketed as Lomotil as an antidiarrheal, but high doses, particularly in infants, often produced opioid-like central activity. An intensive effort to develop a diphenoxylate analog, which would retain the antidiarrheal activity, while having reduced opioid-like central activity, was carried out by Janssen Pharmaceutical. In the course of synthesizing and testing thousands of analogs over a 15-year period [2, 3], loperamide (fig. 1) was discovered [4]. The loperamide series consisted of compounds combining features of haloperidol neuroleptics with those of the isoproamide anticholinergics. A series of analogs with an oxadiazole moiety replacing the dimethylcarboxamide moiety of loperamide represented a further set of antidiarrheals [5].

Loperamide, marketed as Imodium, proved to be a highly satisfactory antidiarrheal drug with a minimum of side effects [2, 3]. Three sites of action have been considered responsible for the antidiarrheal effects of loperamide. The first, as with the action of morphine, is due to blockade of opioid receptors of the gastrointestinal tract resulting in inhibition of peristaltic contractions [2]. Loperamide was about 50-fold more potent than morphine in vitro and the effects were very long-lasting. The opioid antagonist naxolone blocked the effects of loperamide less effectively than those of morphine. Loperamide blocked binding of tritiated naxolone to opioid receptors with high affinity [2, 6] and is a potent agonist with nanomolar affinity for human mu opioid receptors [7]. Specific binding of tritiated loperamide apparently has not been assessed, perhaps due to a high level of nonspecific uptake and binding [8, 9]. Such uptake probably provides a pool of loperamide close to sites of action in the gastrointestinal tract.

* Corresponding author.

In rodents, about 85% of oral-administered loperamide was retained in the gastrointestinal tract [10]. There was little penetration into the central nervous system. In humans, loperamide at oral antidiarrheal dosages of 4 mg attains plasma levels of only about 0.002 nM with nearly all of the drug retained and, thus, at higher concentrations in the gastrointestinal tract [2]. The unusual pharmacokinetics of this drug effectively preclude side effects in brain and other organs. The lack of uptake of this hydrophobic drug into brain appears correlated with a high cross-sectional area when oriented at a hydrophilic/hydrophobic interface [11].

The second site of gastrointestinal action for loperamide is proposed to be an inhibition of calmodulin function. Loperamide inhibits binding of tritiated trifluoperazine to calmodulin [12] and inhibits calmodulin-sensitive phosphodiesterases [13]. Loperamide, with an IC_{50} of about 5 μM, is a potent calmodulin antagonist,

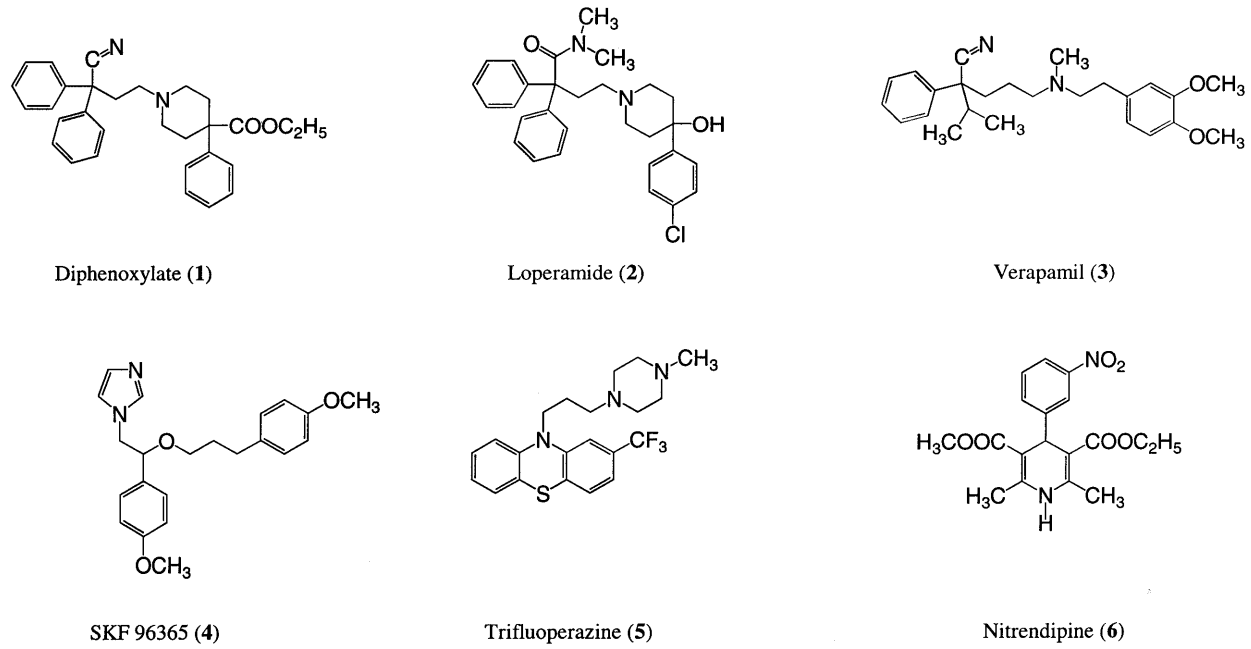

Figure 1. Structures of diphenoxylate, loperamide, and verapamil and of store-operated calcium (SOC) channel blockers SKF 96365, trifluoperazine, and nitrendipine.

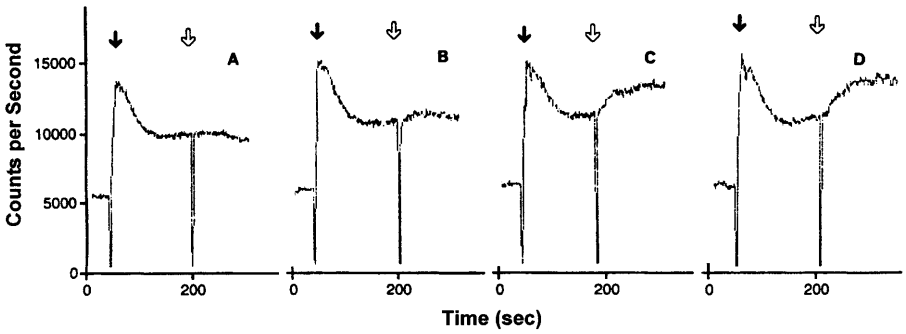

Figure 2. Dose-response effects of loperamide on SOC-channel-dependent elevated levels of intracellular calcium in differentiated HL60 cells. ATP (10 μM) was added as indicated by the solid arrow followed by loperamide indicated by the open arrow at the following micromolar concentrations 1 (*A*), 3 (*B*), 10 (*C*), and 30 (*D*). The incubation temperature was 22 °C. Cells had been previously loaded with fluo-3 and calcium-dependent fluorescence was measured as described by Daly et al. [24]. Data from ref. 24.

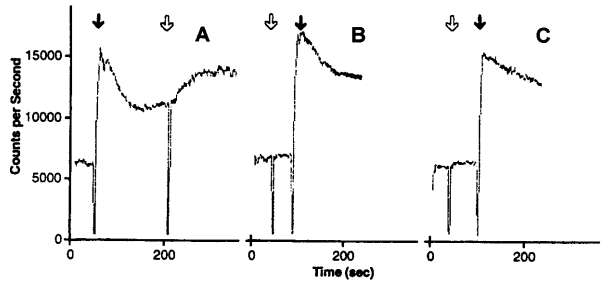

Figure 3. Effect of loperamide on ATP-elicited SOC-channel-dependent elevation of intracellular calcium in differentiated HL60 cells. Loperamide was added (open arrow), either after (*A*, 30 μM) or before (*B*, 10 μM; and *C*, 30 μM) the addition of 10 μM ATP (solid arrow). The incubation temperature was 22 °C. Cells had been previously loaded with fluo-3 and calcium-dependent fluorescence was measured as described by Daly et al. [24]. Data from ref. 24. (*C*) Note the marginal effect on basal levels of the addition of 30 μM loperamide before the addition of ATP.

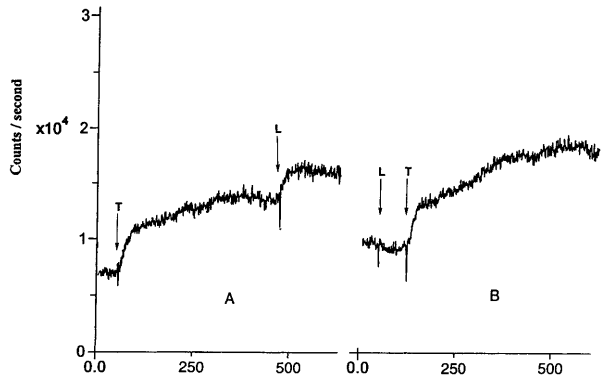

Figure 4. Effect of loperamide on SOC-channel-dependent sustained elevation of levels of intracellular calcium in differentiated HL60 cells: tharpsigargin (T, 15 nM) was added followed by loperamide (L, 30 μM); (*A*) loperamide was added followed by thapsigargin (*B*). The incubation temperature was 22 °C. Cells had been previously loaded with fluo-3 and calcium-dependent fluorescence was measured as described by Harper et al. [51]. Data from ref. 51.

as recently confirmed with brain calcium-dependent phosphodiesterases (unpublished data). In contrast, there has been one report of loperamide as a relatively weak calmodulin antagonist [14]. The effects of loperamide as an antidiarrheal [12], on secretion in a rat colon preparation [15], on adrenocorticotrophic hormone secretion in rat anterior pituitary cells [16], in inhibition of renal secretion of dideoxycytidine [12], and in stimulation of chloride flux in ileum [17, 18] have been proposed to be linked to inhibition of calmodulin-dependent functions. A third mechanism proposed to be involved in the antidiarrheal activity of loperamide is inhibition of

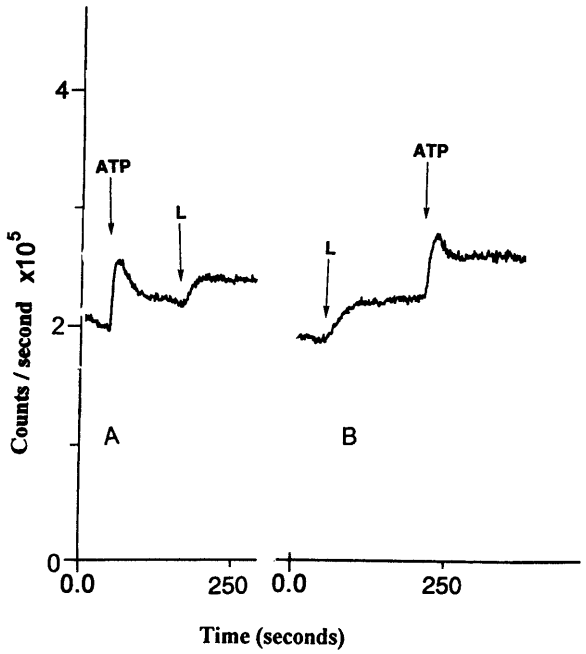

Figure 5. Effect of loperamide on intracellular levels of calcium in astrocytoma 1321N cells: loperamide (L, 30 μM) was added after ATP (10 μM) (*A*) or before ATP (*B*). The incubation temperature was 22 °C. Cells had been previously loaded with fura-2 and calcium-dependent fluorescence was measured as described by Harper et al. [51]. Data from ref. 51. (*B*) Note the loperamide-elicited elevation of levels of calcium prior to addition of ATP and activation of SOC channels. Such effects of loperamide alone were not manifest in differentiated HL60 cells, DDT-MF_2 cells, NIH 3T3 fibroblasts, GH_4C_1 cells, or RBL-2H3 cells [51].

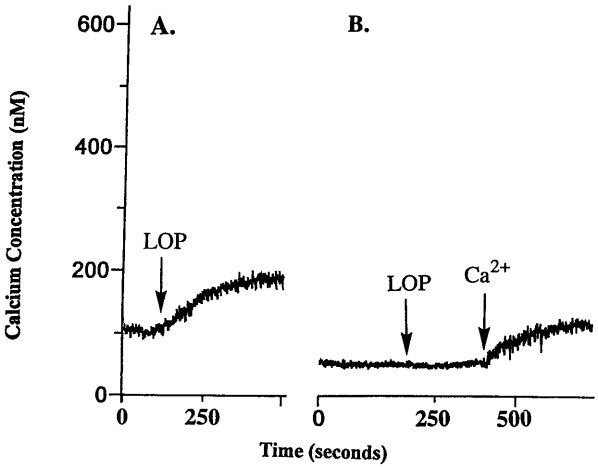

Figure 6. Effect of loperamide on levels of intracellular calcium in undifferentiated HL60 cells. Loperamide (LOP, 30 μM) was added in the presence of extracellular calcium (*A*) or in the absence of extracellular calcium followed by addition of 2 mM calcium chloride (*B*). The incubation temperature was 22 °C. Cells had been previously loaded with fura-2 and the calcium-dependent fluorescence ratio converted to calcium concentration as described by Harper and Daly [53]. Representative results are depicted.

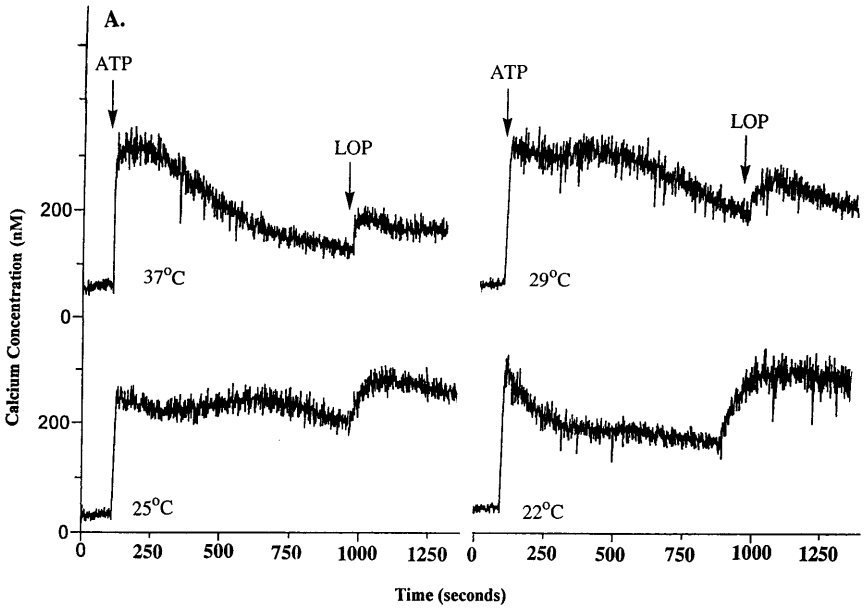

Figure 7. Temperature-dependent effects of ATP and loperamide on elevations of intracellular calcium in differentiated HL60 cells. ATP (10 μM) was added prior to loperamide (LOP, 30 μM) at the indicated temperatures. Cells had been previously loaded with fura-2 and the calcium-dependent fluorescence converted to calcium concentration as described by Harper and Daly [53]. Representative results are depicted.

voltage-dependent calcium channels [19]. Loperamide at submicromolar concentrations blocks L-type calcium channels and inhibits binding of verapamil (fig. 1) to such channels. The antisecretory properties of loperamide may be due in part to blockade of calcium channels [20].

Other sites of action for loperamide have been documented. Loperamide at high micromolar concentrations blocks neuronal voltage-dependent calcium channels [21], N-methyl-D-aspartate-evoked currents [21] and forskolin-elicited secretion [16]. No local anesthetic activity at frog sciatic nerve was reported for 100 μM loperamide [20], but loperamide is a potent (IC_{50}, 0.3 μM) blocker of tritiated batrachotoxinin-A benzoate binding to voltage-dependent sodium channels [22] and at 30 μM markedly inhibits $^{22}Na^+$ influx through batrachotoxin-activated sodium channels in brain preparations (A. Flower, K. Inoueme, J. W. Daly, unpublished results). Locally administered, loperamide has potent antinociceptive activity in several inflammatory pain models [7]. The effect appeared mediated by local opioid receptors, although such peripheral antinociceptive effects of loperamide were less sensitive to blockade by opioid antagonists than those of morphine [23].

Loperamide and capacitative calcium influx

Loperamide with an IC_{50} of 1.6 μM proved to be one of the most potent calcium channel blockers in fibroblast cells in which calcium influx was stimulated by maitotoxin [24]. Diphenoxylate was also very potent, while verapamil was at least ten-fold less potent. Imidazoles, such as miconazole and SKF 96365 (fig. 1), which are widely used as blockers of capacitative calcium influx, were as potent as loperamide in blocking maitotoxin-elicited calcium influx [24]. Indeed, an earlier report had suggested that maitotoxin-activated channel might be similar to the channel subserving capacitative calcium influx, since both were readily blocked by SKF 96365 [25]. Loperamide was, therefore, investigated for effects on capacitative calcium influx in differentiated leukemia HL60 cells [24]. Such cells had been previously used for studies on the so-called store-operated calcium (SOC) channels that are responsible for capacitative calcium influx [26–28]. Recent reviews on factors involved in elicitation and control of capacitative calcium influx are available [29–31]. SOC channels have also been referred to as receptor-operated calcium channels, as calcium-release-activated calcium channels and as store-operated calcium entry channels. The SOC channels appear to be members of a TRP family of calcium channels [31, 32], originally discovered in *Drosophilia*, where they were called transient receptor potential channels.

The opening of SOC channels is triggered by release and hence depletion of inositol trisphosphate (IP_3)-sensitive stores of calcium from endoplasmic reticulum, as first proposed by Putney [33] in 1986. Such depletion can be

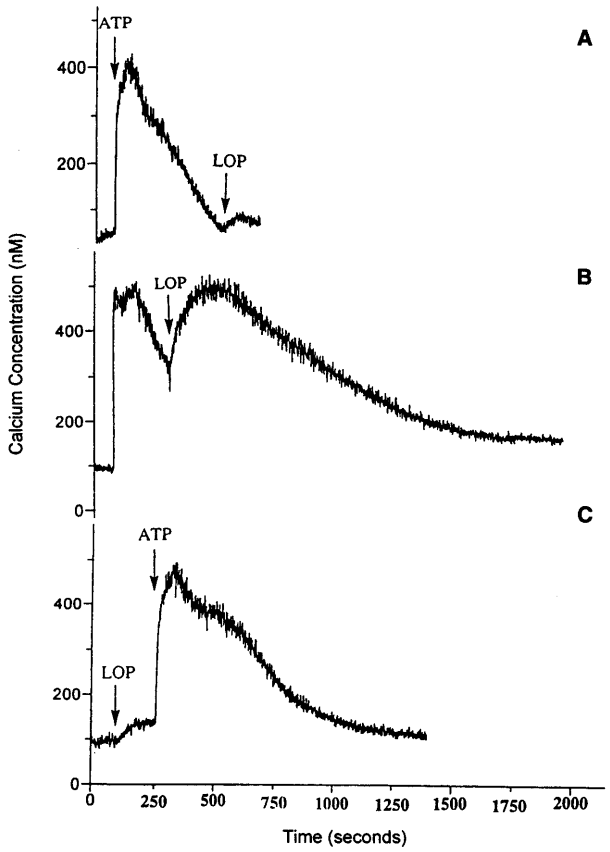

Figure 8. *A–C* Effect of loperamide on ATP-elicited elevation of levels of intracellular calcium in undifferentiated HL60 cells. Loperamide (LOP, 30 μM) and ATP (10 μM) were added as indicated. The incubation temperature was 37 °C. Cells had been previously loaded with fura-2 and the calcium-dependent fluorescence converted to calcium concentration as described by Harper and Daly [53]. Representative results are depicted.

Table 1. Loperamide and SOC-channel-elicited elevation of intracellular calcium in cultured cells.

Cell line	Calcium-elevating agent	Effect of loperamide on calcium levels [24, 53]
Leukemic HL60 cells (differentiated)[a]	ATP	↑
	fMLP	↑
	histamine	↑
	thapsigargin	↑
	ionomycin	↑
	EGTA[b]	↑
	sphingosine	no effect
NIH 3T3 fibroblasts	Thapsigargin	↑
Astrocytoma 1321N cells[a]	ATP	↑
Smooth muscle DDT-MF2 cells	ATP	↑
	thapsigargin	↑
		↑
Pituitary GH_4C_1 cells	ionomycin	
RBL-2H3 mast cells	NECA	↑

fMLP, N-formyl-met-leu-phe; EGTA, ethyleneglycol-bis-(β-aminoethylether)-N,N,N′N′-tetraacetic acid; NECA, N-ethylcarboxamidoadenosine

[a] In undifferentiated HL60 cells and astrocytoma cells, loperamide alone caused an increase in basal levels of intracellullar calcium [51].

[b] EGTA followed by 2 mM $CaCl_2$.

elicited by activation of receptors that through G proteins activate phospholipase C to generate IP_3, which in turn opens an IP_3-sensitive calcium-release channel in the endoplasmic reticulum. Depletion of the calcium stores can also be elicited through selective inhibition of the Ca^{2+}-ATPase that serves to transport calcium into endoplasmic reticulum. Thapsigargin and cyclopiazonic acid are selective inhibitors of such Ca^{2+}-ATPases, resulting, presumably through 'leak' currents, in a depletion of calcium from intracellular stores [34] and, thus, activation of capacitative calcium influx [35–37]. However, cyclopiazonic acid also blocks SOC channels at higher concentrations [36]. The molecular mechanism(s) whereby depletion of calcium stores in the endoplasmic reticulum elicits an opening of SOC channels in the plasma membrane remains elusive. Two currently favored mechanisms are activation through direct conformational contact with calcium-depleted endoplasmic reticulum [30, 38, 39] or by a calcium influx factor, as an intracellular messenger [40, 41; see also ref. 42]. Other possible pathways involving cytochrome P450 [43, 44], G proteins [45], tyrosine kinase [46], protein kinase C [47], or nitric oxide [48] have also been proposed. Similarly, the mechanisms that control inactivation of SOC channels are unclear. Feedback inhibition by calcium and inactivation due to refilling of calcium stores in the endoplasmic reticulum are two possible mechanisms involved in the inactivation of SOC channels [49, 50]. The relative importance of inhibition and inactivation of SOC channels are unclear. Such mechanisms clearly differ in different cell types [50].

The effect of loperamide on SOC-channel-dependent elevation of intracellular calcium levels in HL60 cells [24] was entirely unexpected. Instead of the inhibition that loperamide manifests on voltage-dependent calcium channels and on maitotoxin-elicited influx of calcium, loperamide augmented calcium levels that were being sustained by influx through SOC channels after depletion of IP_3-sensitive calcium stores by the P_{2y} receptor agonist ATP [24, 51]. The augmentation was dose dependent with a maximal loperamide response at 30 μM [fig. 2]. Loperamide alone had no effect on calcium levels, but when added before ATP, the subsequent SOC-channel-dependent elevation of intracellular

calcium was significantly greater than that after ATP alone [fig. 3]. Loperamide caused similar augmentation of SOC-channel-dependent elevation of extracellular calcium after thapsigargin- or ionomycin-elicited depletion of calcium stores [51]. Typical effects of loperamide in combination with thapsigargin are shown in figure 4. Loperamide also augmented the SOC-channel-dependent level of intracellular calcium seen after N-formyl-met-leu-phe (FMLP) or histamine in HL60 cells [51] and after ATP in both astrocytoma 1321N cells and DDT-MF_2 cells (fig. 5 and unpublished data). Augmentations of calcium levels by loperamide also occurred in NIH 3T3 fibroblasts and DDT-MF_2 cells after thapsigargin, in GH_4C_1 cells after inomycin, and in RBL-2H3 cells after N-ethylcarboxamide plus an antigen [51]. In all the above cells, with the exception of the astrocytoma 1321N cells (fig. 5), loperamide alone had no effect on calcium levels, only doing so after SOC channels had been activated by depletion of calcium stores. Remarkably, loperamide, while having no effect alone in differentiated HL60 cells, did cause an increase in levels of calcium in undifferentiated HL60 cells (fig. 6, and unpublished data). Thus, there is no effect of loperamide alone in most cells except in the undifferentiated HL60 cells and astrocytoma 1321N cells, where an increase in basal levels is elicited by 30 μM loperamide [51, and unpublished data]. The elevation of calcium by loperamide alone in these cells was dependent on extracellular calcium (fig. 6). It is possible that there is a lower threshold for activation of SOC channels by loperamide in undifferentiated HL60 cells and in astrocytoma 1321N cells.

Sphingosine causes an elevation of calcium in cells that does not involve activation of SOC channels; indeed, sphingosine inhibits SOC channels [52]. Loperamide did not augment the sustained elevation of intracellular calcium that had been elicited by sphingosine in HL60 cells [51]. Thus, the lack of any effect of loperamide on sphingosine-elicited elevation of intracellular calcium provides evidence that loperamide targets only elevations of calcium caused by influx through SOC channels in HL60 cells.

The stimulatory effect of loperamide on SOC-channel-dependent elevated levels of calcium has now been found to be markedly dependent on temperature (fig. 7). In differentiated HL60 cells, the loperamide-elicited augmentation of SOC-channel-dependent calcium levels after ATP was maximal at about 22 °C. Similar temperature dependency for the loperamide response after ATP occurred with undifferentiated HL60 cells and DDT-MF_2 cells (unpublished results). Thus, investigation of effects of loperamide on SOC-channel-dependent elevation of intracellular calcium are best done at about 22 °C, since the effects are much less at higher temperatures. The SOC-channel-dependent elevation of intracellular calcium after ATP was sustained longer in differentiated HL60 cells compared to undifferentiated cells where levels declined rapidly to basal [unpublished data; compare fig. 7A at 37 °C with fig. 8]. In both differentiated and undifferentiated cells, the SOC-channel-dependent elevation returned more rapidly to basal levels at higher temperatures. Loperamide caused the greatest augmentation when calcium levels were highest after ATP (fig. 8). Thus, the magnitude of the effect of loperamide seems to correlate with the magnitude of SOC-channel-dependent elevation of intracellular calcium.

The mechanism involved in the augmentation by loperamide of SOC-channel-dependent elevation of intracellular calcium remains unknown. Several possible mechanisms can be considered: (i) Loperamide might allosterically enhance conduction through SOC channels; (ii) loperamide might inhibit calcium-dependent deactivation of SOC channels; (iii) loperamide might interfere with storage of calcium entering through SOC channels. The marked temperature dependence for loperamide responses would be compatible with an allosteric effect that augments a conformational activation of SOC channel conductance at 22 °C, while at 37 °C, the conformational change might be nearly completely manifest in the absence of loperamide. A direct effect of loperamide on the SOC channel is also compatible with interactions between SOC channel blockers and loperamide. Thus, the addition of loperamide appears to counteract the effects of SOC channel blockers, such as SKF 96365, trifluoperazine, and nitrendipine (fig. 1), on SOC-channel-dependent elevations in intracellular calcium [51, 53]. Such effects are illustrated in figure 9. The channel blockers, in particular the imidazoles miconazole and econazole, have been proposed to act indirectly through inhibition of cytochrome P450 [43, 44] but have other activities including inhibition of calmodulin function [54] and inhibition of Ca^{2+}-ATPases [55]. Trifluoperazine, another putative SOC channel blocker [53], is a potent calmodulin antagonist, as is loperamide. Trifluoperazine has been reported to enhance calcium influx in rat thymocytes, but only with cells in which calcium stores were not

Figure 9. Effect of loperamide on blockade of SOC-channel-dependent elevation of intracellular calcium in differentiated HL60 cells. Loperamide (L, 30 μM) was added before or after an imidazole blocker (SKF 96365, 30 μM) (*A*), a phenothiazine blocker (trifluoperazine, Tf, 10 μM) (*B*), or a dihydropyridine (nitrendipine, N, 10 μM) blocker (*C*). ATP (10 μM) was added as indicated. The incubation temperature was 22 °C. Cells had been previously loaded with fluo-3 and calcium-dependent fluorescence was measured as described by Harper and Daly [53]. Data from ref. 53; see also ref. 24.

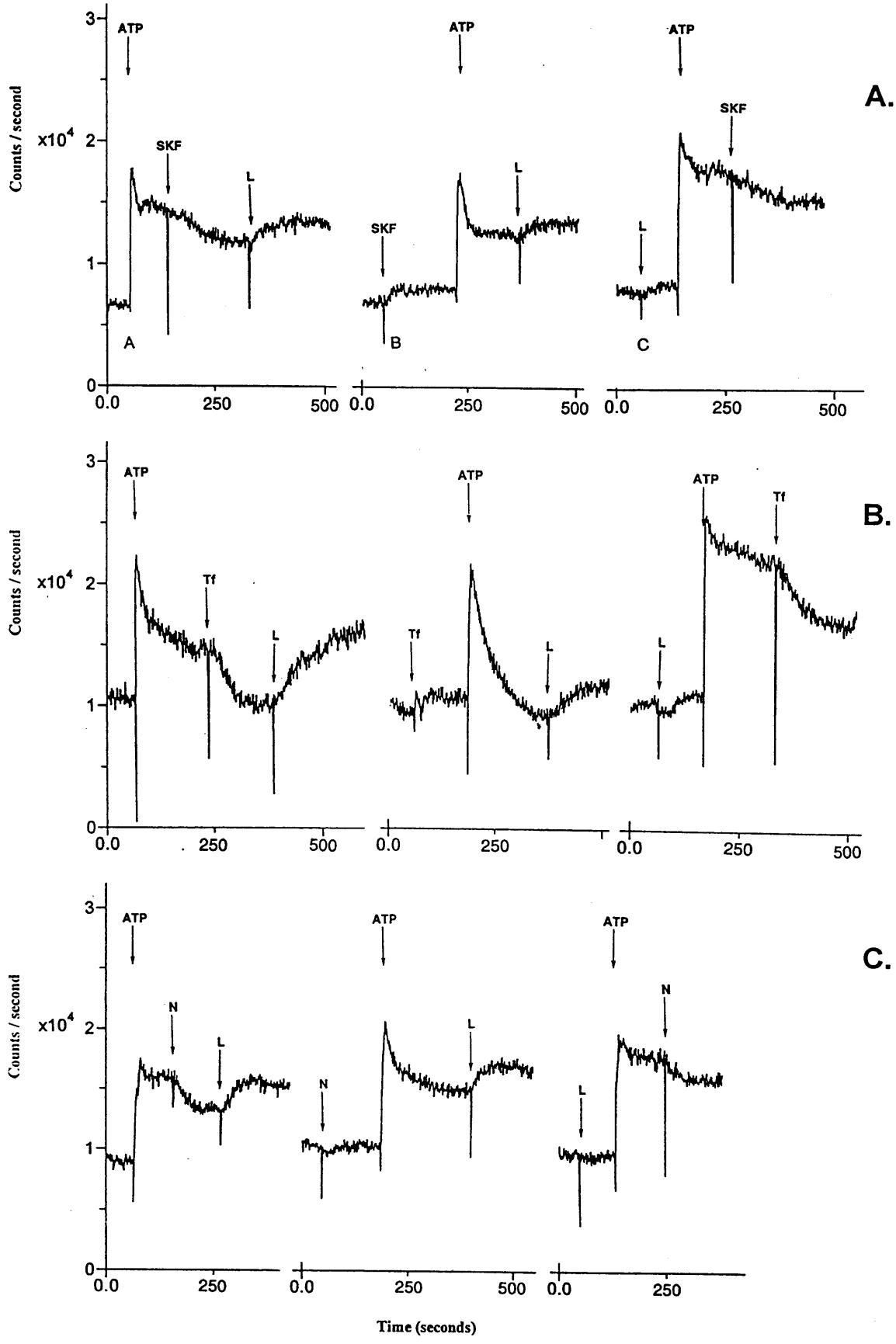
A.
B.
C.
ATP
SKF
L
Tf
N
A
B
C
Counts / second
x10^4
Time (seconds)
0.0
250
500

Fig. 9.

depleted [43, 44]. The effect was blocked by the imidazole econazole. Nitrendipine is an L-type calcium channel blocker, but also has other sites of action at concentrations greater than 10 μM. It appears unlikely that effects on cytochrome P450 or calmodulin represent a common pathway for the effects of imidazoles, trifluoperazine, nitrendipine, and loperamide on SOC-channel-dependent elevations of intracellular calcium. However, TRP and TRP-like proteins do bind calmodulin, thereby enhancing cation flux [56, 57], and presumably calmodulin also interacts with and modulates SOC channels.

The effects of loperamide on SOC-channel-dependent elevations have to date been assessed in several cell types (table 1). The loperamide response appears to be dependent on both the degree of SOC channel activation and on temperature. Whether the effect of loperamide is readily reversible has not been assessed. Structure-activity relationships have not been studied because the many structural analogs of loperamide are not accessible. Diphenoxylate and verapamil, two structurally similar compounds (fig. 1), containing not the N,N-dimethylcarboxamide moiety of loperamide, but a nitrile group instead, appear ineffective in either blocking or augmenting SOC channel function [24 and unpublished data].

Further studies are required, particularly since loperamide is an agent which, at micromolar concentrations, will have effects on calmodulin function, other calcium channels, and probably other targets. At present, however, to our knowledge, it is the only agent that causes augmentation of elevated intracellular levels of calcium when these are due to capacitative calcium influx. It does so in a variety of different cell types [51]. A more potent and selective agent would represent an invaluable research tool for studying the physiological function and importance of capacitative calcium influx.

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